

THE INFLUENCE OF BODY SIZE ON METABOLIC RATES, SCALING, TELOMERE
DYNAMICS, AND METAMORPHOSIS IN MEGACHILID BEES

A Dissertation
Submitted to the Graduate Faculty
of the
North Dakota State University
of Agriculture and Applied Science

By

Courtney Corinne Grula

In Partial Fulfillment of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY

Major Department:
Biological Sciences

May 2021

Fargo, North Dakota

North Dakota State University
Graduate School

Title

THE INFLUENCE OF BODY SIZE ON METABOLIC RATES,
SCALING, TELOMERE DYNAMICS, AND METAMORPHOSIS IN
MEGACHILID BEES

By

Courtney Corinne Gula

The Supervisory Committee certifies that this *disquisition* complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

SUPERVISORY COMMITTEE:

Dr. Julia Bowsher

Chair

Dr. Kendra Greenlee

Dr. Britt Heidinger

Dr. Deirdre Prischmann-Voldseth

Dr. Joseph Rinehart

Approved:

June 17, 2021

Date

Dr. Kendra Greenlee

Department Chair

ABSTRACT

Body size is related to many aspects of a bee's life history including foraging distance and pollination efficiency. *Megachile rotundata*, and *Osmia lignaria* are agriculturally important, solitary pollinators. Adult body size in solitary bees is variable because it is determined by the quantity of food available to the developing larva. The goal of this dissertation was to determine the effect of body size on metabolic rates, scaling, telomere dynamics, and entry into metamorphosis. Body size was manipulated under laboratory conditions by manipulating the amount of food provided during the final larval stage. First, I tested the effect of body size on allometry and amount of energy produced, measured indirectly through CO₂ emission. The power required during flight was predicted using biomechanical formulas. I found larger bees had higher absolute metabolic rates at rest and during flight. Smaller bees had higher mass-specific metabolic rates at rest, but not during flight. As bees increased in size, their thorax and abdomens became disproportionately larger, while wings became disproportionately smaller. Smaller bees had more power available during flight as demonstrated by flight biomechanical formulas. Next, I measured telomere length in *M. rotundata* and *O. lignaria* throughout development. I also measured telomere length across a variety of body sizes, and life stages in both bee species, and found that body size does not affect telomere length. I found that telomere length increases in later life stages in both bee species. Lastly, I determined the cue for metamorphosis in *M. rotundata* by determining the critical weight. Entry into metamorphosis involves the insect's ability to monitor its size. I found the cue for metamorphosis in *M. rotundata* is a critical weight which is influenced by nutritional condition. This was confirmed by declines in Juvenile Hormone titers, and upregulation of genes involved in metamorphic molts. This study found smaller-sized bees are not always at a disadvantage. Smaller bees had

increased flight performance based on flight biomechanical formulas, did not show differences in telomere length based on body size, and enter metamorphosis at small sizes. Advantages to small body size may be a response to declines in resource availability.

ACKNOWLEDGEMENTS

I would like to acknowledge my advisor, Dr. Julia Bowsher for her continuous support, guidance and compassion throughout my entire degree. Dr. Bowsher's enthusiasm for research made me excited to come to work every day. She pushed me to be better, and I will always be grateful for her mentorship. I would also like to acknowledge my committee members Dr. Kendra Greenlee, Dr. Joseph Rinehart, Dr. Britt Heidinger, Dr. Deirdre Prischmann-Voldseth for their guidance, feedback and support throughout my degree. I would like to acknowledge my mentors from the USDA, Dr. George Yocum, and Dr. Arun Rajamohan, their support. I would like to thank Dr. Giancarlo Lopez-Martinez and Jeff Kittilson for their help and guidance and expertise. I would like to thank Micki Plamersheim for the bee art work used in this dissertation. Finally, I would like to thank my friends and fellow graduate students, especially the members of the Greenlee/Bowsher labs, the Insect Cryobiology and Ecophysiology group, and all the members of the NDSU biological sciences faculty and all of the undergraduate students for their help and support.

DEDICATION

This dissertation is dedicated to my family. My family has been there for me every step of the way. Thank you to my parents Tom Grula and Linda Grula whose love, guidance, encouragement, and support has been instrumental in my success throughout graduate school and life. I would also like to thank my sister Cara Grula and Jaime Lane for being my support system throughout it all. I would not be where I am without you all!

TABLE OF CONTENTS

ABSTRACT	iii
ACKNOWLEDGEMENTS	v
DEDICATION.....	vi
LIST OF TABLES.....	ix
LIST OF FIGURES	x
CHAPTER 1: INTRODUCTION.....	1
Body Size and Flight.....	5
Body Size and Aging.....	8
Aging and Telomeres in Insects.....	11
Background: Solitary Bee Lifecycle	12
Objective 1: Determine How Body Size Influences Flight-Related Morphology and Metabolic Rate in the Solitary Bee <i>Megachile rotundata</i>	13
Objective 2: Determine How Body Size Impacts Telomere Dynamics Throughout Development and Adult Lifespan in Solitary Bees.....	13
Objective 3: Determine if Starvation is the Cue for Metamorphosis in the Solitary Bee <i>Megachile rotundata</i>	14
References.....	14
CHAPTER 2: BODY SIZE ALLOMETRY IMPACTS FLIGHT RELATED MORPHOLOGY AND METABOLIC RATES IN THE SOLITARY BEE MEGACHILE ROTUNDATA	28
Abstract.....	28
Introduction.....	29
Methods	33
Results.....	39
Discussion	47
Conclusion	53

Acknowledgements	54
References	54
CHAPTER 3: TELOMERE DYNAMICS IN TWO SOLITARY MEGACHILID BEE SPECIES	66
Abstract.....	66
Introduction.....	67
Methods	71
Results.....	75
Discussion	82
References	86
CHAPTER 4: DETERMINING THE CUE FOR METAMORPHOSIS IN THE SOLITARY BEE MEGACHILE ROTUNDATA.....	97
Abstract.....	97
Introduction.....	97
Methods	100
Results.....	103
Discussion	111
References	120
CHAPTER 5: CONCLUSION	127
References	130

LIST OF TABLES

<u>Table</u>	<u>Page</u>
1. Metabolic rate (MR) in relationship to body size	41
2. Relationship of body segments to total body mass	44
3. Allometric scaling relationships to body size	45
4. Post-hoc comparisons of T/S ratios of different developmental stages in <i>M. rotundata</i>	75
5. Comparing T/S ratios of different developmental stages in <i>O. lignaria</i>	77
6. Gene expression data from NanoString analysis	110

LIST OF FIGURES

<u>Figure</u>	<u>Page</u>
1. Metabolic rates of <i>M. rotundata</i> during rest and flight	41
2. The relationship between discontinuous gas exchange and body size	42
3. Scaling relationship between body size, body segments and wings	46
4. Flight performance and body size	47
5. Life cycle of <i>O. lignaria</i> and <i>M. rotundata</i>	72
6. Telomere length across different developmental life stages in <i>M. rotundata</i>	76
7. Telomere length based on body size in <i>M. rotundata</i>	76
8. Telomere length across different developmental stages in <i>O. lignaria</i>	78
9. Telomere length based on sex and weight across different developmental stages in <i>O. lignaria</i>	78
10. Comparing telomere length in days post emergence in adult <i>O. lignaria</i>	79
11. Telomere length based on sex and weight in days post emergence in adult <i>O.</i> <i>lignaria</i>	80
12. Telomere length in days post emergence in fed and starved adult <i>O. lignaria</i>	81
13. Telomere length based on sex and weight in days post emergence in <i>O. lignaria</i> that were exposed to a feeding treatment	81
14. Time to growth cessation.....	104
15. Critical weight.....	105
16. Juvenile hormone titers in larvae across time.....	106
17. Genes involved in JH synthesis and response.....	108
18. Genes involved in Ecdysone synthesis and response	108
19. Genes involved in the ISS/TOR pathway	109

CHAPTER 1: INTRODUCTION

Body size is variable in animals. A given animal species will have a characteristic size, but intraspecific ranges can be vast. Body size affects many different aspects of animal life habits. Larger animals tend to have higher rates of fecundity, metabolism, longevity, and foraging distance (Smith & Lyons 2013). Additional advantages to a larger body size may include a better ability to defend against predators and increased mating and competition success both intra- and interspecifically (Hone & Benton 2005). Being larger also comes with costs, including longer development time and being more conspicuous to predators. Larger individuals may also have higher energy requirements, and reduced agility (Blanckenhorn 2000).

Several evolutionary theories have attempted to explain variation in body size across spatial and temporal scales. Cope's rule states there is selection for larger body size throughout evolutionary time (Cope 1896). This trend can be seen in many mammals (Alroy 1998), although evidence of this trend across orders is weak. Although, this trend has been seen in insects (D'Amico et al. 2001), insects in general are exceptions to this rule. Gigantic insects have been found in the fossil record, most notably odonates, with wingspans up to 71 cm (Shear & Kukalov'a-Peck 1990, Dudley 1998, Wootton & Kukalov'a-Peck 2000). Insects may have been able to reach such extremely large sizes due to the higher concentration of oxygen in the atmosphere (Miller 1966). High oxygen reduced constraints on the insect tracheal system, which relies on the passive diffusion of oxygen to tissues (Dudley 1998). Although recent findings suggest that tracheal limitations on size may only occur in appendages like legs (Harrison et al., 2020). Other theories have been presented to explain why insect body size has decreased over time including reductions in geographic range, thermal tolerance (Huey & Ward 2005), exoskeleton constraint and responses to predation (Shear & Kukalov'a-Peck 1990).

Bergmann's rule states that body size increases with latitude and altitude, which correlates with larger individuals being found in colder climates. This is due to the smaller surface area to volume ratio of larger animals, which allows these animals to stay warmer in cooler temperatures. This trend can be seen in insects (Dillon et al 2006). Although there are many exceptions to this rule (Nylin & Savard 1991) which are termed converse Bergmann clines. These clines occur intraspecifically, in which size decreases with increasing latitude. This has been seen in insect species with a limited geographical range (Gaston et al., 2008), as well as longer foraging period (i.e., larger insects nearer to the equator have food available for longer periods).

Understanding body size is important for understanding maximum fitness and performance outputs in insects. Body size is a particularly important aspect of an insect's life history because body size is often correlated with fitness (Blanckenhorn 2000). Larger females produce larger oocytes (O'Neill et al. 2014), live longer, are able to establish nests faster, invest more in each offspring (Kim 1997), and have an increased foraging range (Greenleaf et al. 2007). Body size is also important for overwintering, with larger individuals tending to have higher survival (Tepedino & Torchio, 1982; Bosch & Kemp, 2004).

Body size determination in insects is complex. An insect is restricted by how large or small it can become due to genetic (Reeve & Fairbairn 1996), developmental, and physiological constraints (Peters 1986, Reiss 1991). Although, a given insect species will have a characteristic size, which is phylogenetically conserved (Chown et al 2002), the intraspecific ranges of body size can be vast. Solitary bee species can have a 10-fold difference in body size (Helm et al., 2017). The most important factors influencing solitary bee body size include temperature and

nutrition (Chown & Gaston 2010; Bosch & Vicens 2002, Wilkaniec et al. 2004, Radmacher & Strohm 2009).

Body size in solitary bees is largely determined by pollen and nectar allocations to the larvae from the mother (Klostermeyer et al. 1973; Fischman et al., 2017; Freeman 1981; Johnson 1988; Strohm 2000; Bosch and Vicens 2002; Bosch and Vicens 2006). Growth in insects can be attributed to resources available to the developing juvenile (Emlen 1997). Nutritional quantity is a better predictor of body size than quality (Radmacher & Strohm 2009). The amount of food provided to sexually dimorphic male and female offspring, with larger females receiving larger provisions than males (Strohm 2000, Seidelmann et al. 2010). This can also be seen experimentally when larvae provided excess food are larger as adults (Wilkaniec et al 2004, Helm et al 2017, Sidelmann 2018).

Temperature and nest size are also important factors influencing body size in solitary bees. The temperature-size rule states ectotherms reared in lower temperatures during development have an increased body size (Atkinson 1994, Davidowitz et al. 2004). This rule seems to hold true in solitary bees (Radmacher & Strohm 2009). In solitary bees, high temperature may be inducing behavioral changes in feeding behavior. Bees that have been exposed to higher rearing temperatures did not consume all of the provided provisions and consumed less food overall, leading to smaller adult body sizes (Radmacher & Strohm 2009). Lower temperatures increase the efficiency of converting nutrition to body mass (Karl & Fischer 2008).

Nesting hole diameter also influences solitary bee body size. Solitary bees lay offspring in preexisting cavities. Bees that have nested in larger nest holes produce larger offspring. Few females will lay their offspring in a nest with a diameter smaller than the standard commercial

nest size (Tepedino & Torchio 1989). This may be because there is less space for the progeny to develop, as well as less space to place pollen/nectar provisions. Both temperature and nest size influence body size by potentially altering the amount of provision the offspring consume, which indicates that provision amount is the primary influencer of adult body size.

In insects that go through metamorphosis, adult body size is determined after the juvenile growth period when the final molt occurs because linear dimensions remain fixed due to sclerotization of the cuticle (Sehnal, 1985, Nijhout et al., 2006). Adult size is predicted by the weight of the larva at the end of the final instar because feeding ceases prior to the pupal phase (Chown and Gaston 2010). There are different ways for insects to determine whether a molt will be from larva-to-larva or larva-to-pupa. Most cues for metamorphosis are related to feeding. In many insects, such as the well-studied caterpillar, *Manduca sexta*, a critical weight is the cue to begin metamorphosis. The critical weight refers to the size at which the larva is physiologically committed to metamorphosis (Davidowitz et al. 2003). Since solitary bees have a finite amount of food provided by the mother, their initiation for metamorphosis does not rely on a critical weight or size. In one solitary bee species, the cue for metamorphosis is the absence of food (Helm et al 2017).

Larval growth rate and duration are both important for determining the final adult size (Nijhout, 1975; Nijhout and Williams, 1974). When to undergo metamorphosis is driven either directly or indirectly by the critical weight at the final larval instar. Metamorphosis in holometabolous insects begins during the larval-pupal transition. Juvenile Hormone (JH) titers will begin to decline during the last larval instar. A larva-pupa molt will occur when JH has been cleared from the system. In the absence of JH, a Prothoracictropic hormone (PTTH) spike, and 20-Hydroxyecdysone (20E) pulse will begin the metamorphic molt to the pupal stage. The

nutrient sensing pathways such as the insulin/insulin-like growth factor sensing pathway (ISS) and target of rapamycin (TOR) pathway are important for regulating larval growth (Layalle et al. 2008; Mirth and Shingleton, 2012; Ohhara et al., 2017) and are also important in regulating metamorphic molts via regulation of 20E (Koyama et al., 2014; Mirth et al., 2005; Ohhara et al., 2017).

Solitary bees can have intraspecific variation in size due to environmental conditions experienced by the mother bee. The Optimal Allocation Theory predicts that females will allocate available resources to maximize fitness returns for the offspring (Seidelmann 2014). Thus, females will produce more males under stressful resource conditions, because males are less costly to produce, due to their smaller body size (Bosch 2008). Females may also produce tiny female offspring under stressful conditions although small females tend to have lower survival rates (Bosch 2008). One explanation for why this occurs is that females may provide smaller amounts of provisions to close up cells faster to protect the nest cells from predators and parasites (Bosch 2008). In *M. rotundata*, females will invest more in female offspring when resources are high. They will produce more females (as opposed to smaller males), produce more nest cells per day, and females will be larger when resources are high (Kim 1999).

Body Size and Flight

Body size is correlated with aspects of flight performance, including flight foraging distance, predator avoidance, parasitism, ability to mate, and ability to carry provisions (Bushwald & Dudley 2010). This is because body size affects the aerodynamics during flight. Larger individuals tend to have decreased flight maneuverability (Fry et al. 2003), which influences predator avoidance and mating ability. As body size increases, foraging distance increases (Greenleaf et al. 2007).

Flight is particularly important for solitary bees to collect provision materials for their nest. Female *M. rotundata* carry leaf pieces (Pitts-Singer & Cane 2011), and *O. lignaria* carry mud balls (Bosch & Kemp 2001) to line and cap their nests. Solitary bee females build a cell for each offspring and provision it with nectar and pollen. Females must collect and carry nest and provision materials back to their nest with each foraging bout; each cell may take several flight bouts. A solitary female bee can spend around 5 hours a day foraging for food. Each nest can require around 120 leaf pieces to complete a nest (15/nest cell) (Matea & Adachi 2005, Klostermeyer & Gerber, 1969). Pollen weight carried by bees positively correlates with body mass (Giejdasz, 1998) with larger females carrying heavier loads.

Interspecifically, larger bees also tend to have the ability to fly farther (Greenleaf, 2007), which is advantageous for finding resources. Foraging distance determines the scale across which bees can pollinate (Kremen, 2005) and thus collect provisioning materials. Foraging distance is important on the community level because it increases pollination, dispersal, and can influence plant diversity (Ritchie & Olf 1999, Roland & Taylor 1997).

Flight performance is determined by how effectively and efficiently an individual can fly based on their mass plus the load mass. The ability of a bee to successfully lift and transport loads during flight is an important aspect of flight performance. The maximum load that an animal can lift during take-off is relative to body mass (Marden 1994) in many different animals. Flight performance can be assessed by using flight-related morphometrics to predict performance outcomes. The allometry of adult morphology in solitary bees does not increase isometrically. As bees increase in size, the thorax size gets hyperallometrically larger, while the wing area increases hypoallometrically (Helm 2021 in revision). These allometric relationships may influence flight performance. Smaller bees have improved flight performance this may be due to

larger bees having a smaller wing area in relation to body size, which might compromise flight performance (Helm 2021 in revision). Based on measurements of body mass, thorax mass, and wing area, larger bees have decreased flight performance. Larger bees may be spending more of their available flight power to lift their own mass to maintain hovering flight (Dillon & Dudley 2004, Buchwald & Dudley 2010, Sidelmann 2014).

Flight is incredibly energetically costly and requires a high metabolic demand. Insect flight muscles use the highest rate of mass-specific oxygen compared to any other tissue used in the animal kingdom for locomotion (Dudley 2002). The majority of an insect's energy metabolism during flight is directed to flight muscles (Niven & Scharlemann 2005). Flight metabolic rates in insects can vary dramatically within a species (Chown et al., 2007).

Larger animals tend to have higher metabolic rates, but there is a negative correlation between body size and mass-specific metabolic rate. Mass-specific metabolic rate is the metabolic rate per gram of body tissue (Rubner, 1908; Kleiber, 1947). This can be seen in the “mouse-to-elephant curve” where the larger elephant has a lower mass-specific metabolic rate than the smaller mouse (Brody, 1945). This is also seen in invertebrates (Altman and Dittimer, 1968; Schmidt-Nielsen, 1984) including insects (Reinhold, 1999, Lehmann et al., 2000, Niven and Scharlemann, 2005; Coelho and Moore, 1989). Across taxa, metabolic rate in insects scales as $\text{mass}^{0.75}$, once corrected for phylogeny. Intraspecific scaling exponents can range from 0.67 to 1.0. (Chown et al 2007). Differences in scaling exponents are likely due to physiological, taxonomic, or environmental factors (Glazier 2005). General differences in metabolic rate between and within species may be due to selection and heritability (Pettersson et al. 2018), body size (Speakman et al. 2004, Chown et al. 2007), growth (Sadowska et al., 2009), season (Smit & McKechnie 2010), and availability of food resources (Sadowska et al., 2009). Metabolic rates

can vary intraspecifically (Chown et al. 2007). Since solitary bees can be experimentally manipulated to have a 10-fold difference in body size (Helm et al 2017), it makes them an interesting model for investigating differences in intraspecific metabolic rate. Previous studies have not shown a correlation between body size and flight mass-specific metabolic rate in *Megachile rotundata* (Bennett et al. 2013). However, this lack of correlation could be explained by the small range of body masses within the study.

In insects, body size may be limited by the ability of the tracheal system to effectively transport oxygen. The ability to diffuse oxygen to all vital organs within the body (diffusive capacity) of an insect may be limited by size and can possibly affect the flight metabolism of larger insects (Dudley 2002). There may be an upper limit to which the tracheae size can successfully allow for passive diffusion of oxygen through the insect body and maintain the ability to meet the metabolic demands of flight (Callier et al. 2011). To overcome these limitations, Hymenopterans are able to enhance gas transport by flapping their wings, which causes deformation of the cuticle and drives ventilation of the respiratory system. This allows for more oxygen to enter the trachea and be delivered to the tissue (Weis-Fogh 1964). Another method of expanding the air flow to the branches of the tracheal system is through abdominal pumping which may increase air ventilation during flight (Weis-Fogh 1964).

Body Size and Aging

How and why organisms age are big questions in organismal biology. One of the first hypotheses explaining aging originated from Medawar (1952), called the mutation accumulation hypothesis, and notes that natural selection is not efficient against post-reproductive mutations. Williams (1957) further developed Medawar's theory. Williams' Theory of Antagonistic Pleiotropy posits that there is a trade-off between early life-history traits and late in life repair.

This is the idea that genes that control multiple traits in an organism may increase success of early life traits such as development and reproduction, and the trade-off will be the genes may cause a detrimental effect later in life. Another early aging theory, the Disposable Soma Theory, was proposed by Kirkwood (1977). This is idea that there is a tradeoff of allocation of resources into growth, and reproduction, and DNA repair. An increase in investment into growth and reproduction will lead to a decrease in resource available for DNA repair, thus causing an accumulation of damage over time. Another theory, termed the free radical theory of aging hypothesizes that aging is caused by the accumulation of free radicals over time. Free radicals, which occur naturally during cellular metabolism, are uncharged molecules that are highly reactive, causing cellular and molecular damage via oxidative stress (Harman 1956; Betteridge 2000), leading to cellular senescence (Campisi & di Fagagna 2007).

Cellular aging may be understood through the mechanism of telomere attrition over time. Telomeres are repetitive sequences of non-coding DNA located at the end of chromosomes, which protect coding DNA during replication, and delineate the ends of chromosomes (Blackburn 2005). There is a limit to the number of times a cell can divide. This limit is termed the Hayflick limit (Hayflick 1965), which is the number of times a cell successfully mitotically divides before it begins senescing (Wright and Shay 2000). One of the causes of cells to stop dividing is when the telomeres have reached a critically short length (Blackburn 2005) leading to cellular senescence. Cellular senescence occurs when the ability to normally replicate is decreased, and the metabolic function of cells declines (Sahin et al. 2011). This can lead to problems such as chronic inflammation or organ failure (Wong et al 2003). To avoid problematic telomere shortening, cells trigger a DNA damage signal to stop replication

(d'Adda di Fagagna et al., 2003, Takai et al., 2003). Accumulation of damaged senescent cells may lead to aging effects such as declines in overall health (Krtolica et al., 2001, Campisi, 2005)

Although larger animals tend to live longer, intraspecifically larger individuals have shorter lifespans (Selman et al. 2013). This trend can be seen in many animals including; mice, snakes, and domesticated dogs (Miller et al. 2002, Bronikowski & Vleck 2010, Kraus et al. 2013). Factors which contribute to longer lifespan within a species include slow growth rates, and low body mass (Kraus et al. 2013) which slow the pace of aging. Rapid growth in larger individuals can be detrimental downstream because it is associated with higher levels of oxidative stress, and shorter telomeres (Nussey et al. 2009). Shorter telomere lengths have been found in larger individuals, with shorter lifespans (Fick et al. 2012). Body size influences telomere dynamics, which function differently in animals with different life expectancies. This may be due to the rate of expression of telomerase. The enzyme telomerase adds nucleotides to the end of telomeres, which help elongate telomere sequences (Greider 1996). Telomerase is often downregulated in adult somatic tissues, and is typically upregulated in stem cells, immune cells and germline tissue (Monaghan 2010, Weng 2008). Differential telomerase expression may be an adaptation to balance critically short telomeres and tumorigenesis (Campisi, 2001). Expression of telomerase varies among organisms. Small mammals like mice for example, express telomerase in their somatic cells (Prowse and Greider, 1995) and larger mammals like humans tend to only express telomerase in germline tissue, and immune cells, and not in somatic cells (Bekaert et al., 2004). The expression of telomerase in mouse tissue differs from other mammals due to differences in body mass, and rates of extrinsic mortality (Wright and Shay, 2000, Forsyth et al., 2002). It is hypothesized that the absence of telomerase in the somatic tissue of larger mammals may be because in larger, longer lived individuals, the downregulation of

telomerase may be a mechanism to suppress tumor growth by limiting cell proliferation (Seluanov et al., 2006). Comparing telomerase expression in different rodent species with different body sizes and life expectancies shows that telomerase regulation is associated with body size, not lifespan (Seluanov et al., 2007). Telomerase expression in somatic tissues is present in mammals below 2 kg but is not present in animals larger than 5 kg (Gorbunova and Seluanov 2009). Larger animals tend to have lower telomerase expression (Seluanov et al., 2007). Indicating that tumor suppression is essential in larger individuals as opposed to longer-lived individuals. The hypothesized explanation for this phenomenon may be the increased number of cells in larger mammals increases the risk of cancer to develop, therefore the need for tumor-suppressing mechanisms increases (Nunney, 1999; Leroi et al., 2003).

Aging and Telomeres in Insects

Insects are a good model system for understanding aging. In general, insects have short lifespans, small body size, high fecundity, and low laboratory maintenance costs. These factors facilitate the study of these organisms (Lee & Kim 2015). Despite their relatively short lifespans, insects show declines in performance with age (McFarlane 1967, Giraldo & Traniello 2014), and studies in insects have helped to determine signaling pathways that affect lifespan (Lee & Kim 2015).

Hymenopterans are a good system to study telomere dynamics. Telomerase is expressed in bees in a similar way to other insects (Robertson & Gordon 2006, Frydrychová et al. 2004). Telomere dynamics have been studied in hymenopterans, these studies focus on honey bees and ants, which are eusocial. Studying telomeres in these species pose an interesting research question in the framework of aging biology because these organisms have experience extreme plasticity of longevity between castes, with queens typically living 1-3 years, but some queens

have been shown to live up to 8 years (Bozina 1961). In honeybees, previous studies have found no difference in telomere length between different developmental stages or castes (Korandová & Frydrychová 2016). Honeybees do show differential expression of telomerase. The longer-lived queens have higher expression of telomerase (Korandová & Frydrychová 2016).

Background: Solitary Bee Lifecycle

This dissertation focuses on two solitary bee species; *Megachile rotundata* and *Osmia lignaria*. Both bee species are both in the family Megachilidae, which have a defining characteristic of carrying pollen on the ventral side of their abdomen in dense, hairy pollen carrying structures called scopa (Michener 2000). These bees are cavity nesting bees and construct and line their nests with leaf pieces (*M. rotundata*) or mud caps (*O. lignaria*). These bees pollinate agriculturally important crops, including alfalfa for seed and orchard crops (Bosch & Kemp 2001, Pitts-Singer & Cane 2011).

Bees are holometabolous, and complete the egg, larvae, pupae and adult life stages (Michener 2000). In both species, bees will undergo five larval instars (de Oliveira 1960). The larvae will begin to consume their provisions during the second instar, and by the end of the fifth instar, they will consume the entire provision (Trostle and Torchio 1994). The sole source of food available for the larvae is the provision within its brood cell. After the final larval instar, the bee will begin to spin silk to create a cocoon where it will continue to develop until it is prepared to overwinter as a prepupae (*M. rotundata*, Pitts-Singer & Cane 2011) or as an adult (*O. lignaria*, Bosch and Kemp, 2001). *O. lignaria* transition to the pupal phase during late summer, and then will diapause as an adults, where they will remain until they emerge in early spring (Sgolastra, et al., 2012). *M. rotundata* diapause as a prepupae beginning in the late summer and early fall.

They transition to the pupal and adult stages the following summer, although non-diapausing *M. rotundata* can emerge as a second generation in the summer (Pitts-Singer & Cane 2011).

Objective 1: Determine How Body Size Influences Flight-Related Morphology and Metabolic Rate in the Solitary Bee *Megachile rotundata*

Body size is a predictor of metabolic rate in many organisms (Brody, 1945). My goal was to determine how body size influenced resting and flight metabolic rates and flight related morphology within a single species. I manipulated the amount of food given to the developing larva to produce a wide range of adult body sizes. This allowed for the measurement of the effect of body size on allometry, metabolic rates, and power required for flight. I predicted that larger bees would have higher absolute metabolic rates, and lower mass-specific metabolic rates. I also predicted that there would be an investment tradeoff as demonstrated by allometric scaling of differing body segments. Finally, I predicted based on flight biomechanical formulas that smaller bees would have more power available during flight.

Objective 2: Determine How Body Size Impacts Telomere Dynamics Throughout Development and Adult Lifespan in Solitary Bees

Telomere attrition has been linked to senescence in many organisms (Blackburn 2015; Haussman and Vleck 2002) and exposure to stress can increase the rate of telomere loss (Epel 2004). In social bees, telomere length does not change over time (Korandová & Frydrychová 2016; Koubová et al. 2021; Koubová et. al 2019). How stress impacts telomere length in bees has not been established. To date telomere length has not been measured in solitary bees. My goal was to determine how body size impacts telomere dynamics throughout the life span of two solitary bee species *M. rotundata* and *O. lignaria*. I aimed to establish the patterns of telomere dynamics throughout development, overwintering, and post emergence. Telomere length was

measured in larvae, prepupae, pupae, and multiple adult stages. I also measured changes in telomere length in response to stress. A nutritional stressor was implemented in adult *O. lignaria*. I measured relative telomere length using qPCR. I predicted that there would be an increase in telomere loss with age and in response to stress exposure. I also predicted telomere length would negatively correlate with body size.

Objective 3: Determine if Starvation is the Cue for Metamorphosis in the Solitary Bee

Megachile rotundata

There are different ways in which insects sense the timing for entry into metamorphosis. These cues include triggers from stretch receptors post-blood meal (Anwyl 1972), a critical weight (Davidowitz et al. 2003), or a starvation period (Helm et al., 2017). Little research on the cue for metamorphosis has been done in solitary bees. It has been determined in the solitary bee, *Osmia lignaria*, that the removal of food is the cue for metamorphosis. The goal of our study was to determine if the removal of food is the cue for metamorphosis in the solitary bee *M. rotundata*. I determined entry into metamorphosis through three factors, the critical weight, decline in JH titers, and gene expression. I hypothesized that food removal would be the cue to entry into metamorphosis, which would be demonstrated by the absence of a critical weight, decline in JH titers, and the upregulation of genes involved in molting and metamorphic commitment.

References

- Alroy, J.** (1998). Cope's rule and the dynamics of body mass evolution in North American fossil mammals. *Science* **280**, 731–734.
- Anwyl, R.** (1972). The structure and properties of an abdominal stretch receptor in *Rhodnius prolixus*. *J Insect Physiol*, **18**, 2143-2153.

- Atkinson, D.** (1994). Temperature and organism size: a biological law for ectotherms?. *Adv Ecol Res*, **25**, 1-58.
- Austad SN, Fischer KE** (1991). Mammalian aging, metabolism, and ecology: evidence from the bats and marsupials. *J. Gerontol.* **46** , B47–B53.
- Austad SN** (1997). Comparative aging and life histories in mammals. *Exp. Gerontol.* **32** , 23–38.
- Barrett, E. L., Burke, T. A., Hammers, M., Komdeur, J., & Richardson, D. S.** (2013). Telomere length and dynamics predict mortality in a wild longitudinal study. *Mol Ecol*, **22**, 249-259.
- Bekaert S, Derradji H, Baatout S** (2004). Telomere biology in mammalian germ cells and during development. *Dev. Biol.* **274**, 15–30.
- Bennett, M. M., Petersen, K., Yocum, G., Rinehart, J., Kemp, W., & Greenlee, K. J.** (2013). Effects of extended prepupal storage duration on adult flight physiology of the alfalfa leafcutting bee (Hymenoptera: Megachilidae). *J Econo Entomol*, **106**, 1089-1097.
- Betteridge, D. J.** (2000). What is oxidative stress?. *Metabolis*, **49**, 3-8.
- Blanckenhorn, W. U.** (2000). The evolution of body size: what keeps organisms small? *Q Rev Biol*, 385-407.
- Blackburn EH** (2005). Telomeres and telomerase: Their mechanisms of action and the effects of altering their functions. *FEBS Lett.* **579**,859–862.
- Bosch, J., & Vicens, N.** (2002). Body size as an estimator of production costs in a solitary bee. *Ecol Entomol*, **27**, 129-137.
- Bosch, J.** (2008). Production of undersized offspring in a solitary bee. *Anim Behav*, **75**, 809-816.
- Bosch, J., & Kemp, W. P.** (2001). How to manage the blue orchard bee as an orchard pollinator. *Sustainable Agriculture Network/National Agricultural Library*.

- Bozina, K.D.** (1961). How long does the queen live?. *Pchelovodstvo* **38**, 13.
- Bronikowski, A., & Vleck, D.** (2010). Metabolism, Body Size and Life Span: A Case Study in Evolutionarily Divergent Populations of the Garter Snake (*Thamnophis elegans*). *Integr Comp Biol*, **50**, 880–887.
- Buchwald, R. & Dudley, R.** (2010). Limits of vertical force and power production in bumblebees (Hymenoptera: *Bombus impatriens*). *J Exp Biol*, **213**, 426–432.
- Buckner, J.S., Kemp, W.P., Bosch, J.** (2004). Characterization of triacylglycerols from overwintering prepupae of the alfalfa pollinator *Megachilidae rotundata* (Hymenoptera: Megachilidae). *Arch Insect Biochem*, **57**, 1–14.
- Callier, Viviane, & Nijhout, F. H.** (2011). Control of body size by oxygen supply reveals size-dependent and size-independent mechanisms of molting and metamorphosis. *P Natl A Sci*. **108**, 14664-14669.
- Cane, J. H.** (1987). Estimation of bee size using intertegular span (Apoidea). *J Kansas Entomol Soc*, 145-147.
- Campisi, J.** (2001). Cellular senescence as a tumor-suppressor mechanism. *Trends Cell Biol*, **11(11)**, S27-S31.
- Campisi, J.** (2005). Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors. *Cell* **120**, 513–522
- Campisi, J., & di Fagagna, F. D. A.** (2007). Cellular senescence: when bad things happen to good cells. *Nat Rev Mol Cell Biol*, **8**, 729.
- Cattan, V., Mercier, N., Gardner, J.P., Regnault, V., Labat, C., Mäki-Jouppila, J., Nzietchueng, R., Benetos, A., Kimura, M., Aviv, A. and Lacolley, P.** (2008). Chronic

- oxidative stress induces a tissue-specific reduction in telomere length in CAST/Ei mice. *Free Radical Bio Med*, **44**, 1592-1598.
- Chown, S. L., Addo-Bediako, A., & Gaston, K. J.** (2002). Physiological variation in insects: large-scale patterns and their implications. *Comp Biochem Phys*, **131**, 587-602.
- Chown, S. L., Marais, E., Terblanche, J. S., Klok, C. J., Lighton, J. R. B., & Blackburn, T. M.** (2007). Scaling of insect metabolic rate is inconsistent with the nutrient supply network model. *Funct Ecol*, **21**, 282-290.
- Chown, S. L., & Gaston, K. J.** (2010). Body size variation in insects: a macroecological perspective. *Biol Rev*, **85**, 139-169.
- Cope, E. D.** (1896). The primary factors of organic evolution. *Open Court Publishing*, Chicago, IL
- D'Amico, L. J., Davidowitz, G., and Nijhout, H. F.** (2001). The developmental and physiological basis of body size evolution in an insect. *Proc. R. Soc. Lond. B*, **268**, 1589–1593.
- Davidowitz, G., D'Amico, L. J. & Nijhout, H. F.** (2004). The effects of environmental variation on a mechanism that controls insect body size. *Evol Ecol Res*, **6**, 49–62.
- de Oliveira, B. L.** (1960). Mudanças ontogenéticas em larvas de *Melipona Nigra* Schenck gribodo: (Hymenoptera-Apoidea).
- di Fagagna, F.D.A., Reaper, P.M., Clay-Farrace, L., Fiegler, H., Carr, P., Von Zglinicki, T., Saretzki, G., Carter, N.P. and Jackson, S.P.** (2003). A DNA damage checkpoint response in telomere-initiated senescence. *Nature*, **426**, 194.
- Dillon, M. E., & Dudley, R.** (2004). Allometry of maximum vertical force production during hovering flight of neotropical orchid bees (Apidae: Euglossini). *J Exp Biol*, **207**, 417-425.

- Dillon, M. E., Frazier, M.R. & Dudley, R.** (2006). Into thin air: physiology and evolution of alpine insects. *Integr Comp Biol*, **46**, 49-61
- Dudley, R.** (1998). Atmospheric oxygen, giant paleozoic insects and the evolution of aerial locomotor performance. *J Exp Biol*, **201**, 1043–1050.
- Dudley, R.** (2002). The biomechanics of insect flight: form, function, evolution. *Princeton University Press*
- Emlen, D. J.** (1997). Diet alters male horn allometry in the beetle *Onthophagus acuminatus* (Coleoptera: Scarabaeidae). *P R Soc Lond B Bio*, **264**, 567-574.
- Epel, E. S.** (2009). Psychological and metabolic stress: a recipe for accelerated cellular aging?. *Hormones*, **8**, 7-22.
- Fick, L., Fick, G., Li, Z., Cao, E., Bao, B., Heffelfinger, D., Parker, H. Ostrander, E. Riabowol, K.** (2012). Telomere Length Correlates with Life Span of Dog Breeds. *Cell Reports*, **2**, 1530–1536.
- Fischman, Brielle J., Theresa L. Pitts-Singer, and Gene E. Robinson.** (2017). Nutritional regulation of phenotypic plasticity in a solitary bee (Hymenoptera: Megachilidae). *Environ Entomol*, **46**, 1070-1079.
- Fischer, K. & Fiedler, K.** (2002). Reaction norms for age and size at maturity in response to temperature: a test of the compound interest hypothesis. *Evol Ecol* **16**, 333–349.
- Forsyth, N. R., Wright, W. E., & Shay, J. W.** (2002). Telomerase and differentiation in multicellular organisms: turn it off, turn it on, and turn it off again. *Differentiation*, **69**, 188-197.
- Freeman B.E.,** (1981). Parental investment, maternal size and population dynamics of a solitary wasp. *Am Nat* **117**, 357–362

- Frydrychová, R., Grossmann, P., Trubac, P., Vítková, M., & Marec, F. E. (2004).**
Phylogenetic distribution of TTAGG telomeric repeats in insects. *Genome*, **47**, 163-178.
- Fry, S. N., Sayaman, R., & Dickinson, M. H. (2003).** The aerodynamics of free-flight maneuvers in *Drosophila*. *Science*, **300**, 495-498.
- Gaston, K. J., Chown, S. L. & Evans, K. L. (2008).** Ecogeographic rules: elements of a synthesis. *J Biogeogr*, **35**, 483–50
- Giejdasz K. (1998).** The amount of pollen load collected and stored in brood cells by the female of *Osmia rufa* L. (Apoidea, Megachilidae). *Pszczelnicze Zeszyty Naukowe*, **42**, 33-34.
- Giraldo, Y. M., & Traniello, J. F. (2014).** Worker senescence and the sociobiology of aging in ants. *Behav Ecol Sociobiol*, **68**, 1901-1919.
- Glazier, D. S. (2005).** Beyond the ‘3/4-power law’: variation in the intra-and interspecific scaling of metabolic rate in animals. *Biol Rev*, **80**, 611-662.
- Gorbunova, V., & Seluanov, A. (2009).** Coevolution of telomerase activity and body mass in mammals: from mice to beavers. *Mech Ageing Dev*, **130**, 3-9.
- Graakjaer J, Der-Sarkissian H, Schmitz A et al. (2006).** Allele-specific relative telomere lengths and inheritance. *Hum Genet*, **119**, 344–350.
- Greenleaf, S. S., Williams, N. M., Winfree, R., & Kremen, C. (2007).** Bee foraging ranges and their relationship to body size. *Oecologia*, **15**, 589-596.
- Greenlee, K. J. & Harrison, J. F. (2004).** Development of respiratory function in the American locust *Schistocerca americana* I. Across-instar effects. *J Exp Biol*, **207**, 497–508.
- Greider, C.W. (1996).** Telomere length regulation. *Annual Rev. Biochem.* **65**, 337–365.
- Harrison, J. F., Wagner J. M., Aivazian V., Duell M. E., Klok C. J., Weed M., Munoz E., Fezzaa K., Socha J. J., and VandenBrooks J. M. (2020).** How to be a Giant:

- Hypermetric Scaling of the Leg in Cockroaches and Scarab Beetles Suggests Oxygen Transport to the Legs Limits Maximal Insect Size. *FASEB J*, **34**, 1-1.
- Harman D** (1956). Aging: a theory based on free radical and radiation chemistry. *J. Gerontol.* **11**, 298–300.
- Hayflick, L.** (1965). The limited in vitro lifetime of human diploid cell strains. *Exp. Cell Res*, **37**, 614-636.
- Hausmann, M. F., & Marchetto, N. M.** (2010). Telomeres: Linking stress and survival, ecology and evolution. *Curr Zool*, **56**.
- Helm, B. R., Rinehart, J. P., Yocum, G. D., Greenlee, K. J., & Bowsher, J. H.** (2017). Metamorphosis is induced by food absence rather than a critical weight in the solitary bee, *Osmia lignaria*. *P Natl A Sci*, **114**, 10924-10929.
- Hepburn, H.R., Radloff, S.E., Steele, G.R. & Brown, R.E.** (1998). Dimensional aspects of flight in the honey bees of Africa. *J. Apicult Res*, **37**, 147–154.
- Hone, D. W., & Benton, M. J.** (2005). The evolution of large size: how does Cope's Rule work? *Trends Ecol Evol*, **20**, 4-6.
- Houben JMJ, Moonen HJJ, Van Shooten FJ, Hageman GJ.** (2008). Telomere length assessment: biomarker of chronic oxidative stress? *Free Radical Bio Med*, **44**, 235–246.
- Huey, R. B., & Ward, P. D.** (2005). Hypoxia, global warming, and terrestrial Late Permian extinctions. *Science*, **308**, 398-401.
- Johnson M.D.,** (1988). The relationship of provision weight to adult weight and sex ratio in the solitary bee, *Ceratina calcarata*. *Ecol Entomol* **13**, 165–170.
- Karl, I., & Fischer, K.** (2008). Why get big in the cold? Towards a solution to a life-history puzzle. *Oecologia*, **155**, 215-225.

- Kim, J. Y.** (1997). Female size and fitness in the leaf-cutter bee *Megachile apicalis*. *Ecol Entomol*, **22**, 275-282.
- Kim, J. Y.** (1999). Influence of resource level on maternal investment in a leaf-cutter bee (Hymenoptera: Megachilidae). *Behav Ecol*, **10**, 552-556.
- Kirkwood, T.B.L.** (1977). Evolution of ageing. *Nature* **270**, 301–304.
- Klostermeyer, E. C., Mech Jr, S. J., & Rasmussen, W. B.** (1973). Sex and weight of *Megachile rotundata* (Hymenoptera: Megachilidae) progeny associated with provision weights. *J Kansas Entomol Soc*, 536-548.
- Klostermeyer EC, Gerber HS.** (1969). Nesting behavior of *Megachile rotundata* (Hymenoptera: Megachilidae) monitored with an event recorder. *Ann. Entomol. Soc. Am.* **62**, 1321–26
- Korandová, M., & Frydrychová, R. Č.** (2016). Activity of telomerase and telomeric length in *Apis mellifera*. *Chromosoma*, **125**, 405-411.
- Kraus, C., Pavard, S., and Promislow, D.E.L.** (2013). The size-life span trade-off decomposed: why large dogs die young. *Am. Nat.* **181**, 492–505.
- Kremen C.** (2005). Managing ecosystem services: what do we need to know about their ecology? *Ecol Lett* **8**, 468–479.
- Krtolica, A., Parrinello, S., Lockett, S., Desprez, P. Y., & Campisi, J.** (2001). Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. *P Natl A Sci*, **98**, 12072-12077.
- Ku, H. H., Brunk, U. T., & Sohal, R. S.** (1993). Relationship between mitochondrial superoxide and hydrogen peroxide production and longevity of mammalian species. *Free Radical Bio Med*, **15**, 621-627.

- Lee, H. Y., Lee, S. H., & Min, K. J.** (2015). Insects as a model system for aging studies. *Entomol res*, **45**, 1-8.
- Leroi, A. M., Koufopanou, V., & Burt, A.** (2003). Cancer selection. *Nat Rev Cancer*, **3**, 226-231.
- Li, S., Rosenberg, J. E., Donjacour, A. A., Botchkina, I. L., Hom, Y. K., Cunha, G. R., & Blackburn, E. H.** (2004). Rapid inhibition of cancer cell growth induced by lentiviral delivery and expression of mutant-template telomerase RNA and anti-telomerase short-interfering RNA. *Cancer res*, **64**, 4833-4840.
- Maeta Y, Adachi K.** (2005). Nesting behaviors of the alfalfa leaf-cutting bee, *Megachile (Eutricharaea) rotundata* (Fabricius) (Hymenoptera, Megachilidae). *Chugoku Kontyu* **18**, 5-21
- Marden, J.** (1994). From damselflies to pterosaurs: how burst and sustainable flight performance scale with size. *Am J Physiol.* **266**, R1077-R1084.
- Marden, J. H.** (1987). Maximum lift production during takeoff in flying animals. *Journal of experimental Biology*, **130**, 235-258.
- McFarlane, J. E.** (1967). Aging in an adult insect heart. *Can J Zool*, **45**, 1073-1081.
- Medawar, P.B.** (1952). *An Unsolved Problem of Biology*. Lewis, London.
- Michener, C. D.** (2000). *The bees of the world* (Vol. 1). JHU press, p.5
- Miller, P. L.** (1966). The supply of oxygen to the active flight muscles of some large beetles. *J Exp Biol*, **45**, 285-304.
- Miller, R., Harper, J., Galecki, A., Burke, D., & Miller, R.** (2002). Big mice die young: early life body weight predicts longevity in genetically heterogeneous mice. *Aging Cell*, **1**, 22-29.

- Miller, R. A.** (2004). ‘Accelerated aging’: a primrose path to insight? *Aging Cell*, **3**, 47-51.
- Monaghan, P.** (2010). Telomeres and life histories: the long and the short of it. *Ann NY Acad Sci*, **1206**, 130-142.
- Nijhout, H. F., Davidowitz, G. & Roff, D. A.** (2006). A quantitative analysis of the mechanism that controls body size in *Manduca sexta*. *Journal of Biology* **5**, 1–15.
- Niven, J. E., & Scharlemann, J. P.** (2005). Do insect metabolic rates at rest and during flight scale with body mass?. *Biol Letters*, **1**, 346-349.
- Nussey, D.H., Pemberton, J.M., Pilkington, J.G., and Blount, J.D.** (2009). Life history correlates of oxidative damage in a free-living mammal population. *Funct. Ecol.* **23**, 809–817
- Nunney L** (1999). Lineage selection and the evolution of multistage carcinogenesis. *Proc. Biol. Sci.* **266**, 493–498.
- Nylin, S. & Svard, L.** (1991). Latitudinal patterns in the size of European butterflies. *Holarctic Ecol* **14**, 192–202.
- O’Neill, K. M., Delphia, C. M., & O’Neill, R. P.** (2014). Oocyte size, egg index, and body lipid content in relation to body size in the solitary bee *Megachile rotundata*. *PeerJ*, **2**, 314.
- Pitts-Singer, T. L., & Cane, J. H.** (2011). The alfalfa leafcutting bee, *Megachile rotundata*: the world's most intensively managed solitary bee. *Annu rev entomol*, **56**, 221-237.
- Peters, R. H.** (1986). The ecological implications of body size. **2**. *Cambridge University Press*.
- Pettersen, A. K., Marshall, D. J., & White, C. R.** (2018). Understanding variation in metabolic rate. *J Exp Biol*, **221**, jeb166876.
- Prowse, K. R., & Greider, C. W.** (1995). Developmental and tissue-specific regulation of mouse telomerase and telomere length. *P Natl A Sci*, **92**, 4818-4822.

- Radmacher, S., & Strohm, E.** (2010). Factors affecting offspring body size in the solitary bee *Osmia bicornis* (Hymenoptera, Megachilidae). *Apidologie*, **41**, 169-177.
- Reeve, J. P., & Fairbairn, D. J.** (1996). Sexual size dimorphism as a correlated response to selection on body size: an empirical test of the quantitative genetic model. *Evolution*, 1927-1938.
- Reiss, M. J.** (1991). The allometry of growth and reproduction. Cambridge University Press.
- Risques, R. A., & Promislow, D. E.** (2018). All's well that ends well: why large species have short telomeres. *Philos T R Soc B*, **373**.
- Ritchie, M. E., & Olf, H.** (1999). Spatial scaling laws yield a synthetic theory of biodiversity. *Nature*, **400**, 557.
- Robertson, H. M., & Gordon, K. H.** (2006). Canonical TTAGG-repeat telomeres and telomerase in the honey bee, *Apis mellifera*. *Genome res*, **16**, 1345-1351.
- Roland J., Taylor PD.** (1997). Insect parasitoid species respond to forest structure at different spatial scales. *Nature* **386**, 710–713.
- Rubner M.** (1908). Das Problem der Lebensdauer und Seine Beziehungen Zu Wachstum und Ernahrung. R. Oldenburg, Muenchen, Germany. *Samartsev*.
- Sadowska, E. T., Baliga-Klimczyk, K., Labocha, M. K., & Koteja, P.** (2009). Genetic correlations in a wild rodent: grass-eaters and fast-growers evolve high basal metabolic rates. *Evolution: International Journal of Organic Evolution*, **63**, 1530-1539.
- Sahin E., Colla S., Liesa M. et al.** (2011). Telomere dysfunction induces metabolic and mitochondrial compromise. *Nature*, 470.
- Seidemann, K.** (2014). Optimal progeny body size in a solitary bee, *Osmia bicornis* (Apoidea: Megachilidae). *Ecol entomol*, **39**, 656-663

- Seidelmann, K.** (2018). Optimal resource allocation, maternal investment, and body size in a solitary bee, *Osmia bicornis*. *Entomol Exp Appl*, **166**, 790-799.
- Seidelmann, K., Ulbrich, K., & Mielenz, N.** (2010). Conditional sex allocation in the Red Mason bee, *Osmia rufa*. *Behav Ecol Sociobiol*, **64**, 337-347.
- Selman, C., Nussey, D., & Monaghan, P.** (2013). Ageing: It's a Dog's Life. *Curr Biol*, **23**, R451–R453.
- Seluanov A, Chen Z, Hine C, Sasahara TH, Ribeiro AA, Catania KC, Presgraves DC, Gorbunova V.** (2007). Telomerase activity coevolves with body mass not lifespan. *Aging Cell*, **6**, 45–52.
- Shear, W.A. & Kukalov' a-Peck, J.** (1990). The ecology of Palaeozoic terrestrial arthropods: the fossil evidence. *Can J Zool*, **68**, 1807–1834.
- Seluanov, Andrei, Zhuoxun Chen, Christopher Hine, Tais HC Sasahara, Antonio ACM Ribeiro, Kenneth C. Catania, Daven C. Presgraves, and Vera Gorbunova.** (2007). Telomerase activity coevolves with body mass not lifespan. *Aging cell* **6**, **1**, 45-52.
- Sgolastra, F., Kemp, W. P., Buckner, J. S., Pitts-Singer, T. L., Maini, S., & Bosch, J.** (2011). The long summer: pre-wintering temperatures affect metabolic expenditure and winter survival in a solitary bee. *J Insect Physiol*, **57**, 1651-1659.
- Sgolastra F., Hinarejos S., Pitts-Singer T., Boyle N., Joseph T., Lückmann J., Raine N., Singh R., Williams N., Bosch J.** (2019). Pesticide Exposure Assessment Paradigm for Solitary Bees. *Environ Entomol*, **48**, 22–35,
- Smit, B., & McKechnie, A. E.** (2010). Avian seasonal metabolic variation in a subtropical desert: basal metabolic rates are lower in winter than in summer. *Funct Ecol*, **24**, 330-339.

- Smith, F. A., & Lyons, S. K. (Eds.).** (2013). Animal body size: linking pattern and process across space, time, and taxonomic group. University of Chicago Press.
- Speakman, J. R., E. Krol, and M. S. Johnson.** (2004). The functional significance of individual variation in basal metabolic rate. *Physiol. Biochem. Zool.*, **77**, 900–915.
- Speakman, J. R.** (2005). Body size, energy metabolism and lifespan. *J Exp Biol*, **208**, 1717-1730.
- Strohm, E.** (2000). Factors affecting body size and fat content in a digger wasp. *Oecologica*, **123**, 184-191.
- Takai, H., Smogorzewska, A., & de Lange, T.** (2003). DNA damage foci at dysfunctional telomeres. *Curr Biol*, **13**, 1549-1556.
- Tepedino V. J., Torchio P.F.** (1989). The influence of nest-hole selection on sex ratio and progeny size in *Osmia lignaria propinqua* (Hymenoptera: Megachilidae). *Ann. Entomol. Soc. Am.* **82**, 355-360.
- Trostle, G., & Torchio, P. F.** (1994). Comparative nesting behavior and immature development of *Megachile rotundata* (Fabricius) and *Megachile apicalis* Spinola (Hymenoptera: Megachilidae). *J Kansas Entomol Soc*, 53-72.
- Weis-Fogh, T.** (1964). Diffusion in insect wing muscle, the most active tissue known. *J Exp Biol*, **41**, 229-256.
- West, G. B., Brown, J. H., & Enquist, B. J.** (1999). The fourth dimension of life: fractal geometry and allometric scaling of organisms. *Science*, **284**, 1677-1679.
- West, G. B., Brown, J. H., & Enquist, B. J.** (1997). A general model for the origin of allometric scaling laws in biology. *Science*, **276**, 122-126.
- Weng, N. P.** (2008). Telomere and adaptive immunity. *Mech ageing dev*, **129**, 60-66.

- Wigglesworth, V.B.** (1972). *The Principles of Insect Physiology*. London: Methuen
- Williams, G.C.** (1957). Pleiotropy, natural selection and the evolution of senescence. *Evolution* **11**, 398–411.
- West-Eberhard, M. J.** (1989). Phenotypic plasticity and the origins of diversity. *Annual review of Ecology and Systematics*, **20**, 249-278.
- Wilkaniec, Z., Giejdasz, K., & Fliszkiewicz, M.** (2004). The influence of food amount consumed during the larval development on the body weight of the imago of the red mason bee (*Osmia rufa* L., Megachilidae). *J. Apic. Sci*, **48**, 29-36.
- Wong KK, Maser RS, Bachoo RM et al.** (2003). Telomere dysfunction and Atm deficiency compromises organ homeostasis and accelerates ageing. *Nature*, **421**, 643–648.
- Wootton, R.J.& Kukalov'a-Peck, J.** (2000). Flight adaptations in Palaeozoic Palaeoptera (Insecta). *Biol Rev* **75**, 129–167
- Wright W.E., Shay J.W.** (2000). Telomere dynamics in cancer progression and prevention: fundamental differences in human and mouse telomere biology. *Nat. Med.* **6**, 849–851.

**CHAPTER 2: BODY SIZE ALLOMETRY IMPACTS FLIGHT RELATED
MORPHOLOGY AND METABOLIC RATES IN THE SOLITARY BEE MEGACHILE
ROTUNDATA¹**

Abstract

Body size is related to many aspects of life history, including foraging distance and pollination efficiency. In solitary bees, manipulating the amount of larval diet produces intraspecific differences in adult body size. The goal of this study was to determine how body size impacts metabolic rates, allometry, and flight-related morphometrics in the alfalfa leafcutting bee, *Megachile rotundata*. By restricting or providing excess food, we produced a range of body sizes, which allowed us to test the effect of body size on allometry, the power required for flight, and amount of energy produced, as measured indirectly through CO₂ emission. The power required during flight was predicted using the flight biomechanical formulas for wing loading and excess power index. We found larger bees had higher absolute metabolic rates at rest and during flight, but smaller bees had higher mass-specific metabolic rates at rest. During flight, bees did not have size-related differences in mass-specific metabolic rate. As bees increase in size, their thorax and abdomens become disproportionately larger, while their wings (area, and length) become disproportionately smaller. Smaller bees had more power available during flight as demonstrated by flight biomechanical formulas. Smaller body size was advantageous because of a reduced power requirement for flight with no metabolic cost.

¹ The material in this chapter was co-authored by Courtney C. Grula, Joseph P. Rinehart, Kendra J. Greenlee, and Julia H. Bowsher and is in review for the Journal of Insect Physiology in March 2021 under the title “Body size allometry impacts flight-related morphology and metabolic rates in the solitary bee *Megachile rotundata*” and adapted here with permission. Courtney C. Grula had primary responsibility for all of the methods described in this chapter including Metabolic Rate Analysis, and Allometric Scaling analysis. Courtney C. Grula drafted and revised all versions of this chapter. Julia H. Bowsher proofread the text written by Courtney C. Grula.

Introduction

Body size is a critical determinate of performance and fitness in insects. Body size in insects correlates with aspects of flight ability, including increased foraging distance (Greenleaf et al., 2007), predator avoidance, mating ability (Sugiura, 1994), ability to carry provisions (Buchwald and Dudley, 2010), and provisioning rates (Kim, 1997; Sugiura and Matea, 1989; Bosch and Vicens, 2006). Larger body size in solitary female bees is associated with higher rates of fecundity, production of larger oocytes (O'Neill et al., 2014), increased longevity (Sugiura and Maeta, 1989), greater overwintering survival (Tepedino and Torchio, 1989; Bosch and Vicens, 2006) and increased investment in each offspring (Kim, 1997). Despite these many advantages, larger body size also comes with costs such as higher energy requirements and reduced agility (Blanckenhorn, 2000). Thus, smaller body sizes may have an advantage for some performance measures by avoiding these costs.

As organisms grow and develop, all body parts may not grow proportionally. The scaling relationship between a morphological trait and the whole-body size of an organism is termed allometry (Huxley and Tessier, 1936). Adult body size in insects is a phenotypically plastic trait determined during the juvenile growth period (Koyama et al. 2013). Final adult size remains the same after the final molt occurs, because dimensions of most structures are fixed due to sclerotization of the cuticle (Nijhout et al., 2006). Nutritional conditions lead to differences in resource allocation, which can result in allometric trait growth. Examples of this are the exaggerated, hyperallometric growth of the fiddler crab claw (Huxley, 1924) and beetle horns (Emlen, 1997; Emlen, 1994; Emlen et al., 2007). When morphological traits grow in proportion to whole-body size, it is termed isometry.

Allometric trait growth occurs when insects differentially invest in body parts associated with flight morphology or fecundity. Development and maintenance of flight mechanisms is energetically costly, and this investment is thought to come at a cost in egg production. This pattern occurs in wing-dimorphic insects (Guerra, 2011; Mole and Zera, 1993; Roff, 1986; Roff and Bradford, 1996; Tanaka and Suzuki, 1998). When resources are low some insects allocate growth to flight, exhibited by an increase in thorax size (Saastamoinen et al. 2010), or wing size which lead to increased flight performance (Tigereos et al. 2013). Typically, when resources are high, insects devote more resources toward increased body size and fecundity (Forrest, 1987; Zera and Harshman, 2001).

In solitary bees, adult bee body size is largely determined by larval provisions of pollen and nectar, which can vary widely based on environmental, and maternal conditions (Klostermeyer et al., 1973). Variation in adult body size can be generated experimentally (Wilkaniec et al., 2004; Helm et al., 2017; Seidelmann, 2018; Fischman et al., 2017) and has been observed in the field (Bosch, 2008). In the solitary bee *Megachile rotundata* (F.), when resources are high, females invest more to produce female offspring, which are larger than males and require more provisions (Kim, 1999). Diet manipulations during the larval stage have been shown to produce a wide range of adult body sizes in *Osmia lignaria*, a bee in the same family (Megachilidae) (Helm et al., 2017). A wide range of body sizes within a bee species may have implications for performance, including metabolic rate.

Across animal species, body size is a strong predictor of metabolic rate, with larger individuals having higher metabolic rates. However, when corrected for body mass, known as mass-specific metabolic rate, the metabolic rate per gram of body tissue shows a negative trend, with larger organisms having a lower metabolic rate per gram (Rubner, 1908; Kleiber, 1947).

The “mouse-to-elephant curve” demonstrates this phenomenon, in which the large elephant has a lower mass-specific basal metabolic rate than the small mouse (Brody, 1945). This trend also exists in invertebrates (Altman and Dittimer, 1968; Schmidt-Nielsen, 1984), including insects (Reinhold, 1999, Lehmann et al., 2000, Niven and Scharlemann, 2005; Coelho and Moore, 1989) and specifically bees (Casey et al., 1985; Darveau, 2005). This trend extends intraspecifically, as metabolic rate increases with mass (Billardon and Darveau, 2019; Darveau et al. 2014; Skandalis and Darveau, 2012; Greenlee and Harrison 2005; Greenlee and Harrison 2004; Combes et al. 2020) and mass-specific metabolic rate shows a negative trend (Roberts et al. 2004).

For the solitary bee species *M. rotundata* previous studies have not shown a correlation between body size and mass-specific metabolic rate (Bennett et al., 2013, Abdelrahman et al., 2014; Owings et al., 2014), although this lack of correlation could be explained by the narrow range of body masses used in those studies, which had approximately a two-fold change in mass.

Patterns of scaling are even more important for flight metabolic rate, since flight metabolic rate is energetically costly (Dudley 2002). The majority of an insect’s energy allocation during flight is directed to flight muscles (Niven and Scharlemann, 2005). Flight metabolic rates in insects can vary dramatically within a species (Chown et al., 2007). However, intraspecific studies often use a narrow range of body sizes and do not represent body sizes at the extreme ends of the possible size range. Our study measured metabolic rates in bees that had a four-fold change in body size which is a larger range in body size than has previously been measured in the solitary bee *M. rotundata* (Bennett et al., 2013, Abdelrahman et al., 2014; Owings et al., 2014).

Flight, and thus metabolic rate, are important traits for solitary bees because offspring success depends on the resource acquisition by a single female bee (Bosch and Vicens, 2006).

Solitary bee females provision their offspring sequentially, building each offspring a brood cell and providing individual food provisions for each one. Females must collect and carry this material back to their nests, taking multiple foraging bouts to build and provision one brood cell (Pitts-Singer and Cane, 2011). *M. rotundata* uses leaf pieces to line and cap her brood cells (Pitts-Singer and Cane, 2011), and each nest requires around 120 leaf pieces, an average of 15 pieces per brood cell (Klostermeyer and Gerber, 1969; Royauté et al., 2018). Bees also carry nectar and pollen to provide nutrition for their developing offspring. Thus, an investment in the thorax as body size increases could influence reproductive fitness in solitary bees.

The weight of pollen loads carried by bees is positively correlated with body mass (Giejdasz, 1998; Ramalho et al., 1998; Goulson et al., 2002; Kerr et al., 2019) with larger females carrying heavier loads than their smaller conspecifics. The maximum load that an animal can lift during take-off flights is positively correlated with body mass (Marden, 1994). However, larger bees may be spending more of their available flight power to lift their mass and maintain hovering flight (Dillon and Dudley, 2004; Buchwald and Dudley, 2010; Seidelmann, 2014), leading to a reduction in power available for flight. Wing loading, which is body mass divided by wing area, is higher in larger species, and is linked to a higher metabolic cost (Casey 1976, Darveau et al., 2005; Skandalis and Darveau, 2012, Dillon, 2004). Body size also affects aerodynamics during flight. Larger individuals tend to have decreased flight maneuverability (Fry et al., 2003). Body mass and the mass of the load carried determines how effectively and efficiently an individual can fly. The thorax of insects is generally used for locomotion; it contains the legs and wings, and 95% of its mass is muscle tissue (Marden, 1987).

We manipulated provision quantity in *M. rotundata* (as previously done in Helm et al., 2017) to examine the effect of body size on metabolic rates, flight-related morphology, and

growth scaling in a large range of body sizes. We measured bees with a four-fold difference in body size for metabolic rate analysis, and a 12.75-fold change in body size for allometry analysis, a larger body-size range than previously measured in *M. rotundata*. During resting metabolic rate measurements, it was observed that bees were exhibiting discontinuous gas exchange. Therefore, we measured time spent in each phase, as well as CO₂ emission. We predicted that there would be a decreasing mass-specific metabolic rate with increasing body mass, matching the general pattern observed for insects and other animals (Rubner, 1908; Kleiber, 1947; Schmidt-Nielsen, 1984; Chown et al., 2007; Niven and Scharlemann, 2005). For bees to maximize their fitness, they must invest in flight, as well as fecundity, as body size increases. When resources are high, we predicted that thorax and abdomen size would increase hyperallometrically with body size. Within the parameters of our study, we define flight performance as the predicted flight ability based on wingbeat frequency, wing loading, excess power, and metabolic rate during tethered flight. We predicted large bees would have a lower wing beat frequency, decreased lift (higher wing loading), and less flight power (lower EPI) compared with smaller bees, as determined by the wing loading and excess power index.

Methods

Bee Body Size and Rearing. Nests containing larval bees were collected from a polystyrene nesting board (Megablock, Beaver Plastics, Canada) throughout the summer of 2017 in Fargo, ND (46°52'17" N 96°53'54" W; 930 ft elevation). Adult bees used for nesting were sourced from JWM Leafcutters, Inc. of Nampa, ID, US. Each brood cell was removed from its nesting straw and housed in a 96-well plate. The brood cell cap was removed to allow access to the larva and its provision. At the fifth instar larval stage, as described by Trostle and Torchio (1994), larval food quantity was manipulated to generate adults of different body sizes (Helm et

al., 2017). The feeding treatments were: starved (n=90), in which the entire provision was removed from the brood cell; control (n=83), in which the provision was not manipulated; and fed (n=109), in which bees were given approximately double the amount of their provision. Larvae in the fed treatment were provided with leftover provisions from bees placed in the starved treatment. Once assigned a treatment, bees, along with their brood cells, were placed into 3D printed plates (125mm x 82mm; height 20 mm) to provide sufficient room for growth. Each plate had 77 holes (well diameter =7mm and volume = 770mm³). Bees were incubated at 29°C in an environmental chamber to complete larval development then stored as prepupae in fluctuating overwintering temperatures (6°C for 21 hours 20°C for one hour - with one hour ramp up and one-hour ramp down cycle) beginning in October 2017. This fluctuating thermal regime increases survival and lowers thermal stress (Rinehart et al., 2013; Colinet et al., 2018). Bees were transferred to a 29°C environmental chamber in July 2018 to initiate development and adult emergence. Once adults emerged, a subsample was individually placed into separate deli cups with mesh lids and fed ad libitum on a 50% sucrose solution for 24 hours before measuring metabolic rates. Bees that were not used in metabolic rate experiments were stored at -80°C for subsequent morphometric measurements.

Respirometry. The effect of body size on metabolic rate during rest and flight was tested using flow-through respirometry as done previously (Bennett et al., 2013; Abdelrahman et al., 2014; Owings et al. 2014). We measured CO₂ emission as an indirect measure of metabolic rate for a subsample of bees in the starved (n=11), control (n=13), and fed (n=10) treatments. Incurrent air was dried and scrubbed of CO₂ using a purge gas generator (Balston, Haverhill, MA). Flow rate was controlled using mass flow controller (Model 840L-OV1-SV1-D-V1-S1, Sierra Instruments, Monterey, CA). Excurrent CO₂ was measured using a CO₂ analyzer (LI-7000

CO₂/H₂O Analyzer, LI-COR Biosciences, Lincoln, NE). Voltage outputs from the CO₂ analyzer and mass flow controller were digitized and recorded using Sable Systems hardware (UI2) and software (EXPEDATA version PRO, 1.9.22, Sable Systems). This setup was used for measuring both resting and flight metabolic rates, with different chambers used for each type of measurement. Metabolic rate was calculated by taking the mean parts per million CO₂ emission subtracted from the baseline average parts per million CO₂. This value was then multiplied by the flow rate (microliters per minute) and converted to micromoles using the ideal gas constant (22.4 μmol/μl).

Resting Metabolic Rate. Resting metabolic rate (μmolCO₂h⁻¹) of each bee was measured before flight trials. Adult bees inside their brood cells were placed in complete darkness in a small airtight glass chamber (~14 ml), and an activity monitor (AD-1 Activity Detector, Sable systems) was used during resting metabolic rate measurements. Air was pushed through the chamber at a flow rate of 250ml min⁻¹. CO₂ emission was recorded for 1 hour, and the middle 20 minutes of the recording was taken as resting metabolic rate, only complete cycles were measured. The bee's recorded resting absolute metabolic rate (μmolCO₂h⁻¹) was used for analysis. Mass-specific metabolic rate (μmolCO₂g⁻¹h⁻¹) was calculated by dividing the resting metabolic rate by mass (g).

During rest, it was observed that bees were exhibiting discontinuous gas exchange. We measured the three phases of discontinuous gas exchange: the closed phase, the flutter phase, and the open phase, based on descriptions by Lighton (1996). The closed phase occurs when the spiracles are closed and is indicated by a near zero CO₂ emission. The flutter phase is defined as when the spiracles begin to flutter as seen by a rapidly fluctuating CO₂ emission. The open phase occurs when the spiracles release a peak of accumulated CO₂ (Lighton, 1996). We measured

time spent in each phase, as well as amount of CO₂ produced during the open phase by measuring the area under the curve of resulting CO₂ peaks. Mass-specific metabolic rate was calculated by dividing the metabolic rate by mass (g).

Flight Metabolic Rate. Flight metabolic rate ($\mu\text{molCO}_2 \text{ h}^{-1}$) was measured using tethered flight as done previously (Bennett et al., 2013). Tethered flight was used because bees refused to fly freely in the respirometry chamber. Bees were chilled at 6°C for approximately 5 minutes, to facilitate attachment to the tether. Bees were tethered by looping a polyester string directly posterior to the head. This positioning ensured the tether would not interfere with the wings, legs or thorax during flight. Tethered bees were placed into an airtight glass jar attached to the lid (~550 ml). Air was pushed through the chamber at a flow rate of 375ml min⁻¹. Bees were placed on a 3D printed stage (length= 4 cm) within the chamber that allowed for the bees to become acclimated to the new environment. Bees were allowed to fly for the entire flight bout duration, with 30 seconds rest in between each flight bout to standardize the amount of rest bees got between bouts. Bees would only fly in the presence of a heat lamp (Hotspot, I2R, Cheltenham, PA), and temperature inside the chamber was recorded on a thermocouple (TC-2000 Thermocouple Meter, Sable systems). The average temperature with the heat lamp was 27.83°C \pm 0.2020 (min= 25.46°C, max=30.35°C). For reference, the room temperature was 25.920°C \pm 0.20 (min= 24.29°C, max= 29.873°C). Temperature did not significantly affect flight metabolic rate (p =0.959). Flight was initiated by removal of the stage from the tarsi by rotating it away from the bee, and CO₂ emissions were recorded for 30 minutes which included active flight bouts and non-flight resting periods. Each flight bout was marked at the start and end of the flight period on EXPEDATA software. The first 10 minutes were removed from the analysis to ensure the bee was acclimated to the chamber. Absolute metabolic rate was measured as the

average CO₂ emission of six flight bouts, during the beginning, middle and end of the 20-minute recording period. Bees were weighed after flight metabolic rate measurements to obtain their body mass (g).

Wing Beat Frequency. Wingbeat frequencies were recorded during tethered flight using a high-speed camera (Sony Cyber-shot Dsc-rx10iii digital camera). Three videos were recorded during the beginning, middle and end of the 30-minute sample period for each bee during active flight. The videos were replayed in slow motion to allow for counting individual wing beats. Three 10-second measurements from each video were analyzed to obtain a measurement of wingbeats per second. The original recording speed of the videos was 960 frames per second and were reduced to 29.97 frames per second to facilitate wing beat measurements. Wingbeat frequencies were then converted to hertz (Hz).

Morphometrics and Allometric Scaling. To evaluate the effect of body size on flight, we measured body mass (M), thorax mass (TM), wing area (S), wing length and intertegular span (IT span) of each bee. IT span is the distance between the tegulae on the dorsal side of the thorax and a common proxy for whole-bee body size (Cane, 1987). Bees were dissected into three segments: head, thorax and abdomen. All segments were dried at room temperature to a constant weight. The dry weight of the head, thorax and abdomen, as well as whole body weight, were used in analysis morphometric analysis for EPI, while wet weight was used in morphometric analysis for wing beat frequency, wing loading, and allometric analysis. Wings were dissected from the abdomen and mounted onto slides to measure wing area and wing length. Forewings were photographed under a microscope, and wing area was calculated with Image J (version 2) software. These measurements were used to determine how each body segment and wings scaled with overall body size. The relationship between two traits is

represented by the allometric equation, $y=ax^b$, where the exponent b is the slope of the regression and a is the intercept (Schmidt-Nielsen, 1984), and x and y represent the two traits being measured. The linear relationship can be found between two traits when the parameters are log transformed ($\log y=a\log x^b$), exponent b then becomes the slope of the linear function. When determining how metabolic rate (y) scales with body mass (x) the relationship is often represented as mass^b . When comparing two morphological traits of the same dimensions, the predicted slope if there is isometric growth is 1. Thus, for the head, thorax, and abdomen, the expected slope is 1. Predicted slopes change when comparing different dimensions. When comparing wing area and body size the expected slope is 0.667, and for wing length and IT span, the expected slope is 0.333 (Calder, 1996). These measurements were also used to calculate the wing loading (mg mm^{-2}) and excess power index (EPI) ($\text{mg}(\text{mg}^{-1})/(\text{mm}^2)^{-1}$). Wing loading measures the loaded weight the organism can carry with respect to its wing area (Marden, 1987).

$$\text{Wing loading} = M/S$$

The excess power index measures the maximum power production relative to the power required to maintain steady flight (Hepburn et al., 1998).

$$\text{EPI} = \sqrt{\frac{(TM/M)^2}{M/S}}$$

Statistical Analysis. All statistical analyses were performed using R statistical software (version 3.6.1, Base R package) and graphed using the package ggplot2 (Wickham, 2016). To determine the effect of feeding treatments (starved, control, fed) on adult body mass analysis of variance (2-way ANOVA) which included sex, and the interaction of sex and treatment, in the model. A pairwise comparison (Tukey post-hoc test) was used to determine which treatments were different from each other. Linear models were used to determine the correlation between body mass and CO₂ emission rate (absolute and mass-specific) at rest and during flight. All data

were log-transformed before analysis. Linear regression was also used to determine the correlation between body mass and time spent in the open, closed, and flutter phases of discontinuous gas exchange at rest, as well as absolute and mass-specific CO₂ emission during the open phase. Linear regression was also used to determine the correlation between body size and flight metrics (wing beat frequency, and wing loading). For EPI, values were normalized by using the lowest and highest values ($EPI = [i - \min(x)] / [\max(x) - \min(x)]$) to obtain a range from zero to one. A generalized linear model was used to test the relationship between body size and excess power index. Sex was included as a fixed effect in all the models. To determine scaling exponents of metabolic rates, EPI and wing loading, the data were log-transformed and fit to a linear relationship. To determine allometric scaling of the head, thorax, abdomen, wing length, wing area and IT span. An ANCOVA to test if the slopes of the treatments were different using the car package (Fox, 2019) in R. We used the stmatr package (Warton et al., 2012) to test if the relationship was isometric, testing for a predicted slope of 1 (head, thorax, abdomen), 0.667 (wing area), or 0.333 (wing length, IT span).

Results

Bee Body Size. Body masses ranged from 4 – 51mg. Females were typically larger, and weighed an average of 24.88 ± 1.06 mg. Males were typically smaller, and weighed an average of 21.85 ± 0.74 mg. Bees (both male and female) in the starved treatment had a combined average weight of 11.64 ± 0.57 mg, bees in the control treatment (both male and female) had a combined average weight of 24.30 ± 0.64 mg, and bees in the fed treatment (both male and female) had a combined average weight of 32.23 ± 0.69 mg. Feeding treatment, sex, and the interaction between feeding treatment and sex had significant effects on body size (ANOVA, Treatment $F_{2,276} = 310.09$, $p < 0.001$; Sex $F_{1,276} = 30.51$, $p < 0.001$; Interaction, $F_{2,276} = 8.95$, $p < 0.001$). Bees in

the fed treatment were significantly larger than the control bees (Tukey HSD, fed & control, $p < 0.001$), which were larger than bees in the starved treatment (Tukey HSD, control & starved, $p < 0.001$; fed & starved $p < 0.001$).

Metabolic Rates. For the subsample of bees used in respirometry experiments, the starved treatment had an average weight of 16.84 ± 1.55 mg, the control treatment had an average weight of 24.93 ± 1.947 mg, and the fed treatment had an average of weight of 28.38 ± 1.479 mg. For the subsample of bees, body size varied among the feeding treatments and sex (ANOVA, Treatment $F_{2,19}=6.666$; $p < 0.001$, Sex $F_{1,19}= 10.408$; $p= 0.005$, Interaction= $F_{2,19}=1.962$; $p=0.168$). Starved bees were smaller (Tukey HSD: starved vs control, $p = 0.027$; starved vs fed; $p = 0.005$), but the control and fed treatments did not differ (Tukey HSD: $p = 0.398$). Males were smaller than females. Mass had an effect on absolute metabolic rate during flight (Table 1, Fig. 1A; Linear Model, $T_{2,25} = 5.613$; $p < 0.000$) and at rest (Table 1, Fig. 1A; Linear Model, $T_{2,26} = 3.249$; $p = 0.003$). Metabolic rates increased with larger body size. Resting metabolic rate (slope= 0.603) and flight metabolic rate (slope= 1.080) scaled differently (ANCOVA, Mass, $F_{2,56}=-24.65$; $p < 0.0001$, Sex, $F_{2,56}= -1.04$; $p= 0.303$) based on mass. Mass did not affect mass-specific metabolic rate during flight (Table 1, Fig. 1B; Linear Model, $T_{2,25} = 0.419$; $p = 0.679$) but did affect metabolic rate at rest (Table 1, Fig. 1B; Linear Model, $T_{2,26} = -2.141$; $p = 0.042$), although it only explained 16.4% of the variation. This indicates that bees emitted more CO₂ as size increased, but once corrected for mass, larger bees did not have a higher flight metabolic rate.

Table 1. Metabolic rate (MR) in relationship to body size

	Model Term	Estimate	Std error	T value	Pvalue
Absolute Flight MR	Intercept	2.443	0.314	7.788	<0.001
	Mass	1.081	0.193	5.613	<0.001
	Sex	0.024	0.066	0.354	0.726
Absolute Resting MR	Intercept	1.005	0.312	3.221	0.003
	Mass	0.603	0.186	3.249	0.003
	Sex	-0.013	0.060	-0.215	0.832
Mass-Specific Flight MR	Intercept	2.443	0.314	7.788	<0.001
	Mass	0.081	0.193	0.419	0.679
	Sex	0.024	0.066	0.354	0.726
Mass-Specific Resting MR	Intercept	1.005	0.312	3.221	0.003
	Mass	-0.397	0.186	-2.141	0.042
	Sex	-0.013	0.059	-0.215	0.831

Results from Linear regression.

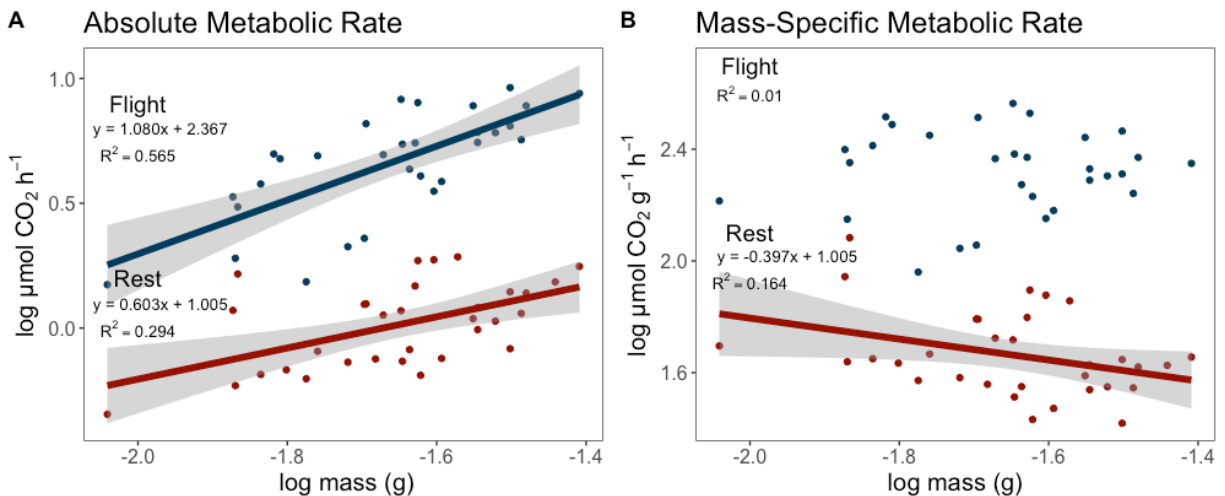


Fig. 1. Metabolic rates of *M. rotundata* during rest and flight. Absolute CO₂ emission (A) during flight (blue line), and at rest (red line). Mass-specific metabolic rate (B) during flight (blue) and at rest (red line). Absolute CO₂ emission (A) of bees during flight ($p < 0.001$) and at rest ($p < 0.003$) increased with body size. Mass-specific CO₂ emission (B) did not differ with body size during flight ($p = 0.679$), but at rest CO₂ emission decreased with body size ($p = 0.042$). Grey shading in all panels indicates the 95% confidence intervals.

Discontinuous Gas Exchange. Bees at rest exhibited discontinuous gas exchange (Fig. 2A). There was no difference in amount of time spent in the open phase (Fig. 2B; Linear Model, Mass, $T_{2,26} = -0.275$; $p = 0.786$; Sex, $T_{2,26} = -1.349$; $p = 0.189$), closed phase (Fig. 2C; Linear Model,

Mass, $T_{2,22} = 0.351$; $p=0.729$; Sex, $T_{2,22} = -0.477$; $p=0.639$) or the flutter phase (Fig. 2D; Linear Model, Mass, $T_{1,16} = 1.368$; $p=0.190$; Sex, $T_{1,16} = 0.176$; $p=0.869$) based on mass (g) or sex. As mass increased, the volume of CO₂ produced during the open phase increased (Fig. 2E; Linear Model, Mass, $T_{2,26} = 4.161$; $p=0.000$; Sex, $T_{2,26} = -0.855$; $p=0.400$). Once corrected for mass, there was no correlation of CO₂ emission with mass or sex (Fig. 2F; Linear Model, $T_{2,26} = -0.630$; $p=0.534$; Sex, $T_{2,26} = -0.855$, $p=0.400$). The slope for absolute resting metabolic rate (Fig. 1A, Table 1, slope = 0.603) was significantly different than the absolute metabolic rate measured during discontinuous gas exchange (Fig. 2E, slope= 0.869) (ANCOVA, $F_{1,61}=6.617$; $p=0.013$)

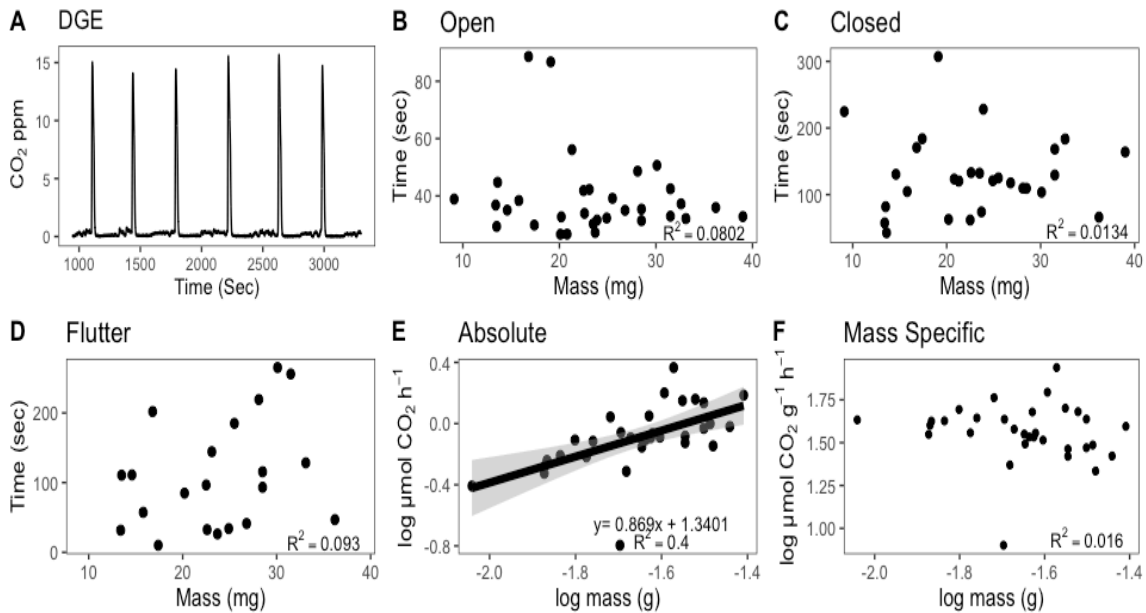


Fig. 2. The relationship between discontinuous gas exchange and body size. An example of a respirometry trace showing discontinuous gas exchange (DGE) including the closed, flutter and open phases (A). Body size does not correlate with the duration of time spent in the (B) open ($p=0.373$), (C) closed ($p=0.981$), or (D) flutter ($p=0.179$) phases of discontinuous gas exchange. Larger bees had a significantly higher CO₂ emission rate (E) ($p<0.001$) but once corrected for mass (F) there was no difference in amount of CO₂ produced ($p=0.496$). Grey shading indicates the 95% confidence intervals.

Allometric Scaling. As body mass increased, head mass (Table 2, Fig. 3A; $p<0.001$), thorax mass (Table 2, Fig. 3B; $p<0.001$) and abdomen mass (Table 2, Fig. 3C; $p<0.001$) increased. There was no difference between the slopes of the treatments (Table 2, head: $p=$

0.0680; thorax: $p = 0.492$; abdomen: $p = 0.740$), which indicates that all body parts increased with size in the same ratio across treatments. As body size increased, wing area (Table 2; Fig. 3D; $p < 0.001$), wing length (Table 2, Fig. 3E; $p < 0.001$), and IT span increased (Table 2, Fig. 3F; $p < 0.001$). The slopes of wing traits and body mass differed by treatment (Table 2, Figs 3D and E, wing area: $p = 0.003$; wing length: $p = 0.022$). There was no relationship between IT span and mass (Table 2, Fig 3F; $p = 0.090$). Sex was a significant factor for head mass (Table 2, ANCOVA $p < 0.001$), thorax mass (Table 2, ANCOVA $p < 0.001$), abdomen mass (Table 2, ANCOVA, $p < 0.001$), and wing area (Table 2, ANCOVA, $p < 0.003$).

We also determined if each body segment and wings scaled isometrically to overall body size by comparing the slopes of each body segment to the expected slope. First, we measured overall scaling patterns of all body segments (Fig. 3, Table 3). The head, thorax, and abdomen were predicted to scale with a reference slope of 1 in relationship to body mass. The head scaled hypometrically with body mass (Fig. 3A). The thorax and abdomen scaled hyperallometrically with body mass (Fig. 3 B and C). Wing area was predicted to scale with a 0.667 relationship to body mass, and wing length was predicted to scale with a 0.333 relationship to mass. Wing area and wing length scaled hypoallometrically with body mass (Fig. 3D and E). IT span was predicted to scale with a 0.333 relationship to mass. IT span scaled hyperallometrically with body mass (Fig 3F). Next, we measured scaling patterns with respect to treatment (Table 3). The head scaled isometrically with body size, with the exception of bees in the control treatment which scaled hypoallometrically with body size (Table 3). Across all treatments, the thorax and abdomen both scaled hyperallometrically with body size (Table 3). Thorax width, which was measured as IT span scaled isometrically in the fed treatment. However, the control and starved treatments had relationships that were hyperallometric (Table 3). Wing length had a significantly

more hypoallometric relationship with body size than expected in the fed and starved treatment but scaled hyperallometrically in the control treatment. Wing area was predicted to scale isometrically in the control and starved treatments, but the fed treatment was significantly more hypoallometric than predicted (Table 3). This suggests that as bees become larger their thoraces and abdomens get disproportionately larger, while wings become disproportionately smaller.

Table 2. Relationship of body segments to total body mass

	Model Term	Slope	Intercept	SS	DF	F value	P value
Head	Log body mass	0.907	-0.419	4.396	1	1019.948	< 0.001
	Treatment			0.023	2	2.7153	0.068
	Sex			0.104	1	24.170	< 0.001
Thorax	Log body mass	1.089	-0.515	6.335	1	1865.341	< 0.001
	Treatment			0.005	2	0.712	0.492
	Sex			0.095	1	27.858	< 0.001
Abdomen	Log body mass	1.202	-0.782	7.578	1	1182.857	< 0.001
	Treatment			0.004	2	0.301	0.740
	Sex			0.162	1	25.230	< 0.001
Wing Area	Log body mass	0.408	0.168	0.853	1	306.967	< 0.001
	Treatment			0.034	2	6.069	0.003
	Sex			0.025	1	9.120	0.003
Wing Length	Log body mass	0.205	0.417	0.205	1	243.440	< 0.001
	Treatment			0.007	2	3.8850	0.022
	Sex			0.003	1	2.9996	0.085
IT Span	Log body mass	0.309	-0.055	0.398	1	290.319	< 0.001
	Treatment			0.007	2	2.432	0.090
	Sex			0.002	1	1.644	0.201

Results from ANCOVA

Table 3. Allometric scaling relationships to body size

	Treatment	Slope	Lower Limit	Upper Limit	P value	Allometry	Reference Slope
Head	Total	0.919	0.887	0.953	<0.001	Hypoallometric	1
	Fed	0.965	0.887	1.050	0.407	Isometric	1
	Control	0.789	0.698	0.893	0.000	Hypoallometric	1
	Starved	1.072	0.973	1.182	0.158	Isometric	1
Thorax	Total	1.113	1.084	1.143	<0.001	Hyperallometric	1
	Fed	1.085	1.016	1.158	0.0160	Hyperallometric	1
	Control	1.168	1.089	1.252	<0.001	Hyperallometric	1
	Starved	1.252	1.158	1.354	<0.001	Hyperallometric	1
Abdomen	Total	1.247	1.207	1.288	<0.001	Hyperallometric	1
	Fed	1.282	1.195	1.375	<0.001	Hyperallometric	1
	Control	1.208	1.085	1.345	0.001	Hyperallometric	1
	Starved	1.346	1.218	1.487	<0.001	Hyperallometric	1
Wing Area	Total	0.524	0.498	0.552	<0.001	Hypoallometric	0.667
	Fed	0.479	0.424	0.543	<0.001	Hypoallometric	0.667
	Control	0.595	0.508	0.697	0.155	Isometric	0.667
	Starved	0.581	0.504	0.669	0.056	Isometric	0.667
Wing Length	Total	0.263	0.248	0.279	<0.001	Hypoallometric	0.333
	Fed	0.282	0.248	0.321	0.012	Hypoallometric	0.333
	Control	0.412	0.346	0.491	0.018	Hyperallometric	0.333
	Starved	0.242	0.210	0.278	<0.001	Hypoallometric	0.333
IT Span	Total	0.375	0.355	0.396	<0.001	Hyperallometric	0.333
	Fed	0.335	0.288	0.391	0.925	Isometric	0.333
	Control	0.466	0.396	0.549	<0.001	Hyperallometric	0.333
	Starved	0.414	0.359	0.479	0.003	Hyperallometric	0.333

Results from the test of isometry comparing the slopes of the head, thorax, and abdomen and treatment to a slope of 1, 0.667 in the case of wing area, and 0.333 for wing length and IT span using the ‘smatr’ package in R.

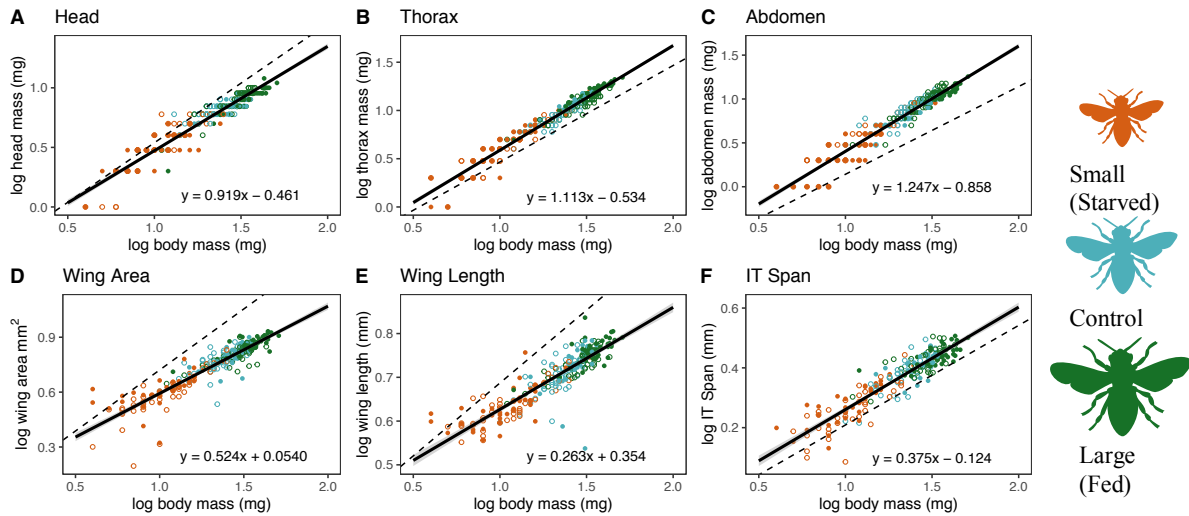


Fig. 3. Scaling relationship between body size, body segments and wings
Allometric scaling relationships for head mass (A), thorax mass (B), abdomen mass (C), wing area (D), wing length (E), and IT span (F). Open circles represent males, and closed circles represent females. The predicted isometric slope is represented by the dashed line. Predicted slope for head mass, thorax mass, and abdomen mass (A-C) is 1, predicted slope for wing area (D) is 0.667, and the predicted slope for wing length and IT span (E & F) is 0.333. Head mass scaled isometrically with body mass. Thorax mass, abdomen mass, and IT span scaled hyperallometrically with body mass. Wing area and wing length scale hypoallometrically with body mass.

Wing Beat Frequency and Flight Morphometrics. Mass and sex did not have an effect on the number of wing beats (Fig. 4A; Linear Model, Mass, $T_{2,23}=0.070$; $p= .945$, Sex, $T_{2,23} = 0.687$; $p = 0.499$). Mass and sex have a significant effect on wing loading (Fig. 4B; Linear Model, Mass, $T_{2,210}= 19.402$; $p < 0.0001$; Sex, $T_{2,210}= 2.375$; $p=0.0184$) and EPI (Fig. 4C; Generalized Linear Model, Mass, $T_{7,210}= -12.978$; $p < 0.0001$; Sex, $T_{7,210}= -3.573$; $p=0.000$). Larger bees had higher wing loading, but lower EPI. Wing loading scaled with $\text{mass}^{0.515}$, and EPI scaled with $\text{mass}^{-0.474}$.

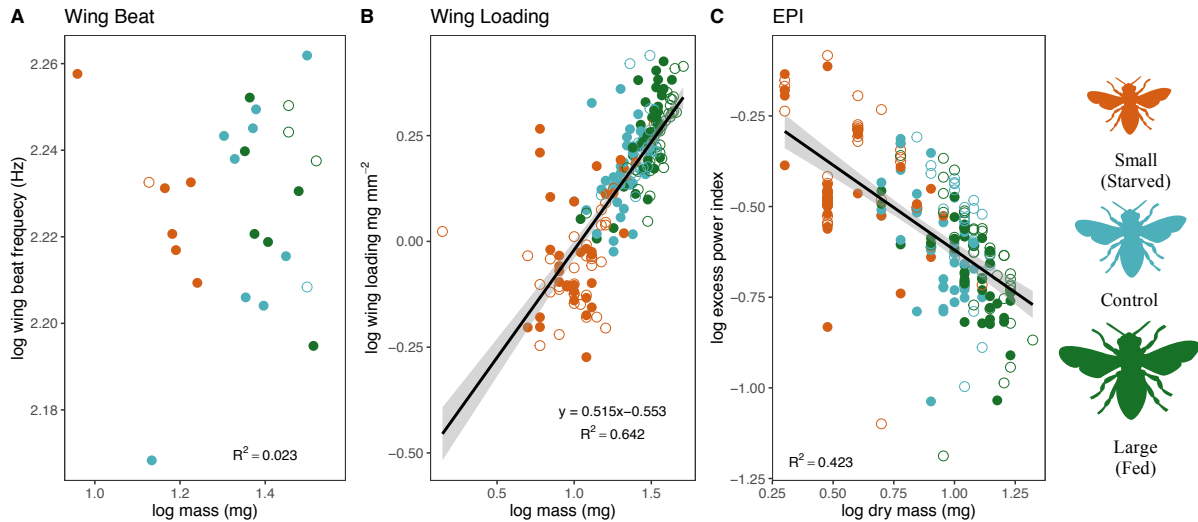


Fig. 4. Flight performance and body size

There is no correlation between body size and wing beat frequency (Hz) ($p = 0.070$) (A) Smaller bees had a lower wing loading ($p < 0.001$) (B) and higher excess power index ($p < 0.0001$) (C) than larger bees. Mass is shown as body mass (mg) for C. Grey shading in all panels indicates the 95% confidence intervals). Open circles represent males, and closed circles represent females.

Discussion

Across insect species, as body size increases, metabolic rate increases (Coelho and Moore, 1989; Bartholomew and Casey, 1977; Darveau et al., 2005; Fielding and DeFoliart, 2008), and there is a negative correlation between body size and mass-specific metabolic rate and body size (Reinhold, 1999, Casey and May, 1985). In contrast, we found that although larger bees had a higher absolute metabolic rate, once corrected for mass there was a correlation between metabolic rate and body size at rest and no correlation during flight. Previous work in *M. rotundata* found the same trend across a two-fold range of body sizes (Bennett et al., 2013) and our study found this trend across a four-fold range of body sizes, which is a larger range of body sizes than previously seen in studies looking at metabolic rate in *M. rotundata*. This suggests both large and small bees are using the same amount of energy per gram during flight, but at rest small bees expend more energy per gram. Other studies on intraspecific metabolic

rates have also found no differences in mass-specific metabolic rates based on body size (Darveau et al., 2014; Darveau et al., 2005; Dillon and Dudley, 2004; Bennett et al., 2013), but intraspecific correlations between body size and absolute metabolic rate have been found (Billardon and Darveau, 2019; Darveau et al. 2014; Skandalis and Darveau, 2012).

Interspecific scaling exponents of flight metabolic rate range from 0.63-0.94 in insects (Harrison and Roberts, 2000). Intraspecific trends in some insect species have scaling exponents higher than 1 (Chappell and Morgan, 1987; Morgan et al., 1985; Harrison and Roberts, 2000). We calculated a scaling exponent of $\text{mass}^{1.080}$ for absolute flight metabolic rate, higher than previously seen in bees (Euglossine bees, 0.64; Casey, 1985) (Euglossine bees 0.69; Darveau et al., 2005) (Honeybees, 0.63; Wolf et al.1989) (*M. rotundata*, 0.92; Bennett et al., 2013) (Worker bumblebees, 0.829; Billardon and Darveau 2019), although a scaling exponent above 1 has been documented in worker bumblebees (1.35; Darveau et al., 2014). Differences in metabolic rate scaling in our study may be due to use of tethering or short flight bouts. Bees were tethered to measure flight metabolic rates, because they would not fly freely in the metabolic chamber. Tethered flight has been successfully used to measure flight metabolic rate in other instances (Brodscheider et al. 2009; Bennett et al. 2013). Bees flew for less than 30 seconds per flight bout which may be a consequence of the environment of the room in which metabolic rates were measured, including artificial light. However, the wing beat frequencies were similar to other insects in the order Hymenoptera, which range from 87–230 Hz (Tercel et al., 2018). The range of wingbeats found in our study was 147.3-182.7 Hz, suggesting that bees were exhibiting flight behavior, supporting our use of tethered flight.

Resting metabolic rate in insects scales as $\text{mass}^{0.75}$, once corrected for phylogeny (Harrison and Roberts, 2000). Intraspecific scaling exponents are more variable and typically

range from 0.67 to 1.0 (Chown et al 2007, Glazier, 2005). Our results show a scaling exponent of $\text{mass}^{0.603}$ at rest. Differences in scaling exponents are likely due to physiological, taxonomic, or environmental factors (Glazier 2005). Differences in resting metabolic rate within and between species may be due to body size (Speakman et al., 2004; Chown et al., 2007), growth rates (Sadowska et al., 2009), and availability of food resources (Sadowska et al., 2009).

Metabolic scaling of bees at rest and during flight differed. In our study, the scaling exponent for flight metabolic rate was $\text{mass}^{1.080}$, and the scaling exponent for resting metabolic rate was $\text{mass}^{0.603}$. Our results are similar to data collected for a meta-analysis (Niven and Scharlemann, 2005), which found flight metabolic rate in insects scaled with body mass^{1.10} and resting metabolic rates scaled with body mass^{0.66}. Differences in metabolic scaling may be due to how the insect partitions its metabolic energy. During flight, most of the insect's metabolism will be consumed by flight muscles (Dudley 2000), while metabolism at rest is allocated to function and maintenance (Weibel, 2002; Glazier, 2005).

At rest, adult bees exhibited discontinuous gas exchange (DGE). We measured the amount of time spent in each phase (closed, open, flutter), as well as the amount of CO₂ produced during the open phase. Patterns of cyclic gas exchange in which spiracles do not entirely close have been observed during the pupal (Yocum et al., 2011) and adult stages in *M. rotundata* (Bennett et al., 2013). We found that there was no correlation between body size and the amount of time spent in each phase. Yocum et al. (2011) showed similar results in pupal *M. rotundata*, where mass had no effect on respiratory patterns, although, that study measured CO₂ emission patterns differently than our study. Larger bees produced more CO₂ during the open phase, but once corrected for mass, there was no correlation between body size and CO₂ production. Studies have found no correlation between body mass and the frequency and

duration of the phases of discontinuous gas exchange (Davis et al., 1999, Chappell and Rogowitz, 2000), although a meta-analysis by Terblanche et al. (2008) found the frequency of the phases in discontinuous gas exchange decreases with mass across species. We found the slope for total absolute resting metabolic rate was different than absolute resting metabolic rate measured just during the open phase. This may be due to the influence of the flutter phase, which was included in the analysis of the total absolute resting metabolic rate.

We also analyzed the scaling relationships between body segments and wings to determine whether the relationships were proportional as body size increased. Our study measured bees that had a 12.75-fold change in body size. As bees got larger their thorax and abdomen were larger than expected based on body size. Head size scaled isometrically with body size. IT span, forewing length, and wing area, in the fed treatment, were smaller than expected based on body size. As bees became larger, their thoraces grew proportionately larger, while their wings grew proportionately smaller. There was also a sex effect in which females had larger heads, thoraces, abdomens, and wing area than similarly-sized males. Resource allocation to somatic or reproductive growth can lead to differences in the size of each body segment (Nijhout and Wheeler, 1996). The hyperallometric scaling of the abdomen may reflect an increased investment in reproduction as body size increases. While these scaling relationships were statistically significant, the slopes and confidence intervals for the thorax and abdomen are very close to 1 and may not be biologically significantly different than 1 for the range of body sizes in this study.

Our study showed that wings grew smaller than expected in response to body size. Differential scaling of wings may occur because final wing size is determined by imaginal disc growth, which is separate but interrelated to overall body growth (Nijhout and Callier, 2015).

Wings complete growth after the body has stopped growing, potentially leading to allometric response to size. In response to starvation, in *Manduca sexta* (L.) and *Drosophila melanogaster* (M.), wings reduced proportionally to body size (Nijhout and Grunert, 2010; Shingleton et al., 2008). Here, we saw a different overall pattern where wings grew smaller than expected in response to body size.

The hormonal regulation of wing growth throughout development has been well documented in insects that have a critical weight as a cue for metamorphosis, such as *M. sexta*, and *D. melanogaster*. This cue is an important regulator for imaginal disc growth. Once the larval insect reaches the critical size, a hormonal cascade is initiated influencing imaginal disc growth. Models describing wing imaginal disc growth, and final size are based upon a critical weight (Shingleton et al., 2008). However, there is a different growth dynamic in solitary bees, which may explain the hypoallometric scaling of wing size in bees. Solitary bees do not have a critical weight, but rather rely on the absence of food as the cue for metamorphosis (Helm et al. 2017). The reliance on nutrition as a cue for development, which is not the case for insects such as *D. melanogaster* (De Moed et al., 1999), may be an explanation for the different wing scaling pattern seen in this study.

We found no correlation between body size and wingbeat frequency in *M. rotundata*. For most insects, wing beat frequency varies with body size (Dudley, 2000), with a negative correlation between body size and wingbeat frequency (Tercel et al., 2018; Casey et al., 1985; Darveau et al., 2005). Smaller individuals have a higher wing beat frequency to control navigation through the higher experienced air viscosity (Ellington, 1999; Wang, 2005). In *M. rotundata*, larger bees did not have a lower wing beat frequency, suggesting there is no size compensation based on number of wing beats. There was no correlation between wing beat

frequency and body size, as well as between mass-specific flight metabolic rate and body size. This result may be because the number of times an insect beats its wings influences flight metabolic rate, with metabolic rate increasing with number of wing beats (Casey et al., 1985; Darveau et al., 2005; Billardon and Darveau 2019). Wing beat frequency and wing loading are often positively correlated, insects with a higher wing loading typically have a higher wing beat frequency (Byrne et al., 1988).

In this study, larger bees had higher wing loading and a lower excess power index. Smaller individuals with lower wing loading have the capacity to carry heavier loads per gram of body mass (Marden, 1987) which may decrease cost of flight. Higher wing loading, as we observed in larger *M. rotundata*, indicates that more power is needed to accomplish the same lift as a smaller bee (Marden, 1994). This may be because larger bees in our study have smaller wings than expected based on their mass. Other studies have also observed lower wing loading in smaller individuals (Darveau et al., 2005) and EPI is higher (Seidelmann, 2014). Wing loading, EPI, and wing beat frequency impact how much female bees can carry when building a nest and provisioning offspring. Overall, we observed that smaller bees have more power available to fly beyond hovering flight, and larger bees may be using most of their available flight power to maintain hovering flight.

While being large may have its advantages in some aspects of flight, the advantages to small body size have often been overlooked. Although a larger insect may have increased foraging distance (Greenleaf et al., 2007), smaller individuals may have better performance by other metrics. We found smaller bees had more power available during flight at no apparent metabolic cost. Because there were no differences in mass-specific metabolic rates during flight, it appears that these bees are equally energetically efficient. The smaller bees required less power

during flight, and as bees decreased in size their power requirements for lifting loads also decreased. This study demonstrates that smaller bees may not be at a disadvantage in all aspects of flight performance and may even be at an advantage. Other advantages to small size include increased agility and reductions in costs of development, maintenance, heat stress, and reproduction (Chown and Gatsos, 2010; Blanckenhorn, 2000). When resources are scarce, female solitary bees provide smaller provisions to offspring, leading to the production of “tiny” female offspring (Kim and Thorp, 2001; Bosch, 2008). General decreases in foraging habitat may lead to bee populations with overall smaller body sizes (Renauld et al., 2016). With concern for pollinator habitat loss, which would decrease resource availability, more research focusing on the potential performance outcomes of smaller body sizes is needed.

Conclusion

The goal of our study was to determine how body size affects allometry, metabolic rate, and flight related morphology in a single species, *M. rotundata*. We produced a large range of body sizes by manipulating food quantity to influence adult body size. By using respirometry to measure excurrent CO₂, we measured both resting and flight metabolic rates for a range of bee body sizes. Our results indicate larger bees have higher absolute metabolic rates at rest and during flight. Smaller bees have higher mass-specific metabolic rates at rest, but mass did not impact mass-specific metabolic rate during flight. Differences in nutritional condition led to allometric growth of the body segments (head, thorax, abdomen, IT span) and wings (area and length). As body size increases the thorax and abdomens of bees become disproportionately larger, while their wings become disproportionately smaller compared to mass. These differences in allometry influenced flight-related morphometrics. Larger bees had wings that were smaller than expected based on body size, which may have contributed to these larger bees having less

power available during flight based on flight biomechanical formulas. Our study and others contradict the assumption that performance only increases with body size, or bigger is always better. Instead, smaller bees are not at a disadvantage when it comes to flight performance.

Acknowledgements

We would like to thank George Yocum, Jacob Campbell, and Arun Rajamohan for their help with data analysis. We would like to thank Dev Patel and Josh Rinehart for their help with data collection. We would like to thank Elisabeth Wilson for their help with intellectual feedback on this manuscript. We would like to thank Micki Palmersheim for creating the artwork in the graphical abstract. We would like to thank Charles Darveau for comments on the manuscript. We would also like to thank everyone at the ICE Network in Fargo, ND for their feedback and support with this project. This work was supported by the NSF-IOS-1557040 to JHB, JPR, KJG, and NSF-RII Track-2 FEC 1826835 to JHB, KJG, and JPR. This work was also supported by the Department of Biological Sciences at NDSU and the USDA Agricultural Research Service.

References

Abdelrahman, H., Rinehart, J.P., Yocum, G.D., Greenlee, K.J., Helm, B.R., Kemp, W.P.,

Schulz, C.H. and Bowsher, J.H. (2014). Extended hypoxia in the alfalfa leafcutting bee, *Megachile rotundata*, increases survival but causes sub-lethal effects. *J. of Insect Physiol.* **64**, 81-89.

Altman, P. L., and Dittmer, D. S. (1968). Biological handbooks: metabolism. *Bethesda:*

Federation of American Societies for Experimental Biology, 347.

Bartholomew, G.A., and Casey, T.M. (1978). Oxygen consumption of moths during rest, pre-flight warm-up, and flight in relation to body size and wing morphology. *J. Exp. Biol.* **76**, 11-25.

- Bennett, M. M., Petersen, K., Yocum, G., Rinehart, J., Kemp, W. and Greenlee, K. J.** (2013). Effects of extended prepupal storage duration on adult flight physiology of the alfalfa leafcutting bee (Hymenoptera: Megachilidae). *J. Econ. Entomol.* **106**, 1089-1097.
- Billardon, F., and Darveau, C. A.** (2019). Flight energetics, caste dimorphism and scaling properties in the bumblebee, *Bombus impatiens*. *J. Exp. Biol.* **222**.
- Blanckenhorn, W. U.** (2000). The evolution of body size: what keeps organisms small? *The Q. Rev. Biol.* **75**, 385-407
- Bosch, J.** (2008). Production of undersized offspring in a solitary bee. *Anim. Behav.* **75**, 809-816.
- Bosch, J. and Vicens, N.** (2006). Relationship between body size, provisioning rate, longevity and reproductive success in females of the solitary bee *Osmia cornuta*. *Behav. Ecol. Sociobiol.* **60**, 26-33.
- Brody, S.** (1945). *Bioenergetics and Growth* Reinhold Publ. Co., New York, 265-266.
- Brodshneider, R., Riessberger-Gallé, U., and Crailsheim, K.,** (2009). Flight performance in artificially reared honeybees (*Apis mellifera*). *Apidologie.* **40**, 441-449.
- Buchwald, R. and Dudley, R.** (2010). Limits to vertical force and power production in bumblebees (Hymenoptera: *Bombus impatiens*). *J. Exp. Biol.* **213**, 426-432.
- Byrne, D. N., Buchmann, S. L., and Spangler, H. G.,** (1988). Relationship between wing loading, wingbeat frequency and body mass in homopterous insects. *J. Exp. Biol.* **135**, 9-23.
- Calder, W. A.,** (1996). *Size, function, and life history.* Courier Corporation.
- Cane, J. H.,** (1987). Estimation of bee size using intertegular span Apoidea. *J Kansas Entomol Soc.* 145-147.

- Casey T.M.**, (1976). Flight energetics in sphinx moths: heat production and heat loss in *Hyles lineata* during free flight. *J. Exp. Biol.* **64**, 545-560.
- Casey, T.M., May, M.L. and Morgan, K.R.** (1985). Flight energetics of euglossine bees in relation to morphology and wing stroke frequency. *J. Exp. Biol.* **116**, 271-289.
- Chappell M.A., and Morgan K.R.** (1987). Temperature regulation, endothermy, resting metabolism, and flight energetics of tachinid flies (*Nowickia*, sp.). *Physiol. Zool.* **60**, 550–59
- Chappell, M. A. and Rogowitz, G. L.** (2000). Mass, temperature and metabolic effects on discontinuous gas exchange cycles in eucalyptus-boring beetles (Coleoptera: Cerambycidae). *J. Exp. Biol.* **203**, 3809-3820.
- Chown, S. L., Marais, E., Terblanche, J. S., Klok, C. J., Lighton, J. R. B. and Blackburn, T. M.** (2007). Scaling of insect metabolic rate is inconsistent with the nutrient supply network model. *Funct. Ecol.* **21**, 282-290.
- Chown, S. L. and Gaston, K. J.** (2010). Body size variation in insects: a macroecological perspective. *Biol. Rev.* **85**, 139-169.
- Coelho, J.R. and Moore, A.J.** (1989). Allometry of resting metabolic rate in cockroaches. *Comp. Biochem. Physiol.* **94**, 587-590.
- Colinet, H., Rinehart, J. P., Yocum, G. D. and Greenlee, K. J.** (2018). Mechanisms underpinning the beneficial effects of fluctuating thermal regimes in insect cold tolerance. *J. Exp. Biol.* **221**.
- Combes, S. A., Gagliardi, S. F., Switzer, C. M., and Dillon, M. E.** (2020). Kinematic flexibility allows bumblebees to increase energetic efficiency when carrying heavy loads. *Science advances*, **6**.

- Darveau, C.A., Hochachka, P.W., Welch, K.C., Roubik, D.W. and Suarez, R.K. (2005).**
Allometric scaling of flight energetics in Panamanian orchid bees: a comparative phylogenetic approach. *J. Exp. Biol.* **208**, 3581-3591.
- Darveau, C.A., Billardon, F. and Bélanger, K. (2014).** Intraspecific variation in flight metabolic rate in the bumblebee *Bombus impatiens*: repeatability and functional determinants in workers and drones. *J. Exp. Biol.* **217**, 536-544.
- Darveau, C. A., Hochachka, P. W., Welch, K. C., Roubik, D. W., and Suarez, R. K. (2005).**
Allometric scaling of flight energetics in Panamanian orchid bees: a comparative phylogenetic approach. *J. Exp. Biol.* **208**, 3581-3591.
- Davis, A. L., Chown, S. L. and Scholtz, C. H. (1999).** Discontinuous gas-exchange cycles in *Scarabaeus dung* beetles (Coleoptera: Scarabaeidae): mass-scaling and temperature dependence. *Physiol. Biochem. Zool.* **72**, 555-565.
- De Moed, G. H., Kruitwagen, C. L. J. J., De Jong, G., and Scharloo, W. (1999).** Critical weight for the induction of pupariation in *Drosophila melanogaster*: genetic and environmental variation. *J. Evol. Biol.*, **12**, 852-858.
- Dillon, M. E. and Dudley, R. (2004).** Allometry of maximum vertical force production during hovering flight of neotropical orchid bees (Apidae: Euglossini). *J. Exp. Biol.* **207**, 417-425.
- Dudley, R. (2002).** The biomechanics of insect flight: form, function, evolution. Princeton, New Jersey, Princeton University Press.
- Ellington, C.P. (1999).** The novel aerodynamics of insect flight: applications to micro-air vehicles. *J. Exp. Biol.* **202**, 3439–3448

- Emlen, D. J.** (1997). Diet alters male horn allometry in the beetle *Onthophagus acuminatus* (Coleoptera: Scarabaeidae). *P. Roy. Soc. Lond. B. Bio.* **264**, 567-574.
- Emlen, D. J., Lavine, L. C., and Ewen-Campen, B.** (2007). On the origin and evolutionary diversification of beetle horns. *P. Natl. Acad. Sci. USA.* **104**, 8661-8668.
- Emlen, D. J.** (1994). Environmental control of horn length dimorphism in the beetle *Onthophagus acuminatus* (Coleoptera:Scarabaeidae). *Proc. R. Soc. Lond. B.* **256**, 131–136
- Fielding, D.J. and DeFoliart, L.S.** (2008). Relationship of metabolic rate to body size in Orthoptera. *J. Orthoptera Res.* **17**, 301-307.
- Fischman, B. J., Pitts-Singer, T. L. and Robinson, G. E.** (2017). Nutritional regulation of phenotypic plasticity in a solitary bee (Hymenoptera: Megachilidae). *Environ. Entomol.* **46**, 1070-1079.
- Forrest, T.G.,** (1987). Insect size tactics and developmental strategies. *Oecologia.* **73**, 178–184.
- Fox J., and Weisberg S.** (2019). *An R Companion to Applied Regression*, Third edition. Sage, Thousand Oaks CA.
- Fry, S. N., Sayaman, R. and Dickinson, M. H.** (2003). The aerodynamics of free-flight maneuvers in *Drosophila*. *Science.* **300**, 495-498.
- Giejdasz, K.** (1998). The amount of pollen load collected and stored in the brood cells by the female of *Osmia rufa* L.(Apoidea, Megachilidae). *Pszczelnicze Zeszyty Naukowe*, **2**.
- Glazier, D. S.** (2005). Beyond the ‘3/4-power law’: variation in the intra-and interspecific scaling of metabolic rate in animals. *Biol rev.* **80**, 611-662.

- Goulson D., Peat J., Stout J.C., Tucker J., Darvill B., Derwent L.C., and Hughes W.O.H.** (2002). Can alloethism in workers of the bumblebee, *Bombus terrestris*, be explained in terms of foraging efficiency? *Anim. Behav.* **64**, 123-130.
- Greenleaf, S. S., Williams, N. M., Winfree, R. and Kremen, C.** (2007). Bee foraging ranges and their relationship to body size. *Oecologia.* **153**, 589-596.
- Greenlee, K. J., and Harrison, J. F.** (2004). Development of respiratory function in the American locust *Schistocerca americana* I. Across-instar effects. *J. Exp. Biol.*, **207**, 497-508.
- Greenlee, K. J., and Harrison, J. F.** (2005). Respiratory changes throughout ontogeny in the tobacco hornworm caterpillar, *Manduca sexta*. *J. Exp. Biol.*, **208**, 1385-1392.
- Guerra, P.A.,** (2011). Evaluating the life-history trade-off between dispersal capability and reproduction in wing dimorphic insects: a meta-analysis. *Biol. Rev.* **86**, 813–835.
- Harrison, J.F. and Roberts, S.P.** (2000). Flight respiration and energetics. *Annu. Rev. Physiol.* **62**, 179-205.
- Helm, B. R., Rinehart, J. P., Yocum, G. D., Greenlee, K. J. and Bowsher, J. H.** (2017). Metamorphosis is induced by food absence rather than a critical weight in the solitary bee, *Osmia lignaria*. *P. Natl. Acad. Sci.* **114**, 10924-10929.
- Hepburn, H. R., Radloff, S. E., Steele, G. R. and Brown, R. E.** (1998). Dimensional aspects of flight in the honey bees of Africa. *J. Apicult. Res.* **37**, 147-154.
- Huxley, J. S. and Tessier, G.** (1936). Terminology of relative growth. *Nature.* **137**, 780–781.
- Huxley, J. S.** (1924). Constant differential growth-ratios and their significance. *Nature.* **114**, 895–896.

- Kerr N.Z., Crone E.E., and Williams N.M.** (2019). Integrating vital rates explains optimal worker size for resource return by bumblebee workers. *Funct. Ecol.* **33**, 467-478.
- Kim, J. Y.** (1997). Female size and fitness in the leaf-cutter bee *Megachile apicalis*. *Ecol. Entomol.* **22**, 275-282.
- Kim, J. Y.** (1999). Influence of resource level on maternal investment in a leaf-cutter bee (Hymenoptera: Megachilidae). *Behav. Ecol.* **10**, 552-556.
- Kim JY, and Thorp RW.** (2001). Maternal investment and size-number trade-off in a bee, *Megachile apicalis*, in seasonal environments. *Oecol.* **126**, 451-456.
- Kleiber, M.,** (1947). Body size and metabolic rate. *Physiol. Rev.* **27**, 511-541.
- Klostermeyer, E. C. and Gerber, H. S.** (1969). Nesting behavior of *Megachile rotundata* (Hymenoptera: Megachilidae) monitored with an event recorder. *Ann. Entomol. Soc. Am.* **62**, 1321-1325.
- Klostermeyer, E. C., Mech Jr, S. J. and Rasmussen, W. B.** (1973). Sex and weight of *Megachile rotundata* (Hymenoptera: Megachilidae) progeny associated with provision weights. *J. Kansas Entomol. Soc.* **46**, 536-548.
- Koyama, T., Mendes, C. C., and Mirth C. K.** (2013). Mechanisms regulating nutrition-dependent developmental plasticity through organ-specific effects in insects. *Front. Physiol.* **4**, 263.
- Lehmann, F. O., Dickinson, M.H., and Staunton, J.** (2000). The scaling of carbon dioxide release and respiratory water loss in flying fruit flies (*Drosophila spp.*). *J. Exp. Biol.* **203**, 1613-1624.
- Lighton, J. R.** (1996). Discontinuous gas exchange in insects. *Annu. Rev. Entomol.* **41**, 309-324.

- Marden, J.H.** (1987). Maximum lift production during takeoff in flying animals. *J. Exp. Biol.* **130**, 235-258.
- Marden, J. H.** (1994). From damselflies to pterosaurs: how burst and sustainable flight performance scale with size. *Am. J. Physiol-Reg. I.* **266**, R1077-R1084.
- Mole, S., and Zera, A.J.,** (1993). Differential allocation of resources underlies the dispersal-reproduction trade-off in the wing-dimorphic cricket, *Gryllus rubens*. *Oecologia.* **93**, 121-127.
- Morgan K.R., Shelley T.E., and Kimsey L.S.** (1985). Body temperature regulation, energy metabolism and foraging in light-seeking and shade-seeking robber flies. *J. Comp. Physiol. B* **155**, 561–70
- Nijhout, H. F., and Wheeler, D. E.** (1996). Growth models of complex allometries in holometabolous insects. *Am. Nat.*, **148**, 40-56.
- Nijhout, H. F., and Grunert, L. W.** (2010). The cellular and physiological mechanism of wing-body scaling in *Manduca sexta*. *Science*, **330**, 1693-1695.
- Nijhout, H. F., and Callier, V.** (2015). Developmental mechanisms of body size and wing-body scaling in insects. *Annu. Rev. Entomol.* **60**, 141-156.
- Nijhout, H. F., Davidowitz, G. and Roff, D. A.** (2006). A quantitative analysis of the mechanism that controls body size in *Manduca sexta*. *Journal of Biology.* **5**, 1–15.
- Niven, J. E. and Scharlemann, J. P.** (2005). Do insect metabolic rates at rest and during flight scale with body mass? *Biol. Letters.* **1**, 346-349.
- O’Neill, K. M., Delphia, C. M. and O’Neill, R. P.** (2014). Oocyte size, egg index, and body lipid content in relation to body size in the solitary bee *Megachile rotundata*. *Peer J.* **2**, e314.

- Owings, A.A., Yocum, G.D., Rinehart, J.P., Kemp, W.P. and Greenlee, K.J.** (2014). Changes in respiratory structure and function during post-diapause development in the alfalfa leafcutting bee, *Megachile rotundata*. *J. Insect Physiol.* **66**, 20-27.
- Pitts-Singer, T. L. and Cane, J. H.** (2011). The alfalfa leafcutting bee, *Megachile rotundata*: the world's most intensively managed solitary bee. *Annu. Rev. Entomol.* **56**, 221-237.
- Ramalho M., Imperatriz-Fonseca V.L., and Giannini T.C.** (1998). Within-colony size variation of foragers and pollen load capacity in the stingless bee *Melipona quadrifasciata anthidioides* Lepelletier (Apidae, Hymenoptera). *Apidologie.* **29**, 221-228.
- Renauld, M., Hutchinson, A., Loeb, G., Poveda, K. and Connelly, H.** (2016). Landscape simplification constrains adult size in a native ground-nesting bee. *PLoS One.* **11**, e0150946.
- Rinehart, J. P., Yocum G. D., Kemp W. P., and Greenlee K. J.** (2013). A fluctuating thermal regime improves long-term survival of quiescent prepupal *Megachile rotundata* (Hymenoptera: Megachilidae). *J. Econ. Entomol.* **106**, 1081-1088.
- Reinhold, K.** (1999). Energetically costly behavior and the evolution of resting metabolic rate in insects. *Funct. Ecol.* **13**, 217-224.
- Roberts, S. P., Harrison, J. F., and Dudley, R.** (2004). Allometry of kinematics and energetics in carpenter bees (*Xylocopa varipuncta*) hovering in variable-density gases. *J. Exp. Biol.* **207**, 993-1004.
- Roff, D.A.,** (1986). The evolution of wing dimorphism in insects. *Evolution.* **40**, 1009–1020.
- Roff, D.A., and Bradford, M.J.,** (1996). Quantitative genetics of the trade-off between fecundity and wing dimorphism in the cricket *Allonemobius socius*. *Heredity.* **76**, 178–185.

- Royauté, R., Wilson, E.S., Helm B. R., Mallinger, R.E., Prasifka, J., Greenlee, K.J., and Bowsher J.H.** (2018). Phenotypic integration in an extended phenotype: among-individual variation in nest-building traits of the alfalfa leafcutting bee (*Megachile rotundata*). *J. Evolution. Boil.* **31**, 944-956.
- Rubner, M.** (1908). Das Problem der Lebensdauer und seine Beziehungen zu wachstum und ernährung. Oldenbourg.
- Saastamoinen, M., van der Sterren, D., Vastenhout, N., Zwaan, B.J., and Brakefield, P.M.,** (2010). Predictive adaptive responses: condition-dependent impact of adult nutrition and flight in the tropical butterfly *Bicyclus anynana*. *Am. Nat.* **176**, 686–698
- Sadowska, E. T., Baliga-Klimczyk, K., Labocha, M. K. and Koteja, P.** (2009). Genetic correlations in a wild rodent: grass-eaters and fast-growers evolve high basal metabolic rates. *Evolution.* **63**, 1530-1539.
- Seidelmann, K.** (2014). Optimal progeny body size in a solitary bee, *Osmia bicornis* (Apoidea: Megachilidae). *Ecol. Entomol.* **39**, 656-663.
- Seidelmann, K.** (2018). Optimal resource allocation, maternal investment, and body size in a solitary bee, *Osmia bicornis*. *Entomol. Exp. Appl.* **166**, 790-799.
- Schmidt-Nielsen, K.,** (1984). Scaling: why is animal size so important?. Cambridge university press.
- Shingleton, A. W., Mirth, C. K., and Bates, P. W.** (2008). Developmental model of static allometry in holometabolous insects. *P. R. Soc. B.* **275**, 1875-1885
- Skandalis, D.A. and Darveau, C.A.** (2012). Morphological and physiological idiosyncrasies lead to interindividual variation in flight metabolic rate in worker bumblebees (*Bombus impatiens*). *Physiol. Biochem. Zool.* **85**, 657-670.

- Speakman, J. R., Król, E. and Johnson, M. S.** (2004). The functional significance of individual variation in basal metabolic rate. *Physiol. Biochem. Zool.* **77**, 900-915.
- Sugiura, N. and Maeta, Y.** (1989). Parental investment and offspring sex ratio in a solitary Mason bee, *Osmia cornifrons* (Radoszkowski) (Hymenoptera, Megachilidae). *Japanese Journal of Entomology*, **57**, 861–875.
- Sugiura, N.** (1994). Parental investment and offspring sex ratio in a solitary bee, *Anthidium septemspinosum* Lepeletier (Hymenoptera: Megachilidae). *J.Ethol.* **12**, 131-139.
- Tanaka, S., and Suzuki, Y.,** (1998). Physiological trade-offs between reproduction, flight capability and longevity in a wing-dimorphic cricket, *Modicogryllus confirmatus*. *J. Insect Physiol.* **44**, 121–129.
- Tepedino, V. J. and Torchio, P. F.** (1989). Influence of nest hole selection on sex ratio and progeny size in *Osmia lignaria propinqua* (Hymenoptera: Megachilidae). *Ann. Entomol. Soc. Am.* **82**, 355-360.
- Terblanche, J. S., White, C. R., Blackburn, T. M., Marais, E., and Chown, S. L.** (2008). Scaling of gas exchange cycle frequency in insects. *Biol. letters*, **4**, 127-129.
- Tercel, M. P., Veronesi, F. and Pope, T. W.** (2018). Phylogenetic clustering of wingbeat frequency and flight-associated morphometrics across insect orders. *Physiol. Entomol.* **43**, 149-157.
- Tigeros, N., Sass, E.M., and Lewis, S.M.,** (2013). Sex-specific response to nutrient limitation and its effects on female mating success in a gift-giving butterfly. *Evol. Ecol.* **27**, 1145–1158.

- Trostle, G. and Torchio, P.F.**, (1994). Comparative nesting behavior and immature development of *Megachile rotundata* (Fabricius) and *Megachile apicalis* Spinola (Hymenoptera: Megachilidae). *J. Kansas Entomol. Soc.* 53-72.
- Wang, Z.J.** (2005). Dissecting insect flight. *Annu. Rev. Fluid. Mech.* **27**, 183–210.
- Warton, David I., Duursma, Remko A., Falster, Daniel S. and Taskinen, Sara** (2012). smatr 3 - an R package for estimation and inference about allometric lines. *Methods Ecol. Evol.* **3**, 257-259.
- Weibel, E. R.** (2002). The pitfalls of power laws. *Nature.* **417**, 131–132.
- Wickham, H.**, (2016). Ggplot2: Elegant Graphics for Data Analysis.
<https://ggplot2.tidyverse.org>.
- Wilkaniec, Z., Giejdasz, K. and Fliszkiewicz, M.** (2004). The influence of food amount consumed during the larval development on the body weight of the imago of the red mason bee (*Osmia rufa* L., Megachilidae). *J. Apic. Sci.* **48**, 29-36.
- Wolf, T. J., Schmid-Hempel, P., Ellington, C. P., and Stevenson, R. D.** (1989). Physiological correlates of foraging efforts in honey-bees: oxygen consumption and nectar load. *Funct. Ecol.* 417-424.
- Yocum, G.D., Greenlee, K.J., Rinehart, J.P., Bennett, M.M. and Kemp, W.P.**, (2011). Cyclic CO₂ emissions during the high temperature pulse of fluctuating thermal regime in eye-pigmented pupae of *Megachile rotundata*. *Comp. Biochem. Phys. A.* **160**, 480-485.
- Zera, A., and Harshman, L.**, (2001). The physiology of life history trade-offs in animals. *Annu. Rev. Ecol. Syst.* **32**, 95–126.

CHAPTER 3: TELOMERE DYNAMICS IN TWO SOLITARY MEGACHILID BEE SPECIES

Abstract

Adult insects have relatively short lifespans and show declines in performance with age. The mechanisms that underlie these age-associated declines in performance are not well understood. Cellular aging may depend on life stage and time spent in that stage, especially if that life stage is extended, as during diapause. Telomeres are repetitive sequences of non-coding DNA located at the end of chromosomes, which protect coding DNA during replication. Decreasing telomere length is associated with increased age. This study aimed to determine how body size impacts telomere dynamics throughout development, overwintering, and post emergence, in the solitary bee species *Osmia lignaria* and *Megachile rotundata*. Relative telomere length was measured using qPCR in larvae, prepupae, pupae, and multiple adult stages. Our results show surprising trends. First, telomere length increased after adult *O. lignaria* emerged from diapause. Second, telomere length was shorter in prepupal *M. rotundata* compared to other life stages. Third, telomere length did not change during the eight months of diapause in either species. We also tested whether telomere length was influenced by days post emergence and whether stress exposure after emergence affected telomere length. Telomere length was not affected up to fifteen days post-emergence for either species, nor was telomere length influenced by sex or body size. In *M. rotundata*, exposure to food restriction as a stress did not influence telomere length. These results suggest that telomere dynamics in bees differ from what would be expected from vertebrate species and suggest that insect diapause has specialized the life stages for aging dynamics.

Introduction

The rate and manner of how individuals age is highly variable (AihieSayer et al. 1999). Differences in biological age compared to chronological age make it possible for an individual older in years to be physiologically similar to a younger individual (Gunn et al. 2009). Biological age is defined by an organism's fecundity and performance (Mitnitski et al. 2013), which decrease over time, termed senescence. Senescence can refer to deterioration of the whole organism or to individual cells and tissues. Cellular senescence occurs when the metabolic function of cells declines (Campisi and d'Adda di Fagagnan 2007), associated with changes in gene expression (Sahin et al. 2011) the ability to replicate ceases. Cellular senescence can lead to problems such as chronic inflammation and organ failure (Wong et al 2003). Stress can accelerate senescence (Eisenberg et al., 2009) contributing to age-associated diseases such as cardiovascular disease and cancer (Boonekamp et al. 2013, Haycock et al. 2014, Wentzensen et al. 2011, Willet 2014). This occurs due to a weakened response to stress, and reduced ability for maintenance and repair (De Loof 2011).

Telomere dynamics are one mechanism for understanding cellular senescence because telomere shortening is a mechanism for cellular division cessation. Telomere dynamics can be defined as changes in telomere length over time. Telomeres are repetitive sequences of non-coding DNA located at the end of chromosomes, which protect coding DNA during replication (Blackburn 2005). Telomeres shorten naturally during DNA replication due to the inability of RNA polymerase to completely copy the lagging strand (Vaziri H, et al. 1994). Critically shortened telomeres will trigger cells to stop dividing (Blackburn 2005). To avoid problematic telomere shortening, cells trigger a DNA damage signal to prevent potential replication problems (d' Amico et al. 2001; di Fagagna et al., 2003, Takai et al., 2003). Accumulation of damaged

senescent cells may lead to aging effects such as a decline in tissue repair ability, leading to a decline in overall health (Krtolica et al., 2001, Campisi, 2005).

Telomere length is considered one of the “hallmarks of aging” (Lopez-Otin et al. 2013) because telomeres shorten over time (Allsopp et al. 1992; Jennings et al. 1999; Haussmann et al. 2003; Herbig et al. 2006; Jemielity et al. 2007). Telomere length is a good predictor of lifespan, because individuals with longer telomeres early in life having increased longevity (Heidinger 2012, Rudolph et al. 1999; Cawthon et al. 2003; Joeng et al. 2004; Haussmann et al. 2005; Pauliny et al. 2006). Declining telomere length and aging has been detected in a variety of animals, including humans (Blackburn 2015; Harley et al., 1990; Aubert et al., 2008) birds (Haussmann et al., 2002; Hausmann et al., 2003; Haussmann et al., 2008), and fish (Hatakeyama et al., 2008; Hartmann et al., 2009). A meta-analysis of 27 non-model species showed that shorter telomeres were correlated with an increased mortality risk (Wilbourn 2018). While telomere length correlates inversely with chronological age, their rate of shortening is positively related to biological aging, with slower rates of telomere loss in longer lifespans (Tricola et al. 2018).

Aside from the issue of not fully copying the lagging DNA strand during replication, oxidative stress is another primary cause of telomere loss (von Zglinicki, 2002). Oxidative stress is defined as the imbalance of free radicals and antioxidant production, leading to oxidative damage (Monaghan et al., 2009). Humans who experienced high levels of stress had shorter telomeres, lower telomerase levels, and higher oxidative stress (Epel 2004). Individuals with high levels of stress, had higher levels of free radicals, increased oxidative damage, and increased telomere shortening (Monaghan 2014). However, this pattern is not as clear in insects.

In a metanalysis measuring oxidative stress in *Drosophila* did not find a clear pattern between oxidative stress and longevity (Le Bourg, 2001).

Rate of growth is thought to impact telomere length (Kraus et al. 2013). Although, larger vertebrate species tend to have longer lifespans (Selman et al. 2013), intraspecifically, the trend is reversed, with smaller individuals tending to live longer. Examples of this trend have been observed in mice, snakes, and domesticated dogs (Miller et al. 2002, Bronikowski & Vleck 2010, Kraus et al. 2013), with large breeds of dogs tending to have shorter lifespans than small breeds. Mechanisms underlying the slower pace of senescence in small individuals are slower growth rates, low body mass (Kraus et al. 2013), which are associated with lower levels of oxidative stress and reduced rates of telomere loss (Nussey et al. 2009). Larger individuals within a species have shorter telomere lengths, which are correlated with shorter lifespans (Fick et al. 2012). One possible mechanism is telomerase expression, which has been shown to be downregulated in the somatic cells of larger mice, which demonstrates telomerase activity negatively coorelated with body size (Seluanov et al., 2007)

Insects show declines in performance with age, even within their relatively short lifespans (McFarlane, 1967, Giraldo & Traniello, 2014). Examples of insect senescence include reduced performance of muscles and organs (Sohal, 1976; Seehuus et al., 2006), declines in flight duration (Williams et al., 1943), declines in reproduction, and increased probability of death over time. In adult solitary bees there are also visible, physical signs of age including loss of hairs which can impact their ability to collect pollen, and tattered wings which can impact flight performance and cause increased mortality (Torchio & Tepedino 2016; Carter 1992). Insects are a good model system for understanding aging because of their short lifespans, small body size, high fecundity, and low laboratory maintenance costs (Lee & Kim 2015).

Telomere dynamics in eusocial Hymenopterans (bees, wasps and ants) share many similarities to those in vertebrate species in that in ants, longer telomeres are associated with longer-lived reproductive castes (Jemielity et al., 2007). In honeybees, queens typically live 1-3 years, (Bozina 1961), whereas the worker lifespan is less than a year. Overall telomerase levels are higher in the queen caste, in both the larval and adult stages. (Korandová & Frydrychová 2016). Telomerase is differentially expressed over time and season in worker honeybees and is higher in the longer-lived winter honeybee workers (Koubová et al. 2021). In bumblebees, telomerase is upregulated in the fat bodies of queens (Koubová et. al 2019). Telomerase expression is higher in the reproductive caste, which may indicate that in social bees, there is not a longevity-reproduction tradeoff. Telomerase is expressed in bees in a similar way to other insects (Robertson & Gordon 2006, Frydrychová et al. 2004). While telomere dynamics have been measured in social hymenopterans, there have been no studies to date in solitary bees.

Megachile rotundata (F.) and *Osmia lignaria* (Say) are both solitary bees in the family Megachilidae. These bees differ in which stage they overwinter, as well as the amount of time spend as adults (Fig 5). *O. lignaria* overwinters as an adult, and may go through multiple diapause periods, first as prepupae during the late summer, (Sgolastra, et al., 2012) and later as adults during the winter months (Bosch et al., 2010; Sgolastra et al., 2010). Typically, *O. lignaria* bees are in the adult stage for eight months. *O. lignaria* emerges in early spring, and *M. rotundata* emerges in mid-summer. *M. rotundata* overwinters in the prepupal stage, which begins during the late summer (Pitts-Singer & Cane 2011), and the average adult lifespan is about one month. The post-emergence lifespan in *O. lignaria* is also about one month, but their adult lifespan begins before they enter diapause. Our goal was to determine the telomere dynamics in solitary bees. We hypothesize that telomere length will get shorter in later

developmental stages in solitary bees. We wanted to determine if telomeres were an indicator of aging in these organisms. We were also interested in determining how stress would impact telomere length. We predict that individuals that received a stress treatment would have shorter telomeres. Finally, we were interested in determining if body size would impact telomere length, we predict that larger individuals would have shorter telomeres.

Methods

Study System. Solitary bee females nest in cavities and a single mother constructs their offspring brood cells with leaf pieces (*M. rotundata*) or mud caps (*O. lignaria*). Females provide provisions for their offspring within these brood cells. All bees are holometabolous, and complete the egg, larvae, pupae and adult life stages (Michener 2000). After the final larval instar, the bee will begin to spin silk to create a cocoon where it will continue to develop into the prepupal stage. *M. rotundata* diapauses for four to six months as a prepupa (Fig. 5). As *M. rotundata* and *O. lignaria* develop into adults, they go through distinct pupal stages, including the white eye, pink eye, and red eye pupal stages. This takes place during the first week after being exposed to increased temperatures in *M. rotundata*; in *O. lignaria*, pupal development occurs before overwintering. Sclerotization and tanning of the pupa begins in the second and third weeks of development (Helm et al., 2018; Bosch and Kemp 2001). *O. lignaria* begins winter diapause after they have become adults (Fig. 5)

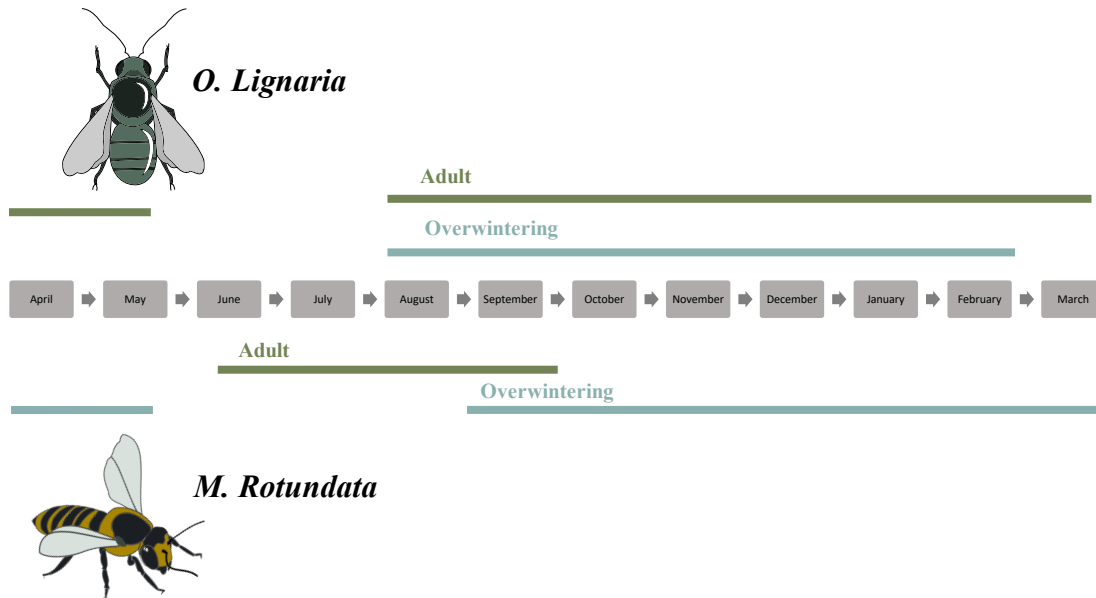


Fig. 5. Life cycle of *O. lignaria* and *M. rotundata*
 Timeline of development and overwintering in *O. lignaria* and *M. rotundata*. We show the entire active season for adults in *M. rotundata*, although the typical adult lifespan is one month. The average lifespan post-emergence for *O. lignaria* is also about one month.

***Megachile rotundata* Rearing and Sample Collection.** Prepupal bees sourced from JWM Leafcutters, Inc. of Nampa, ID, US were taken from 6°C storage, removed from their brood cells, and placed in 96 well plate in a 29°C incubator to initiate development. For comparison of telomere lengths, bees were sampled at the following stages: overwintering prepupae (Arrived March 2020, n=28), white eye pupae (n=28), pink eye pupae (n=28), red eye pupae (n=25), tanning (n=18), newly emerged adult (n=28). Stages are clearly represented in Helm et. al 2018. is termed “body pigmented.” Bees were flash frozen on liquid nitrogen and stored in the -80°C freezer.

***Osmia lignaria* Rearing and Sample Collection.** Freshly capped *O. lignaria* nests from Woodenville, WA (2018), and CA (2021) were kept in an incubator at 25°C and 75% humidity and allowed to develop. Developmental stages were monitored by X-ray. Bees were sampled at the following stages: fifth instar larvae (n=6), prepupae (n=6), pupae (n=13), prewintering adult

(n=18), diapausing adult (date pulled, n=18), post diapausing adults (n=20), emerged adults (n=20). Bees were flash frozen on liquid nitrogen and stored in the -80°C freezer.

***Osmia lignaria* Post-Emergence Adult Sample Collection.** 100 bees from Woodenville, WA were shipped to Fargo, ND. Upon arrival, a subsample of adult bees were immediately frozen at -80°C for the first timepoint. The rest of the adult bees were stored in the incubator at 25°C in separate 15 ml conical tubes and fed a 1:1 sucrose solution. Bees were checked daily for survival. Bees were sampled on day 1 (n=20), day 5 (n=20), day 15 (n=32), frozen on liquid nitrogen and stored in the -80°C for telomere measurement.

***Osmia lignaria* Post-Emergence Adult Nutritional Stress.** We repeated the above experiment measuring telomere length in adult *O. lignaria* with the addition of a nutritional stress treatment. Bees from Logan, UT were shipped to Fargo, ND. Upon arrival, adult bees were immediately frozen on liquid nitrogen, then stored in -80°C. The remaining bees were then placed into feeding treatments 24 hours after arrival. The feeding treatments included a food removal treatment (starved) and a continuously fed treatment (fed). Bees that were continuously fed bees were given a 1:1 sucrose solution, bees in the food removal treatment were given only water for 24 hours. After the 24 hour period, all bees were then given a 1:1 sucrose solution for the remainder of the experimental time period. Bees were stored in the incubator at 25°C in separate 15 ml conical tubes and fed a 1:1 sugar solution. Bees were checked daily for survival. Bees were sampled at day 1 (fed n=30; starved n=25) and day 15 (fed n=31; starved n=23) post-emergence for telomere measurement.

Telomere Measurement. We analyzed telomere lengths from the thorax (adult) or partial bee (juvenile stages). Bees were cut to fit the weight requirement of the kit. We used Machery-Nagel Insect DNA Extraction Kit and measured the quantity and purity of DNA on a

spectrophotometer. We used the quantitative PCR (qPCR) to determine relative telomere length, using the T/S ratio (Cawthon, 2002). The T/S ratio describes the telomere signal (T) relative to a reference single control gene (S), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), relative to a reference control sample used across all plates. This calculates the proportional average of the relative telomere length. The same telomere primer was used for both *M. rotundata* and *O. lignaria*. The following forward and reverse primers were used to amplify the telomere: Telo1 (5'-CGG TTT GTT TGG TTT GGT TTG GTT TGG TTT GGT T-3'), Telo2 (5'-GGC TTG CCT TAC CTT ACC TTA CCT TAC CTT ACC T-3') and bee-specific GAPDH sequences: (*M. rotundata*) GAPDH-F (5'-GACGTAGTGTCTTCCGACTTTAT -3'), (*M. rotundata*) GAPDH-R (5'-CAATCACGCGGCTAGAGTAA-3'). (*O. lignaria*) GAPDH-F (5'-GGCCAATGTCGGGAGATAAAA-3'), (*O. lignaria*) GAPDH- R (5'-GAGACTCTGCTTCGCTTTCA-3'). The telomere and GAPDH reactions were run on two separate plates. The number of PCR cycles required to accumulate a fluorescent signal to cross a threshold was measured. A standard curve was used to determine the reaction efficiency. All samples were run in duplicate, and the standard curve was run in triplicate. Average duplicate values were used to calculate the T/S ratios for each sample relative to the reference sample according to the formula: $2^{\Delta\Delta C_t}$, where $\Delta\Delta C_t = (C_{t_{telomere}} - C_{t_{GAPDH}})_{reference} - (C_{t_{telomere}} - C_{t_{GAPDH}})_{sample}$. Reference samples were a pool of multiple individuals that were used to normalize expression across the multiple plates run for each experiment. Reference samples were different between experiments. Telomere length is a relative measurement based on the reference sample, therefore telomere length cannot be compared between experiments.

Statistical Analysis. All statistical analyses were performed using R statistical software (version 3.6.1, Base R package) and graphed using the package *ggplot2* (Wickham, 2016). To

determine the effect of developmental stage, sex and weight on telomere length a multiple comparisons of linear model tests were performed using the lme4 package (Bates et al., 2015), the MuMIn package (Bartoń 2020) to was used to calculate R², and the multcomp package (Hothorn et al., 2008) was used for Tukey post-hoc comparisons.

Results

***Megachile rotundata* Development.** The prepupal life stage had significantly shorter telomeres than other life stages (Fig. 6, Table 4, Linear Model, $p < 0.0001$). Weight did have an impact on telomere length, although the interaction of weight and life stage was not significant (Fig. 7, Linear Model, weight, $p = 0.0278$; lifestage*weight, $p=0.391$).

Table 4. Post-hoc comparisons of T/S ratios of different developmental stages in *M. rotundata*

Life Stage 1	Life Stage 2	P value
Adult	Pink Eye	0.736
Adult	Prepupa	<0.001
Adult	Red Eye	0.919
Adult	Tanning	0.413
Adult	White Eye	0.923
Pink Eye	Prepupa	<0.001
Pink Eye	Red Eye	0.999
Pink Eye	Tanning	0.979
Pink Eye	White Eye	0.999
Prepupa	Red Eye	<0.001
Prepupa	Tanning	<0.001
Prepupa	White Eye	<0.001
Red Eye	Tanning	0.913
Red Eye	White Eye	1.00
Tanning	White Eye	0.937

Results from Multiple Comparisons of Linear Model test

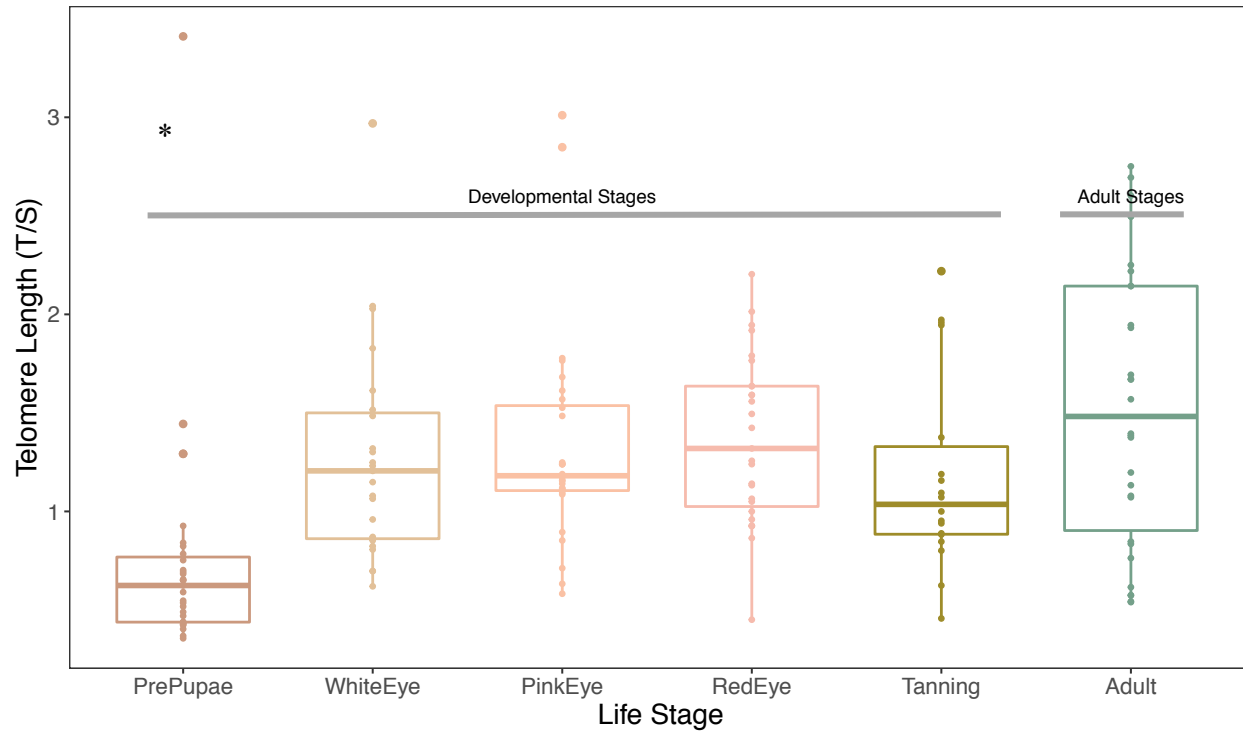


Fig. 6. Telomere length across different developmental life stages in *M. rotundata*. Significance represented by an astrick (*) Results from Multiple Comparisons of Linear Model Test

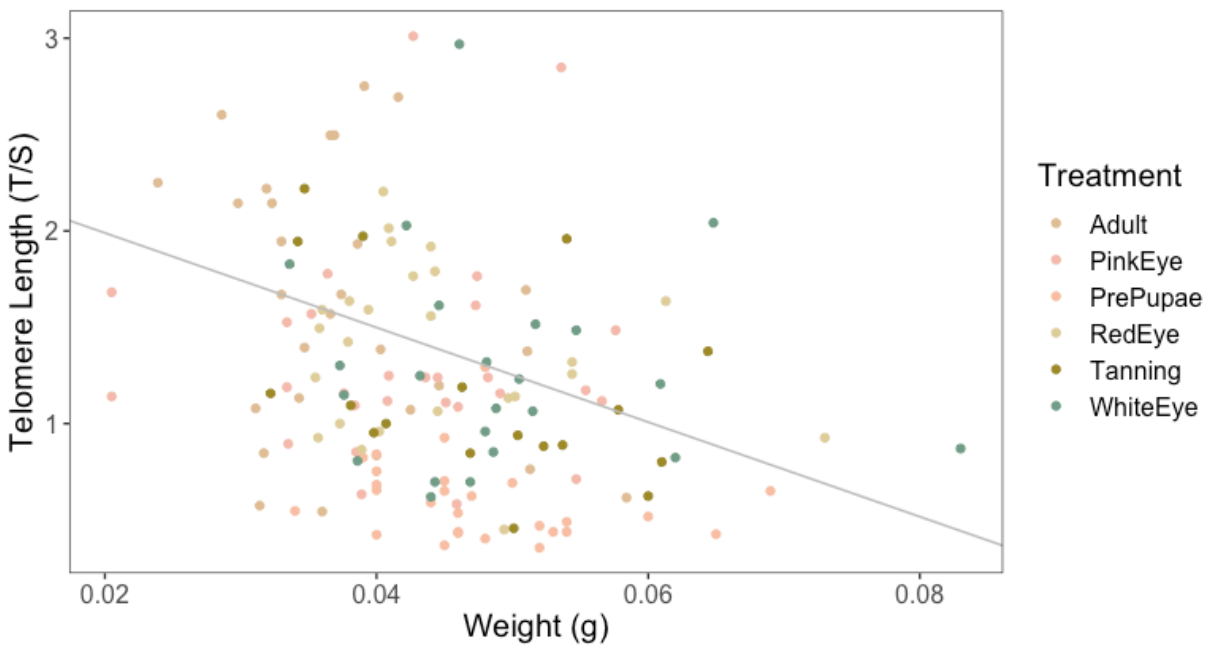


Fig. 7. Telomere length based on body size in *M. rotundata*

***Osmia lignaria* Development.** Emerged bees had significantly longer telomeres than other developmental stages, with the exception of bees in the diapause stage (Fig. 8, Table 5; Linear Model, $p < 0.001$). Weight and sex were only collected for adult bees, and do not affect telomere length, nor did the interaction between weight and sex (Fig. 9, Linear Model, weight, $p = 0.849$; sex, $p = 0.259$; sex*weight, $p = 0.661$).

Table 5. Comparing T/S ratios of different developmental stages in *O. lignaria*

Life Stage 1	Life Stage 2	P value
Diapause	Emergence	<0.001
Diapause	Larva	0.133
Diapause	Post-Diapause	0.835
Diapause	Pre-Winter	0.999
Diapause	Prepupa	0.739
Diapause	Pupa	0.799
Emerged	Larva	<0.001
Emerged	Post-Diapause	<0.001
Emerged	Pre-Winter	<0.001
Emerged	Prepupa	<0.001
Emerged	Pupa	<0.001
Larva	Post-Diapause	0.627
Larva	Pre-Winter	0.270
Larva	Prepupa	0.976
Larva	Pupa	0.785
Post-Diapause	Pre-Winter	0.979
Post-Diapause	Prepupa	0.977
Post-Diapause	Pupa	1.000
Pre-Winter	Prepupa	0.902
Pre-Winter	Pupa	0.959
Prepupa	Pupa	1.000

Results from Multiple Comparisons of Linear Model Test

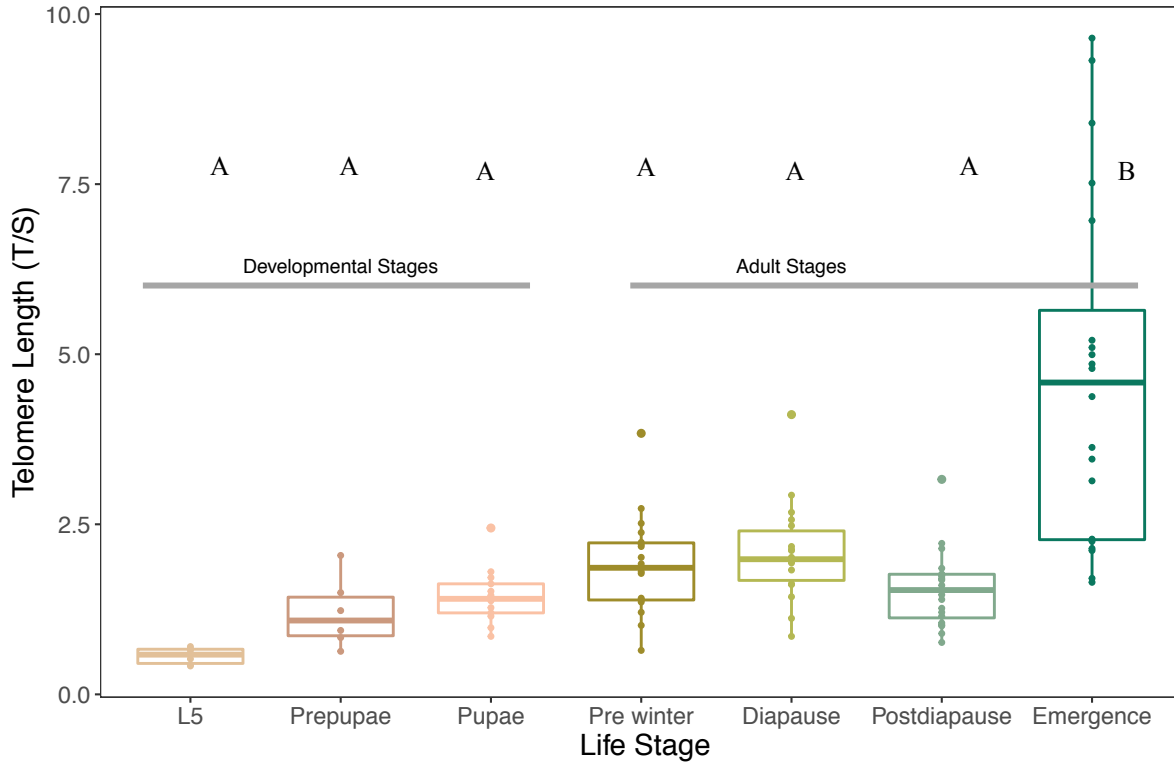


Fig. 8. Telomere length across different developmental stages in *O. lignaria*. Letters represent significant differences between life stages. Results from Multiple Comparisons of Linear Model Test.

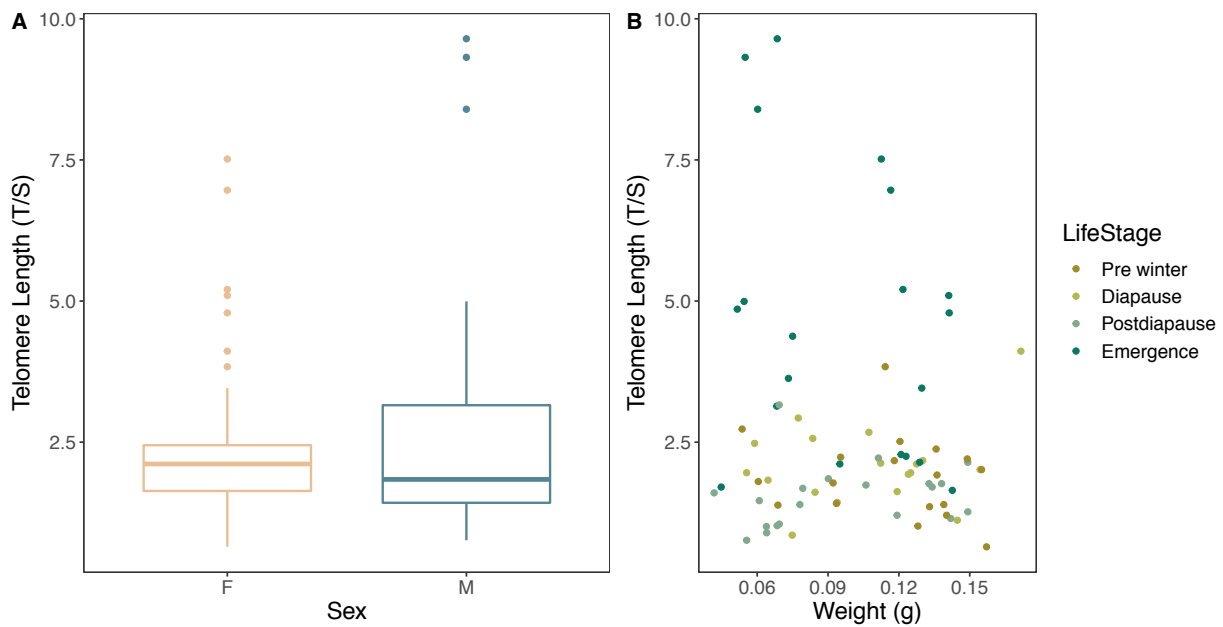


Fig. 9. Telomere length based on sex and weight across different developmental stages in *O. lignaria*

***Osmia lignaria* Post-Emergence Adult Lifespan.** Telomere length did not change throughout the post-emergence adult lifespan (Fig. 10, Linear Model, $p = 0.948$). Sex and mass do not affect telomere length in adults, nor did the interaction of sex and weight (Fig. 11, Linear Model, Sex, $p = 0.916$; Weight $p = 0.821$; Sex*Weight $p = 0.366$).

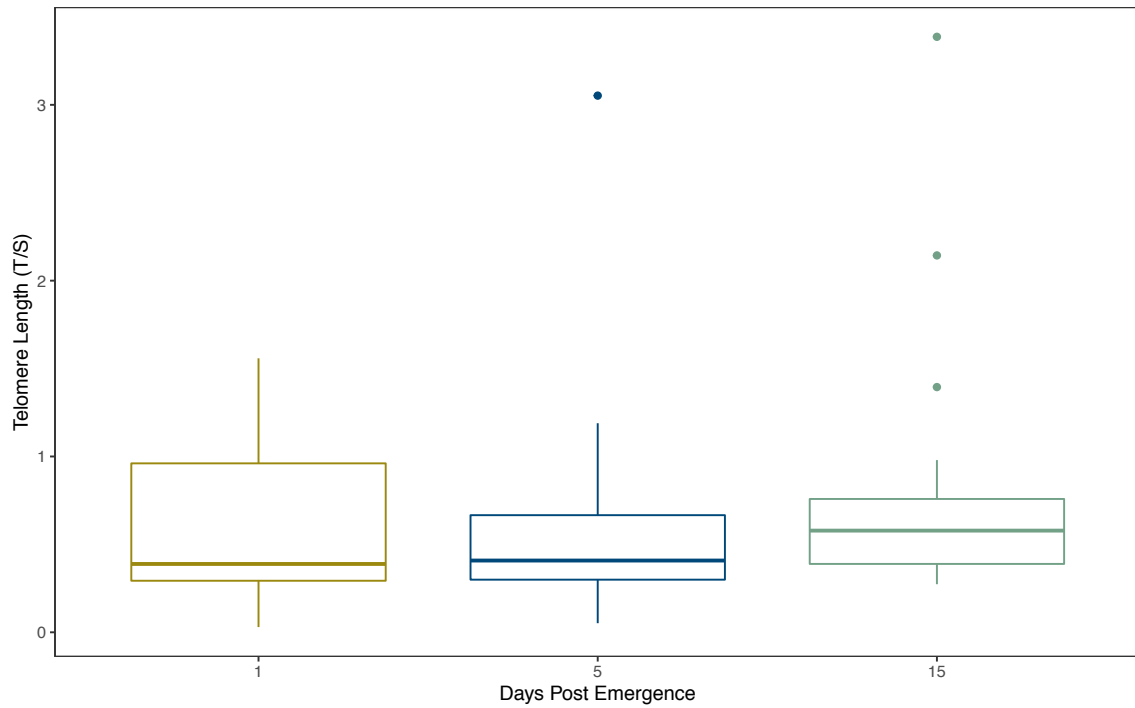


Fig. 10. Comparing telomere length in days post emergence in adult *O. lignaria*

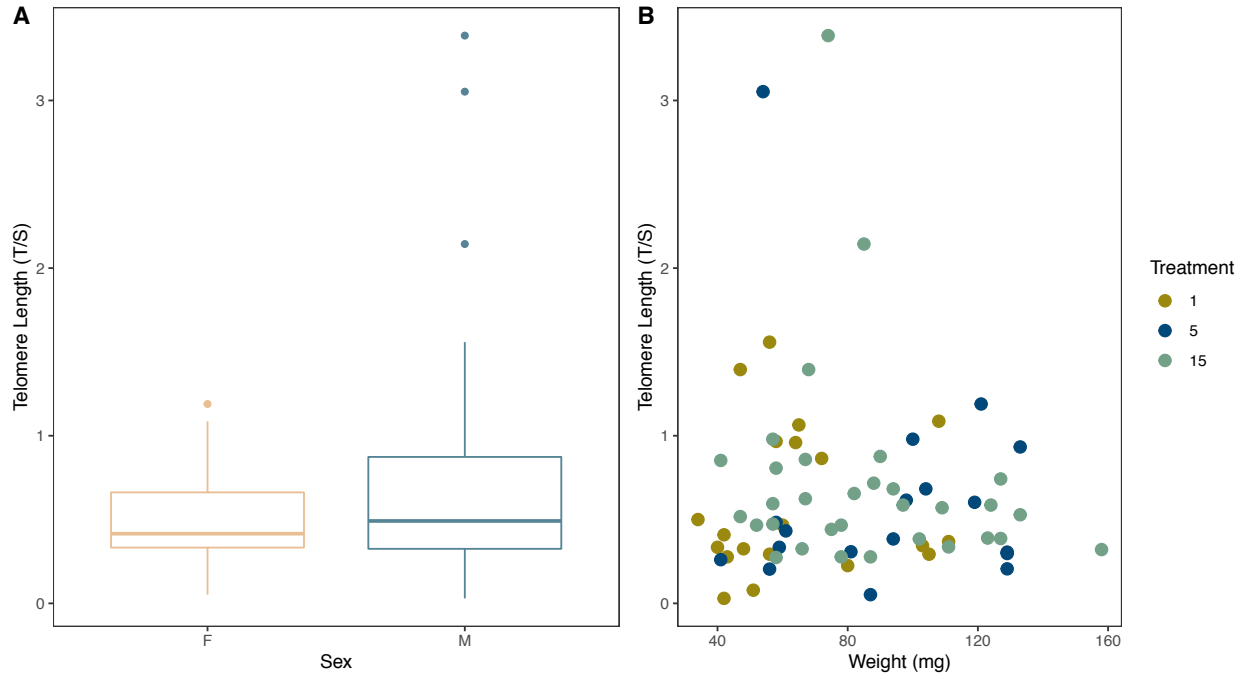


Fig. 11. Telomere length based on sex and weight in days post emergence in adult *O. lignaria*

***Osmia lignaria* Adult Feeding Stress.** Telomere length did not differ throughout the adult lifespan or by feeding treatment, or the interaction of days post emergence or feeding treatment (Fig. 12, Linear Model, days post-emergence, $p=0.523$; feeding treatment, $p=0.966$; days post-emergence*feeding treatment, $p=0.655$) Telomere length did not differ by sex, weight or the interaction of sex and weight (Fig. 13, Linear Model, sex, $p=0.747$; weight, $p=0.955$, sex*weight, $p=0.995$).

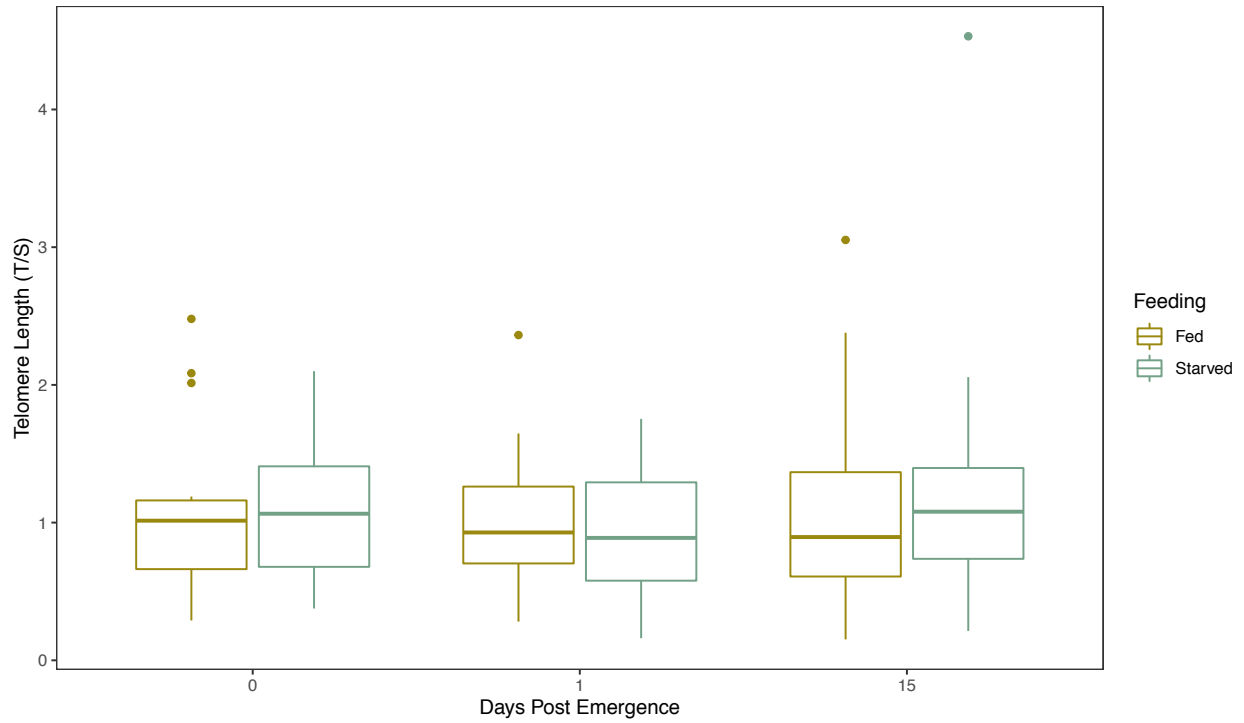


Fig. 12. Telomere length in days post emergence in fed and starved adult *O. lignaria*

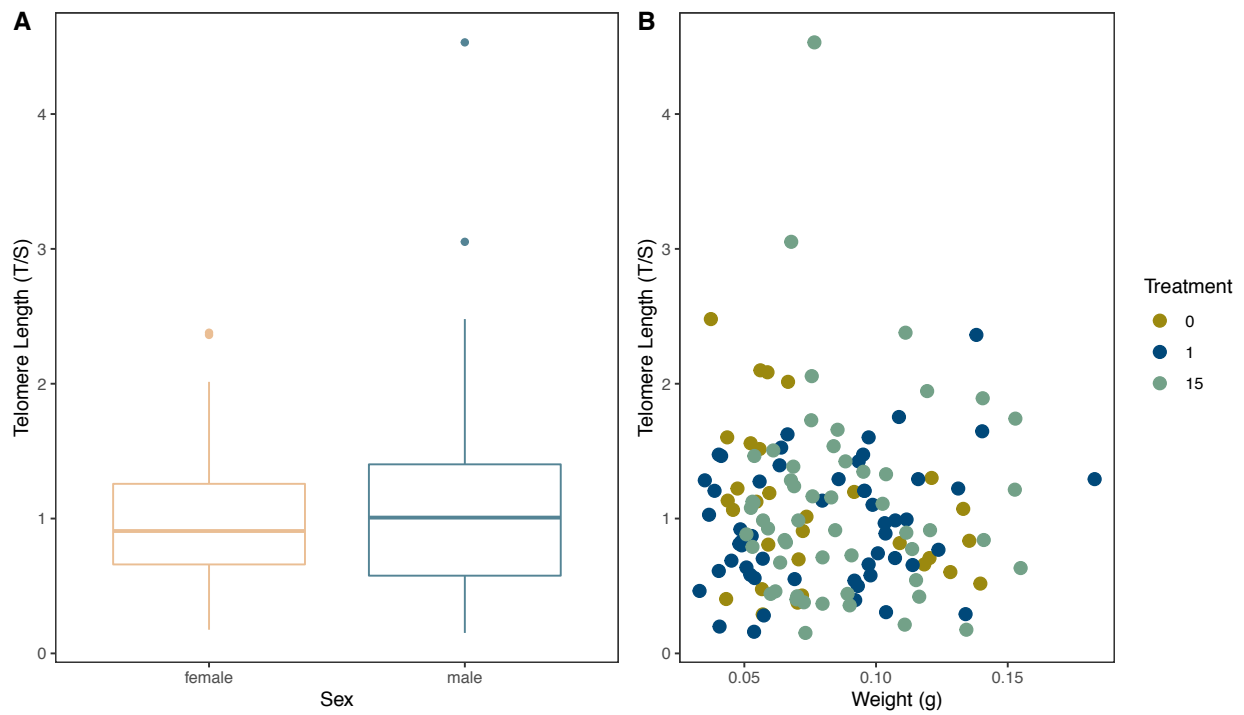


Fig. 13. Telomere length based on sex and weight in days post emergence in *O. lignaria* that were exposed to a feeding treatment

Discussion

We found that telomeres were longer in later developmental stages in both *M. rotundata* and *O. lignaria*. In *M. rotundata* telomeres were the shortest in the prepupal phase and became longer in later developmental stages. In the case of *O. lignaria* telomeres were shorter in early developmental stages including early adult stages and became longer upon adult emergence. The emerged bees had the longest telomeres compared to all other life stages with the exception of the diapausing adult bees. There were no differences in telomere length in adult *O. lignaria* based on days since emergence, or due to nutritional stress. We also saw no differences in telomere length based on size, or between the sexes in the experiments. Our results were cross-sectional, we were unable to measure telomere length in the same individuals across time. These results contradict the theory that telomeres shorten with age; telomeres were significantly longer in later stages in both solitary bees.

Our study did not find a negative correlation between telomere length and age. It is still unclear whether telomere shortening directly contributes to aging or is an artifact of the combination of biological age, stress accumulation, and physical condition (Lansdorp 2008). Others have demonstrated telomere length does not always appear to be directly correlated with age (Lund et al. 2009; Olsson et al., 2010). In a zebrafish, there is no relationship between telomere length and age, likely due to high levels of telomerase expression in somatic tissue (Lund et al. 2009). In lizards, differences in telomere length were based on sex and response to stress, rather than age (Olsson et al., 2010). Factors associated with telomere length may be indirectly associated with senescence.

The rate and timing of telomerase expression may be a cause of increasing telomere length in later stages. Telomerase is a reverse transcriptase that functions in telomere

maintenance by adding nucleotides to chromosome ends to elongate telomeres. In many animals, telomerase is downregulated in somatic tissues and upregulated in embryonic tissue, stem cells, immune cells and germline tissue (Monaghan 2010, Weng 2008). The downregulation of telomerase may be an adaptation to balance short telomeres and unregulated cell proliferation (Campisi, 2001). Expression of telomerase varies among organisms. Some organisms, such as mice, continuously express telomerase in their somatic cells (Prowse and Greider, 1995). The cause of lengthening in our study is likely upregulation of telomerase, but telomerase expression was not directly measured.

Our results differ from other studies on telomere length in bees. Honeybee telomere length does not differ across developmental stages, adult age, caste or time (Korandová & Frydrychová 2016; Koubová et al. 2021). In addition, bumble bees also do not have different telomere lengths based on developmental stage or caste (Koubová et al. 2019). There was a higher expression of telomerase in the longer-lived caste (Korandová & Frydrychová 2016). From these data, it is hypothesized that telomerase expression may be maintaining telomere length (Korandová & Frydrychová 2016; Koubová et al. 2021). Lack of correlation of telomere length and age has been found in cricket species as well (Boonekamp et al., 2021) Although it is unclear whether comparison of telomere dynamics to social hymenopterans is the best representation. While many insects share a similar telomere motif (TTAGG)_n, including the families Formicidae (ants) and Apidae (honeybee) in the order Hymenoptera (Kuznetsova et al., 2019). This motif does not occur in all families in Hymenoptera, 13 families lack this sequence (Menzes et al. 2017) Whether or not solitary bee telomeres are more similar to Apidae and Formicidae or other Hymenopteran families where the TTAGG sequence is absent is not yet determined. While there has been many studies on determining the telomere structure in insects

(Kuznetsova et al., 2019 and citations within), studies focusing on telomere dynamics in insects have been under studied.

In this study, telomere length varied with developmental stage, increasing in later life stages. Although telomere length does not increase in later life stages in social bees, it does appear to be maintained, and there is not a decrease in telomere length with age (Korandová & Frydrychová 2016; Koubová et al. 2021). The difference seen in the bees in our study may be due to the difference in life histories and biology of solitary bees compared to social bees. Solitary bees undergo developmental diapause, whereas honey bees do not (Kapheim 2017). While some *M. rotundata* skip diapause and emerge as a second generation in the summer, those bees were not used in this study. Solitary bees typically live for about one year, most of this time is in a quiescent state associated with overwintering.

Periodic developmental arrest such as hibernation, diapause, or quiescence, can slow or reverse aging. Arrested development during the dauer stage (diapause), *Caenorhabditis elegans* increases longevity and reverse signs of senescence including decrease of metabolic capacity. These signs of aging occur at lower levels in the dauer *C. elegans*, and they can reverse or go away once the organism resumes development (Houthoofd et al., 2002). Hibernating mice have increased telomere length during the active season after hibernation. Rodents that hibernate have longer lifespans than ones that do not (Turbill et al., 2011; Wilkinson and South 2002). The increased telomere length and reduced senescence may be due to somatic maintenance that occurs during hibernation (Turbill et al., 2013). Insects that go through diapause can also have reduced signs of senescence. *D. melanogaster* adults that go through a reproductive quiescence age slower than those that do not, and they show low mortality rates similar to newly eclosed adults (Tatar et al., 2001). Our study demonstrates increases in telomere length in both bee

species after exposure to warm temperatures trigger emergence from the quiescent state. In *M. rotundata*, we see that the prepupal stage has significantly shorter telomeres than other stages. The telomere length in prepupal bees was measured before being placed in warm temperatures, which initiates emergence from diapause. Telomere length in all other life stages in *M. rotundata* were measured after the bees had begun post-quiescent development. The same can be seen in the case of *O. lignaria*. Telomere length increases in the diapausing and emerged adult bees, and this increase occurs during diapause and after the bees were in a post quiescent state. We see a decline in telomere length in the post diapause quiescence stage. This indicates that telomere maintenance is upregulated during diapause, and then again later when the bees emerge. Telomere length appears to be maintained throughout the lifespan of post-emergence adult *O. lignaria*. We did not see declines in telomere length in days post emergence, even when exposed to a stress treatment. This may indicate that telomeres post-quiescence are sufficiently long, or are actively being maintained.

We did not see differences in telomere length based on weight or sex in either species. This is a surprising result because in both species females typically live longer than males. In field conditions, *Osmia* females live for 20 days post emergence (Bosch and Vicens, 2006), and males typically live less than 20 days. With females being the longer-lived of the sexes, we would expect telomere length to be longer in those individuals. In the ant species *Lasius niger*, females live longer and have longer telomeres than males (Jemielity et al. 2007). The sex differences seen in that study may be due to caste differences and extreme differences in longevity between males and females. It was also surprising to see that telomere length did not differ based on body size in this study. Within other species, smaller individuals age at a slower rate than larger individuals (Kraus et al. 2013). We might not see differences in telomere length

based on sex or size because the timing of telomere restoration in these bees occurs right before emergence. It appears from our results in adult *O. lignaria* that telomere length does not change in emerged adults.

In contrast to other studies, telomere length is positively correlated with age in our study. This study demonstrates that telomere loss that occurs during development can be reversed when development resumes. Telomere length was maintained in the adult stage in *O. lignaria* which may indicate that this reverse in cellular senescence is maintained in adult stages. Our results show that telomere length is not connected to chronological age and is not predictive of senescence. While it is clear that telomeres are playing a role in senescence in many organisms, it may not be representative of aging demonstrating that telomeres may play a different role in solitary bees.

References

- AihieSayer A., Osmond C., Briggs R, Cooper C.** (1999). Do all systems age together?
Gerontology **45**, 83–86
- Allsopp, R. C., Vaziri, H., Patterson, C., Goldstein, S., Younglai, E. V., Futcher, A. B., Greider, C. W. & Harley, C. B.** (1992). Telomere length predicts replicative capacity of human fibroblasts. *Proc. Natl Acad. Sci.* **89**, 10114–10118.
- Aubert G, Lansdorp PM.** (2008). Telomeres and aging. *Physiol. Rev.* **88**, 557–579.
- Bates, Douglas, Martin Maechler, Ben Bolker, Steve Walker** (2015). Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software*, 67, 1-48.
- Bartoń, Kamil** (2020). MuMIn: Multi-Model Inference. R package version 1.43.17.
<https://CRAN.R-project.org/package=MuMIn>.

- Blackburn, E. H., Epel, E. S., & Lin, J.** (2015). Human telomere biology: a contributory and interactive factor in aging, disease risks, and protection. *Science*, **350**, 1193-1198.
- Blackburn EH** (2005). Telomeres and telomerase: Their mechanisms of action and the effects of altering their functions. *FEBS Lett.* **579**,859–862.
- Boonekamp J.J., Simons M.J., Hemerik L., Verhulst S.** (2013). Telomere length behaves as biomarker of somatic redundancy rather than biological age. *Aging Cell.* **12**, 330–332.
- Bosch, J., Sgolastra, F., Kemp, W.P.** (2010). Timing of eclosion affects diapause development, fat body consumption and longevity in *Osmia lignaria*, a univoltine, adult-wintering solitary bee. *J. Insect Physiol.* **56**, 1949–1957.
- Bosch, J., & Kemp, W. P.** (2001). How to manage the blue orchard bee. *Sustainable Agriculture Network, Beltsville, MD.*
- Bosch, J., & Vicens, N.** (2006). Relationship between body size, provisioning rate, longevity and reproductive success in females of the solitary bee *Osmia cornuta*. *Behav. Ecol. Sociobiol.*, **60**, 26-33.
- Bozina, K.D.,** (1961). How long does the queen live?. *Pchelovodstvo* **38**, 13.
- Bronikowski, A., & Vleck, D.** (2010). Metabolism, Body Size and Life Span: A Case Study in Evolutionarily Divergent Populations of the Garter Snake (*Thamnophis elegans*). *Integr Comp Biol*, **50**, 880–887.
- Campisi, J.** (2001). Cellular senescence as a tumor-suppressor mechanism. *Trends cell Biol*, **11**, S27-S31.
- Campisi, J.** (2005). Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors. *Cell* **120**, 513–522.

- Cartar, R. V.** (1992). Morphological senescence and longevity: an experiment relating wing wear and life span in foraging wild bumble bees. *J. Anim Ecol*, 225-231.
- Cawthon, R. M.** (2002). Telomere measurement by quantitative PCR. *Nucleic acids research*, 30, e47-e47.
- Colman, R.J., Anderson, R.M., Johnson, S.C., Kastman, E.K., Kosmatka, K.J., Beasley, T.M., Allison, D.B., Cruzen, C., Simmons, H.A., Kemnitz, J.W. and Weindruch, R.** (2009). Caloric restriction delays disease onset and mortality in rhesus monkeys. *Science* 325, 201-204.
- di Fagagna, Fabrizio d'Adda, Philip M. Reaper, Lorena Clay-Farrace, Heike Fiegler, Philippa Carr, Thomas Von Zglinicki, Gabriele Saretzki, Nigel P. Carter, and Stephen P. Jackson.** (2003). A DNA damage checkpoint response in telomere-initiated senescence. *Nature*, 426, 194.
- D'Amico, L. J., Davidowitz, G., and Nijhout, H. F.** (2001). The developmental and physiological basis of body size evolution in an insect. *Proc. R. Soc. Lond. B.* 268, 1589–1593.
- Dinno, A.,** (2017). dunn.test: Dunn's Test of Multiple Comparisons Using Rank Sums.
<https://CRAN.R-project.org/package=dunn.test>
- Eisenberg, T., Knauer, H., Schauer, A., Büttner, S., Ruckstuhl, C., Carmona-Gutierrez, D., Ring, J., Schroeder, S., Magnes, C., Antonacci, L. and Fussi, H.,** (2009). Induction of autophagy by spermidine promotes longevity. *Nat cell biol*, 11, 1305-131
- Epel, E. S., Blackburn, E. H., Lin, J., Dhabhar, F. S., Adler, N. E., Morrow, J. D., & Cawthon, R. M.** (2004). Accelerated telomere shortening in response to life stress. *P Natl A Sci*, 101, 17312-17315.

- Fick, L., Fick, G., Li, Z., Cao, E., Bao, B., Heffelfinger, D., Parker, H. Ostrander, E. Riabowol, K.** (2012). Telomere Length Correlates with Life Span of Dog Breeds. *Cell Reports*, **2**, 1530–1536.
- Frydrychová, R., Grossmann, P., Trubac, P., Vítková, M., & Marec, F. E.** (2004). Phylogenetic distribution of TTAGG telomeric repeats in insects. *Genome*, **47**, 163-178.
- Giraldo, Y. M., & Traniello, J. F.** (2014). Worker senescence and the sociobiology of aging in ants. *Behav ecol sociobiol*, **68**, 1901-1919.
- Gunn D.A., Rexbye H., Griffiths C.E., Murray P.G., Fereday A., Catt S.D., Tomlin C.C., Strongitharm, B.H., Perrett D.I., Catt M.** (2009). Why some women look young for their age. *PLoS ONE*. **4**, e8021
- Harley C.B., Futcher A.B., Greider C.W.** (1990). Telomeres shorten during ageing of human fibroblasts. *Nature*. **345**, 458–460.
- Hartmann, N., Reichwald, K., Lechel, A., Graf, M., Kirschner, J., Dorn, A., Terzibasi, E., Wellner, J., Platzer, M., Rudolph, K.L. and Cellerino, A.,** (2009). Telomeres shorten while Tert expression increases during ageing of the short-lived fish *Nothobranchius furzeri*. *Mech Ageing Dev*. **130**, 290–296
- Hausmann, M.F., Winkler, D.W., O'Reilly, K.M., Huntington, C.E., Nisbet, I.C. and Vleck, C.M.** (2003). Telomeres shorten more slowly in long-lived birds and mammals than in short-lived ones *P Natl A Sci*, **270**, 1387–1392.
- Hausmann, M. F., Winkler, D. W. & Vleck, C. M.** (2005). Longer telomeres associated with higher survival in birds. *Biol. Lett.* **1**, 212–214.
- Hausmann M.F., Mauck R.A.,** (2008). New strategies for telomere-based age estimation. *Mol Ecol Resour* **8**, 264–274.

- Hausmann M.F., Mauck R.A.,** (2008). Telomeres and longevity: Testing an evolutionary hypothesis. *Mol Biol Evol*, **25**, 220–228.
- Hausmann MF, Vleck CM,** (2002). Telomere length provides a new technique for aging animals. *Oecologia*, **130**, 325–328.
- Haycock P.C., Heydon E.E., Kaptoge S., Butterworth A.S., Thompson A., Willeit, P.** (2014). Leucocyte telomere length and risk of cardiovascular disease: systematic review and meta-analysis. *BMJ*. **349**
- Heidinger, B. J., Blount, J. D., Boner, W., Griffiths, K., Metcalfe, N. B., & Monaghan, P.** (2012). Telomere length in early life predicts lifespan. *P Natl A Sci*, **109**, 1743-1748.
- Helm, B. R., Payne, S., Rinehart, J. P., Yocum, G. D., Bowsher, J. H., & Greenlee, K. J.** (2018). Micro-computed tomography of pupal metamorphosis in the solitary bee *Megachile rotundata*. *Arthropod struct dev*, **47**, 521-528.
- Herbig, U., Ferreira, M., Condell, L., Carey, D. & Sedivy, J. M.** (2006). Cellular senescence in aging primates. *Science*, **311**, 1257
- Hothorn, Torsten, Frank Bretz and Peter Westfall** (2008). Simultaneous Inference in General Parametric Models. *Biometrical Journal*, **50**, 346-363
- Houthoofd, K., Braeckman, B. P., Lenaerts, I., Brys, K., De Vreese, A., Van Eygen, S., & Vanfleteren, J. R.** (2002). Ageing is reversed, and metabolism is reset to young levels in recovering dauer larvae of *C. elegans*. *Expl gerontol*, **37**, 1015-1021.
- Jemielity, S., Kimura, M., Parker, K. M., Parker, J. D., Cao, X., Aviv, A., & Keller, L.** (2007). Short telomeres in short-lived males: what are the molecular and evolutionary causes?. *Aging cell*, **6**, 225-233.

- Jennings, B. J., Ozanne, S. E., Dorling, M. W. & Hales, C. N.** (1999). Early growth determines longevity in male rats and may be related to telomere shortening in the kidney. *FEBS Lett.* **448**, 4–8.
- Joeng, K. S., Song, E. J., Lee, K.-J. & Lee, J.** (2004). Long lifespan in worms with long telomeric DNA. *Nat. Genet.* **36**, 607–611.
- Kapheim, K. M.** (2017). Nutritional, endocrine, and social influences on reproductive physiology at the origins of social behavior. *Curr opin insect sci*, **22**, 62-70.
- Keller, L., & Jemielity, S.** (2006). Social insects as a model to study the molecular basis of ageing. *Exp geront*, **41**, 553-556.
- Korandová, M., & Frydrychová, R. Č.** (2016). Activity of telomerase and telomeric length in *Apis mellifera*. *Chromosoma*, **125**, 405-411.
- Koubová, J., Sáblová, M., Brejcha, M., Kodrík, D., & Frydrychová, R. Č.** (2021). Seasonality in telomerase activity in relation to cell size, DNA replication, and nutrients in the fat body of *Apis mellifera*. *Sci rep*, **11**.
- Koubová, Justina, Tomáš Jehlík, Dalibor Kodrík, Michala Sáblová, Peter Šima, Hana Sehadová, Radka Závodská, and Radmila Čapková Frydrychová.** (2019). Telomerase activity is upregulated in the fat bodies of pre-diapause bumblebee queens (*Bombus terrestris*). *Insect biochem molec* **115**, 103241.
- Kraus, C., Pavard, S., and Promislow, D.E.L.** (2013). The size-life span trade-off decomposed: why large dogs die young. *Am. Nat.* **181**, 492–505.
- Krtolica, A., Parrinello, S., Lockett, S., Desprez, P. Y., & Campisi, J.** (2001). Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. *P Natl A Sci*, **98**, 12072-12077.

- Le Bourg, É.** (2001). Oxidative stress, aging and longevity in *Drosophila melanogaster*. *FEBS letters*, **498**, 183-186.
- Lee, H. Y., Lee, S. H., & Min, K. J.** (2015). Insects as a model system for aging studies. *Entomol res*, **45**, 1-8.
- Lopez-Otin C, Blasco MA, Partridge L, Serrano M, Kroemer G.** (2013). The hallmarks of aging. *Cell* **153**, 1194–1217.
- Lund T.C., Glass T.J., Tolar J., Blazar B.R.** (2009). Expression of telomerase and telomere length are unaffected by either age or limb regeneration in *Danio rerio*. *PLoS ONE* **4**, e7688.
- McFarlane, J. E.** (1967). Aging in an adult insect heart. *Can J zoolog*, **45**, 1073-1081.
- Michener, C. D.** (2000). *The bees of the world* (Vol. 1). JHU press.
- Miller, R., Harper, J., Galecki, A., Burke, D., & Miller, R.** (2002). Big mice die young: early life body weight predicts longevity in genetically heterogeneous mice. *Aging Cell*, **1**, 22–29.
- Mitnitski A., Song X., Rockwood K.** (2013). Assessing biological aging: the origin of deficit accumulation. *Biogerontology* **14**, 709–717.
- Monaghan P, Metcalfe NB, Torres R,** (2009). Oxidative stress as a mediator of life history trade-offs: Mechanisms, measurements and interpretation. *Ecol Lett*, **12**, 75–92.
- Nussey, D.H., Pemberton, J.M., Pilkington, J.G., and Blount, J.D.** (2009). Life history correlates of oxidative damage in a free-living mammal population. *Funct. Ecol.* **23**, 809–817.
- Olsson M, Pauliny A, Wapstra E, Blomqvist D.** (2010). Proximate determinants of telomere length in sand lizards (*Lacerta agilis*). *Biol. Lett.* **6**, 651–653.

- Pauliny, A., Wagner, R., Augustin, J., Szep, T. & Blomqvist, D.** (2006). Age-independent telomere length predicts fitness in two bird species. *Mol. Ecol.* **15**, 1681–1687.
- Pitts-Singer, T. L., & Cane, J. H.** (2011). The alfalfa leafcutting bee, *Megachile rotundata*: the world's most intensively managed solitary bee. *Annu rev entomol*, **56**, 221-237.
- Prowse, K. R., & Greider, C. W.** (1995). Developmental and tissue-specific regulation of mouse telomerase and telomere length. *P Natl A Sci*, **92**, 4818-4822.
- Robertson, H. M., & Gordon, K. H.** (2006). Canonical TTAGG-repeat telomeres and telomerase in the honey bee, *Apis mellifera*. *Genome res*, **16**, 1345-1351.
- Rudolph, K. L., Chang, S., Lee, H. W., Blasco, M., Gottlieb, G. J., Greider, C. & DePinho, R. A.** (1999). Longevity, stress response, and cancer in aging telomerase-deficient mice. *Cell* **96**, 701–712.
- Sahin, E., Colla, S., Liesa, M., Moslehi, J., Müller, F.L., Guo, M., Cooper, M., Kotton, D., Fabian, A.J., Walkey, C. and Maser, R.S.** (2011). Telomere dysfunction induces metabolic and mitochondrial compromise. *Nature*, **470**, 359-365.
- Selman, C., Nussey, D., & Monaghan, P.** (2013). Ageing: It's a Dog's Life. *Curr Biol* **23**, R451–R453.
- Seehuus, S. C., Krekling, T., & Amdam, G. V.** (2006). Cellular senescence in honey bee brain is largely independent of chronological age. *Exp gerontol*, **41**, 1117-1125.
- Seluanov A, Chen Z, Hine C, Sasahara TH, Ribeiro AA, Catania KC, Presgraves DC, Gorbunova V.** (2007). Telomerase activity coevolves with body mass not lifespan. *Aging Cell* **6**, 45–52.

- Sgolastra, F., Bosch, J., Molowny-Horas, R., Maini, S., Kemp, W.P.,** (2010). Effect of temperature regime on diapause intensity in an adult-wintering Hymenopteran with obligate diapause. *J Insect Physiol* **56**, 185–194.
- Sgolastra, F., Kemp, W. P., Maini, S., & Bosch, J.** (2012). Duration of prepupal summer dormancy regulates synchronization of adult diapause with winter temperatures in bees of the genus *Osmia*. *J Insect Physiol*, **58**, 924-933.
- Sohal, R. S.** (1976). Aging changes in insect flight muscle. *Gerontology*, **22**, 317-333.
- Takai, H., Smogorzewska, A., & de Lange, T.** (2003). DNA damage foci at dysfunctional telomeres. *Curr Biol*, **13**, 1549-1556.
- Tatar, M., Chien, S. A., & Priest, N. K.** (2001). Negligible senescence during reproductive dormancy in *Drosophila melanogaster*. *Am Nat*, **158**, 248-258.
- Torchio, P. F., & Tepedino, V. J.** (1980). Sex ratio, body size and seasonality in a solitary bee, *Osmia lignaria propinqua* Cresson (Hymenoptera: Megachilidae). *Evolution*, 993-1003.
- Tricola, G.M., Simons, M.J., Atema, E., Boughton, R.K., Brown, J.L., Dearborn, D.C., Divoky, G., Eimes, J.A., Huntington, C.E., Kitaysky, A.S. and Juola, F.A.,** (2018). The rate of telomere loss is related to maximum lifespan in birds. *Philos T Soc B*, **373**, 20160445.
- Turbill, C., Bieber, C., Ruf, T.** (2011). Hibernation is associated with increased survival and the evolution of slow life histories among mammals. *Proc.R. Soc.B* **278**, 3355–3363.
- Turbill, C., Ruf, T., Smith, S., & Bieber, C.** (2013). Seasonal variation in telomere length of a hibernating rodent. *Biology Lett*, **9**, 20121095.

- Vaziri, H., Dragowska, W., Allsopp, R. C., Thomas, T. E., Harley, C. B., & Lansdorp, P. M.** (1994). Evidence for a mitotic clock in human hematopoietic stem cells: Loss of telomeric DNA with age. *Proc Natl Acad Sci*, **91**, 9857–9860.
- Von Zglinicki, T.** (2003). Replicative senescence and the art of counting. *Exp gerontol*, **38**, 1259-1264.
- Weindruch, R.** (1996). The retardation of aging by caloric restriction: studies in rodents and primates. *Toxicol pathol*, **24**, 742-745.
- Wentzensen I.M., Mirabello L., Pfeiffer R.M., Savage S.A.** (2011). The association of telomere length and cancer: a meta-analysis. *Cancer Epidemiol. Prev. Biomarkers* **20**, 1–13.
- Wickham, H.**, (2016). Ggplot2: Elegant Graphics for Data Analysis.
<https://ggplot2.tidyverse.org>,
- Wilbourn, R. V., Moatt, J. P., Froy, H., Walling, C. A., Nussey, D. H., & Boonekamp, J. J.** (2018). The relationship between telomere length and mortality risk in non-model vertebrate systems: a meta-analysis. *Philos T R Soc*, **373**, 20160447.
- Wilkinson, G.S, South, J.M.** (2002). Life history, ecology and longevity in bats. *Aging Cell* **1**, 124–13.
- Willeit, P., Raschenberger, J., Heydon, E.E., Tsimikas, S., Haun, M., Mayr, A., Weger, S., Witztum, J.L., Butterworth, A.S., Willeit, J. and Kronenberg, F.** (2014). Leucocyte telomere length and risk of type 2 diabetes mellitus: new prospective cohort study and literature-based meta-analysis. *PLoS ONE* **9**, e112483.

Williams, C. M., Barnes, L. A., & Sawyer, W. H. (1943). The utilization of glycogen by flies during flight and some aspects of the physiological ageing of *Drosophila*. *Biol Bull*, **84**, 263-272.

Wong KK, Maser RS, Bachoo RM et al. (2003). Telomere dysfunction and Atm deficiency compromises organ homeostasis and accelerates ageing. *Nature*, **421**, 643–648

CHAPTER 4: DETERMINING THE CUE FOR METAMORPHOSIS IN THE SOLITARY BEE *MEGACHILE ROTUNDATA*

Abstract

Body size is important because it correlates to performance and fitness. Adult body size in insects is determined during the larval stage when majority of feeding and growth occurs. Larval feeding stops directly prior to the start of metamorphosis, and adult body size becomes fixed. The physiological regulation of growth and body size has been well studied in the model organisms *Manduca sexta* and *Drosophila melanogaster*, but few studies have determined the developmental basis of size in other insect species. Our goal was to determine the regulation and timing of metamorphosis in the solitary bee *Megachile rotundata*. We measured the critical weight, which is considered a proxy for the physiological commitment to metamorphosis. To confirm critical weight initiates the start of metamorphosis, we measured Juvenile Hormone (JH) titers and genes involved in JH synthesis and response. We also measured genes involved in ecdysone synthesis and response, and genes involved in the ISS/TOR pathway. We found that *M. rotundata* has a critical weight, and that this critical weight initiates the start of metamorphosis. This metamorphic commitment was demonstrated by declines in JH titers, and the upregulation of genes involved in ecdysone synthesis, and genes in the ISS/TOR pathway which are involved in pupal commitment.

Introduction

In holometabolous insects, adult body size is determined by growth during the larval period and directly influenced by the size at metamorphosis. Body size in adults is fixed due to sclerotization of the cuticle (Sehnal, 1985, Nijhout et al., 2006). Larval growth rate and duration are both important for determining final adult size. Achieving adult size involves determining the

rate of change in larval growth over time (growth rate), as well as when larval growth stops, and the insect enters metamorphosis (growth duration) (Nijhout, 1975; Nijhout and Williams, 1974). The rate and duration of larval growth are regulated by different pathways. Larval growth rate is regulated by the nutrient sensing pathways including the insulin/insulin-like growth factor sensing pathway (ISS) and target of rapamycin (TOR) pathway. (Layalle et al. 2008; Mirth and Shingleton, 2012; Ohhara et al., 2017). Larval growth duration is influenced by titers of juvenile hormone (JH) and ecdysone (or 20-Hydroxyecdysone) which influence the end of the larval growth period and beginning of metamorphosis.

There are different ways in which an insect senses its size and determines when to begin a metamorphic molt. One way a molt is induced is via stretch receptors, which has been demonstrated in *Rhodnius prolixus*. If the volume of a blood meal is beyond a critical volume, the abdomen of the insect will become enlarged and the stretch receptors will trigger a molt (Anwyl 1972). In other insects, size is assessed through oxygen limitation, the size of the Prothoracic Gland, and imaginal disc size (Mirth et al. 2005; Callier and Nijhout 2011). The mechanism for sensing size has not been determined for all insects, and there is likely variability in how insects determine size.

There are three factors which influence adult body size and are proxies for the physiological commitment to metamorphosis including: the critical weight, the terminal growth period, and the growth rate during the terminal growth period. The critical weight refers to the size at which the larva is physiologically committed to metamorphosis (Davidowitz et al. 2003). The critical weight has been well-studied in the model species *Manduca*, and *Drosophila*, although regulation of the critical weight and the response to starvation differs between the two. The terminal growth period occurs between reaching the critical weight and growth cessation

(Shingleton et al., 2007; Davidowitz et al. 2003; Nijhout et al., 2014). In some insects, the minimum viable weight is used as a proxy for the critical weight. The minimum viable weight is the minimum size at which a larva can successfully molt to the pupal stage (Mirth et al., 2005; De Moed et al., 1999; Ohhara et al., 2017). Not all insects use a critical weight to regulate metamorphosis. The solitary bee *Osmia lignaria*, for example, does not rely on a critical weight, but rather the cue for metamorphosis is the absence of food (Helm et al., 2017).

The critical weight is associated with hormonal changes that prepare the insect for metamorphosis. In *Manduca*, when the critical weight is reached, JH titers will begin to decline (Browder et al. 2001). JH titers during the last larval instar are relatively low in *Drosophila*, and JH does not affect timing of metamorphosis (Riddiford et al., 2010). The critical weight in *Drosophila* is regulated by the insulin/TOR signaling in the Prothoracic Gland, which regulates ecdysone synthesis (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005; Layalle et al., 2008). The release of Prothoracicotropic Hormone (PTTH) begins the molting process. PTTH will then stimulate the release of ecdysone from the prothoracic glands. Small increases in ecdysone during the last larval instar will begin to prepare the insect for metamorphosis (Warren et al., 2006), and a high pulse of ecdysone at the end of the instar, post-critical weight, will trigger the molt from larva to pupa (the metamorphic molt) (Riddiford, 1993).

Megachile rotundata (F.) is a holometabolous solitary bee. In *M. rotundata*, the prepupal stage occurs once the larva has consumed all of its provision, defecated, and spun its cocoon (Kemp & Bosch, 2000). The cocoon is made of silk-like strands from the mandibles. At this point, a prepupa will enter a diapause period which will continue throughout the winter, or they will continue their development and emerge as adults the same summer (Kemp & Bosch, 2000). This commitment to diapause in *M. rotundata* may influence the metamorphic pathway because some

bees are not going directly into the pupal phase. Although the physiological commitment of the pupal molt will occur before differentiation takes place (Klowden, 2003).

The goal of this study was to determine if a starvation period initiates metamorphosis in the solitary bee *M. rotundata* based on previous findings from Helm et al., 2017. We manipulated nutritional quantity to compare rate of entry into metamorphosis. We measured entry into metamorphosis in three ways, first determining if there is a critical weight by measuring time to growth cessation. Second, we measured JH titers over time after either a starvation or *ad libitum* feeding treatment. Finally, we measured gene expression under those same conditions. We measured genes involved in JH synthesis and response, ecdysone synthesis and response, and genes involved in the ISS/TOR pathway. We predicted the cue for entry into metamorphosis would be starvation, and there will be no critical weight. Second, we predicted JH titers would decrease with time, and would have a steeper slope in the starved treatment, indicating food removal initiates metamorphosis. Finally, we predicted the genes involved in metamorphosis would be upregulated sooner in the starved treatment.

Methods

Bee Rearing. *M. rotundata* nests containing larval bees were collected from a polystyrene nesting board (Megablock, Beaver Plastics, Canada) during the summers of 2019 and 2020 in Fargo, ND. Adult bees used for nesting were sourced from JWM Leafcutters, Inc. of Nampa, ID, US. Each nesting straw was removed from the nesting board, brought back to the laboratory, and the brood cells were removed and placed into 96-well plate. The cap of the brood cell was removed to monitor larval development.

Feeding Treatments. At the final (fifth) instar larval stage, as described by Trostle and Torchio (1994), larval food quantity was manipulated to determine if the removal of food is the

cue for metamorphosis. Bees were placed into three feeding treatments which included the fed treatment in which bees were fed an *ad libitum* amount of food which was approximately triple amount of pollen (2019 average = $0.1079 \text{ g} \pm 0.0096$) (2020 average = $0.0960 \text{ g} \pm 0.003$), control in which the amount of provision was not manipulated (2019 average = 0.0301 ± 0.0016) (2020 average = $0.0339 \text{ g} \pm 0.002$), and the starved, in which the entire provision was removed. Larval bees along with the pollen provided, were placed into 24-well plates with specialized 3D printed inserts to provide sufficient room for growth, as well as have the proper diameter for web spinning. Bees were weighed immediately prior to being placed into treatment. Bees were incubated at 25°C with 75% humidity maintained in darkness in an environmental chamber to complete larval development then stored as prepupae in a 6°C chamber to overwinter beginning in 11/2/20. Development resumed after being placed in warm temperatures on 2/22/21.

Growth Cessation and Critical Weight. Bees were placed into feeding treatment as mentioned above (fed n=156, control n=156, starved n=156). After bees were placed into treatment, their development was monitored under a microscope after 2, 4, 8, 12, 24, 36, 48, 72, and 96 hours. The time of cession of growth was determined by when the individual showed the first signs of web spinning, as indicated by silk strands being produced from the mandible of the bee. We considered web spinning to be when the bee begins to construct their cocoon. This is an indicator of entry into the prepupal stage (Kemp & Bosch, 2000). In 2019 bees were placed into treatment the same day they were in the fifth instar, in 2020 bees were placed into treatment during the fifth instar, but not the first day. Time to growth cessation was determined by the time after treatment that the bee began web spinning.

Juvenile Hormone Titters. Bees were placed into feeding treatments as mentioned above. We collected hemolymph from the larva 0, 2, 4, 8, 12, 18, 24, 36, 48, 72, and 96 hours

post treatment. Hemolymph was collected using Drummond PCR (glass) Micropipettes (Drummond Scientific Company, Broomdal, PA). HPLC-grade methanol was added, and samples were dried under nitrogen. Samples were stored in -20°C freezer for later analysis. Upon analysis, HPLC-grade methanol was added back to the samples and samples were then spiked with Methoprene (company name), a JH analogue. Methoprene was added in consistent concentrations to each sample to be used as a control. Samples were then run through a high-performance liquid chromatography (HPLC) which detected JHIII homologue. Instrument: Waters ACQUITY TQD – Tandem Quadrupole Detector, Waters ACQUITY UPLC – Classic. Columns used: Phenomenex Synergi Hydro RP 30x4.6mm 4 μ (Pt. #: 00A-4375-E0) & Phenomenex Security Guard C18 Guard Column (Pt. #: AJO-4286). JH III homologue was used because it is found in most insect orders and is considered to be the simplest form of JH. JH titers were measured to determine how they changed over time in response to feeding treatments.

Gene Expression. Bees were placed into feeding treatments as mentioned above. Final instar larva were collected 0, 2, 4, 8, 12, 18, 24, 36, 48, 72, and 96 hours post treatment (fed or starved) and were flash frozen on liquid nitrogen. Bees were stored in -80C. RNA extraction was performed using Machery-Nagel NucleoSpin® Set for NuceloZOL kit, and purity of RNA on a spectrophotometer. RNA was diluted and loaded into a 96 well plate. Samples were then sent to Minnesota Genomics Center (Minneapolis, MN) for processing. Gene expression was quantified using an nCounter Analysis System (NanoString Technologies Inc.) with a custom code set for the 34 *M. rotundata* target genes. Gene expression was normalized using the geometric mean of eight control genes, which were chosen because they were not differentially expressed across multiple RNAseq datasets in *M. rotundata* (Yocum et al. 2018, Torson et al. 2015, Torson et al. 2017, Melicher et al. 2019).

Statistical Analysis. All statistical analyses were performed using R statistical software (version 3.6.1, Base R package) and graphed using the package *ggplot2* (Wickham, 2016). To determine the effect of feeding treatments (starved, control, fed) on time to growth cessation, we used an ANOVA. A pairwise comparison (Tukey post-hoc test) was used to determine which treatments were different from each other. To determine the critical weight, a break-point analysis was performed using the Davies test (Davies, 1987; Davies, 2002). This analysis determines a significant change in the slope. Next, we used the *segmented* package (Muggeo, 2003) to confirm the estimated breakpoint from the Davies test. To measure JH titers over time, we used linear regression. In order to determine differences in gene expression between treatments over time from nCounter results, a linear regression was performed, a quadratic regression was run if it was an appropriate fit for the model.

Results

Growth Cessation. Growth cessation was defined as when the bees began to spin their cocoons (Kemp & Bosch, 2000). We found that feeding treatment impacts time to growth cessation (Fig. 14, ANOVA, $F_{2,461} = 78.12$, $p < 0.001$). Bees in the starved treatment began growth cessation earlier than the control treatment ($p < 0.001$). The fed treatment began growth cessation later than both the starved and control treatments (fed v starved, $p < 0.001$) (fed v control, $p < 0.001$).

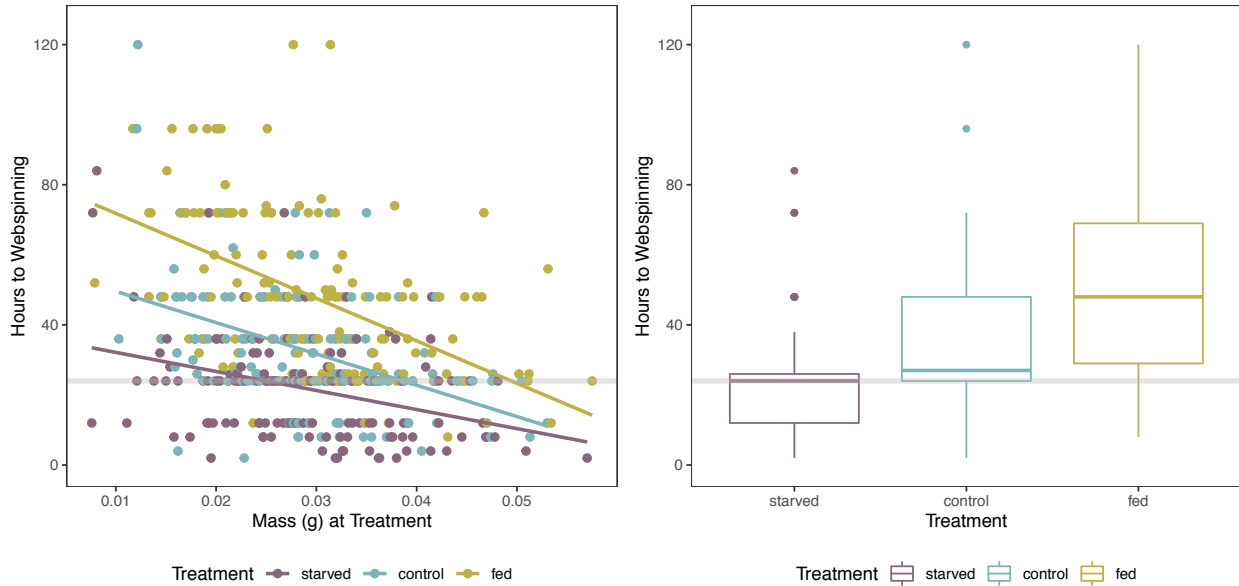


Fig. 14. Time to growth cessation

Time to growth cessation was measured as time to the start of web spinning. The horizontal grey line represents 24 hours. Results from ANOVA.

Critical Weight. Both the starved and control treatments had a significant critical weight breakpoint (Fig. 15, starved, Davies Test, $p= 0.034$) (Fig. 15, control, Davies Test, $p= 0.031$). The critical weight in the starved treatment was predicted to be $0.0125g\pm.002$. Segment analysis was performed to confirm the estimated breakpoint from the Davies test (starved, segment predicted breakpoint= $.0110g$). Fifty percent of bees in the starved treatment began web spinning at 24 hours. The critical weight in the control treatment was $0.0165g\pm.003$. Segment analysis was performed to confirm the estimated breakpoint from the Davies test (control, segment predicted breakpoint= $0.0162g$). Bees that were in the fed treatment did not have a significant break point (Fig. 15, fed, Davies test, $p = 0.124$). We were not able to calculate the minimum viable weight, which is the size at which 50% of larvae develop into adults (Davidowitz et al., 2003). Survival in the starved treatment was below 50%. Survival in the starved treatment was only 12.5%, survival in the fed treatment was 58.3%, survival in the control treatment was 61.1%.

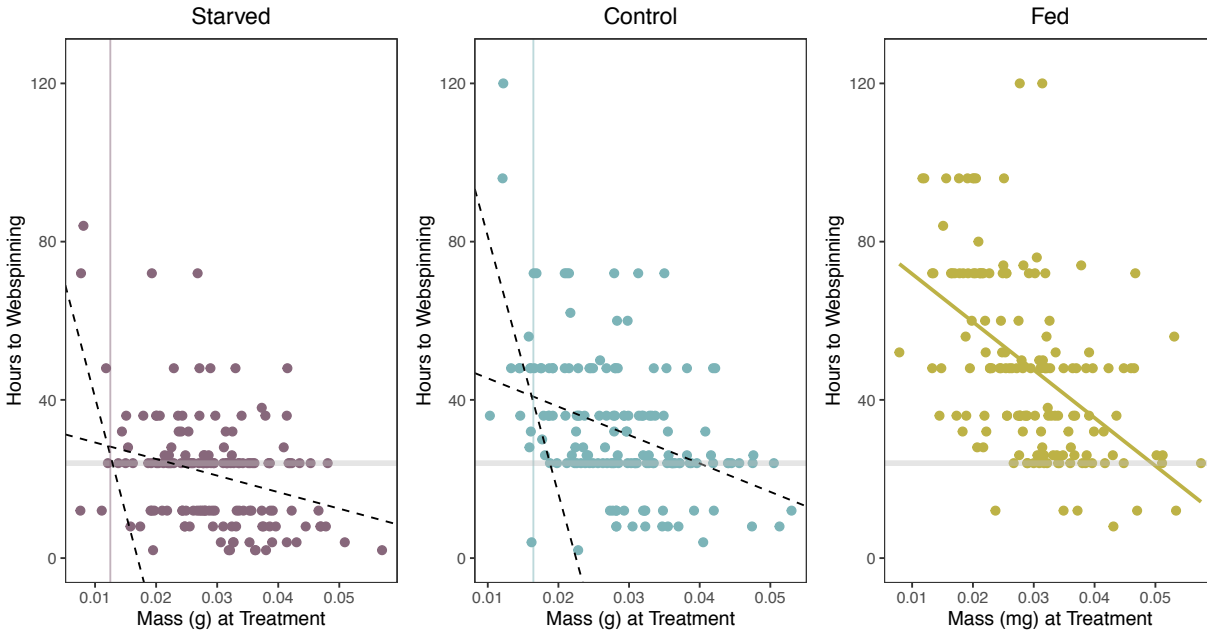


Fig. 15. Critical weight

Critical weight in the different feeding treatments. Results from Davies and segment breakpoint analysis. Horizontal grey line represents 24 hours. Dotted lines indicate the two separate slopes calculated by the segmented package. Vertical lines represent the critical weight.

Hormone Analysis. We found significant relationship between concentration of JH and time in the control treatment (Fig. 16, $p < .001$) and fed treatment (Fig 16, $p = .018$). We did not find a significant relationship between concentration of JH and time in the starved treatment (Fig 16, $p = 0.36$).

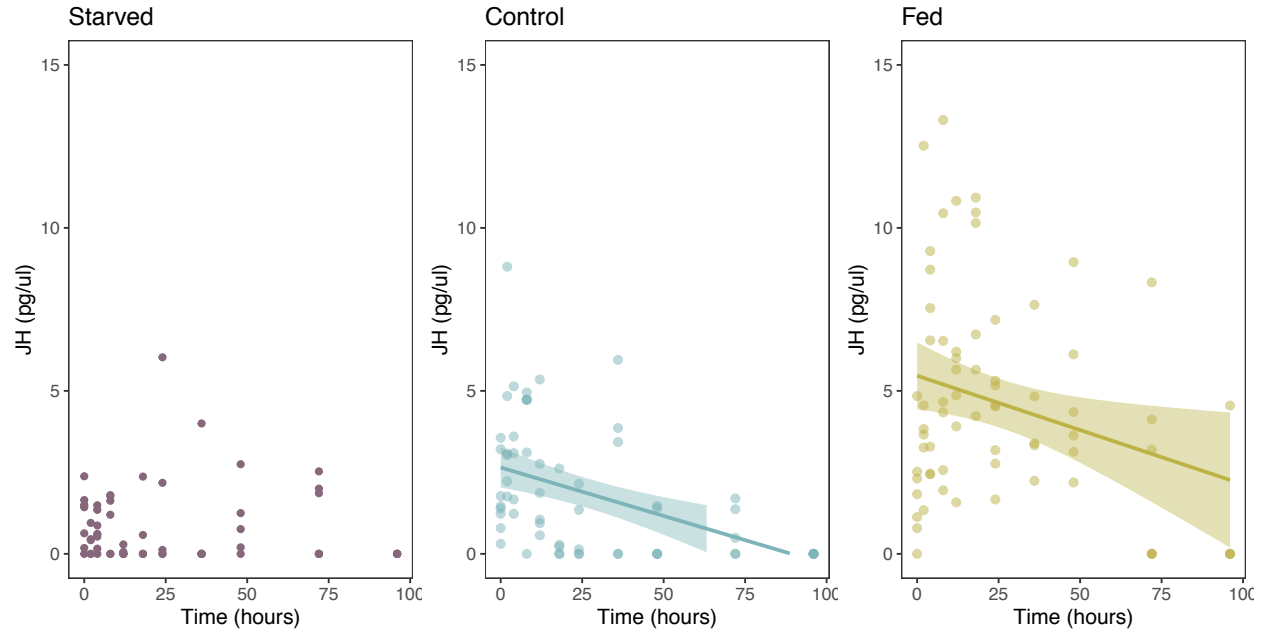


Fig. 16. Juvenile hormone titers in larvae across time
 Juvenile hormone titers from HPLC analysis. Results from linear model

Juvenile Hormone Gene Expression. Expression of genes involved in JH synthesis and response changed significantly with time (Table 6, Fig. 17), with the exception of *methyl farnesoate epoxidase / farnesoate epoxidase* (Mfe). Mfe, a gene involved in JH synthesis, was not affected by time or treatment (time: $p=0.291$, treatment: $p=0.549$). *Krüppel-homolog 1*, an early JH response gene was not affected by treatment ($Kr-h1$, $p=0.535$). Genes involved in JH synthesis including *aldehyde dehydrogenase* (Adh, $p=0.0215$), *farnesyl dehydrogenase* (fd, $p=0.001$), and *juvenile hormone methyl transferase* (jhamt, $p<0.001$) were all upregulated in the fed treatment. The JH receptor *Methoprene-tolerant* was upregulated in the starved treatment (Met, $p<0.001$) *Juvenile hormone epoxide hydrolase* (jhe), a gene involved in the breakdown of JH was upregulated in the fed treatment ($p=0.001$). *Farnesyl diphosphate phosphatase* (fppp, $p=0.090$) did not differ by treatment.

Ecdysone Gene Expression. Many genes associated with ecdysone synthesis and response changed significantly with time (Table 6, Fig. 18). Genes involved in ecdysone

synthesis and activation such as *disembodied* (Dib, $p < 0.001$), and *shade* (shd, $p < 0.001$) were upregulated in the starved treatment. Although *shadow* (sad, $p = 0.105$), and *26-hydroxylase* (heh, $p = 0.385$) were not significantly affected by feeding treatment. The ecdysone receptor (*Ecr*) and *Ultraspiracle* (*Usp*) increased with time but were not affected by feeding treatment (*Ecr* $p = 0.195$), (*Usp* $p = 0.246$). *PTTH* was not significantly affected by feeding treatment ($p = 0.223$). *Broad-complex* (BR-C), *brick-a-brack-tamtrack-broad* (BTB), *E93*, and *E74* which are involved in early ecdysone response and associated with pupal commitment, increased with time. BR-C ($p < 0.001$) was upregulated in the starved treatment. *E74* was upregulated in the fed treatment ($p < 0.001$), BTB ($p = 0.0324$) and *E93* ($p = 0.089$) was not differentially expressed by treatment but increased with time.

Insulin Pathway Gene Expression. Expression of genes involved in the insulin signaling pathway changed significantly with time (Table 6, Fig. 19), with the exception of *insulin-like growth factor 1* (*igf1*). *Igf1* was not affected by time ($p = 0.120$) but was significantly upregulated in the fed treatment ($p < 0.001$). *Serine-threonine protein kinase* (*Akt*, $p < 0.001$) and *phosphoinositide-3-kinase* (*Pi3k*, $p < 0.001$) were also upregulated in the fed treatment. The *insulin-like receptor* (*Inr*, $p < 0.001$), *Forkhead box protein O* (*FOXO*, $p < 0.001$) and *3-phosphoinositide-dependent protein kinase 1* (*pdk*, $p < 0.001$) were upregulated in the starved treatment. *Phosphatase and tensin homolog* (*PTEN*), a negative regulator of the insulin pathway, was not differentially expressed by treatment ($p = 0.224$).

Target of Rapamycin (TOR) Pathway. Expression of all genes involved in the Target of Rapamycin (TOR) pathway changed significantly with time (Table 6, Fig. 19). *Target of Rapamycin* (*TOR*, $p < 0.001$), and the regulator-associated protein of TOR (*Raptor* $p = 0.010$)

were upregulated in the starved treatment. and *ribosomal protein kinase 6* (s6k p=0.101)

expression did not differ by treatment.

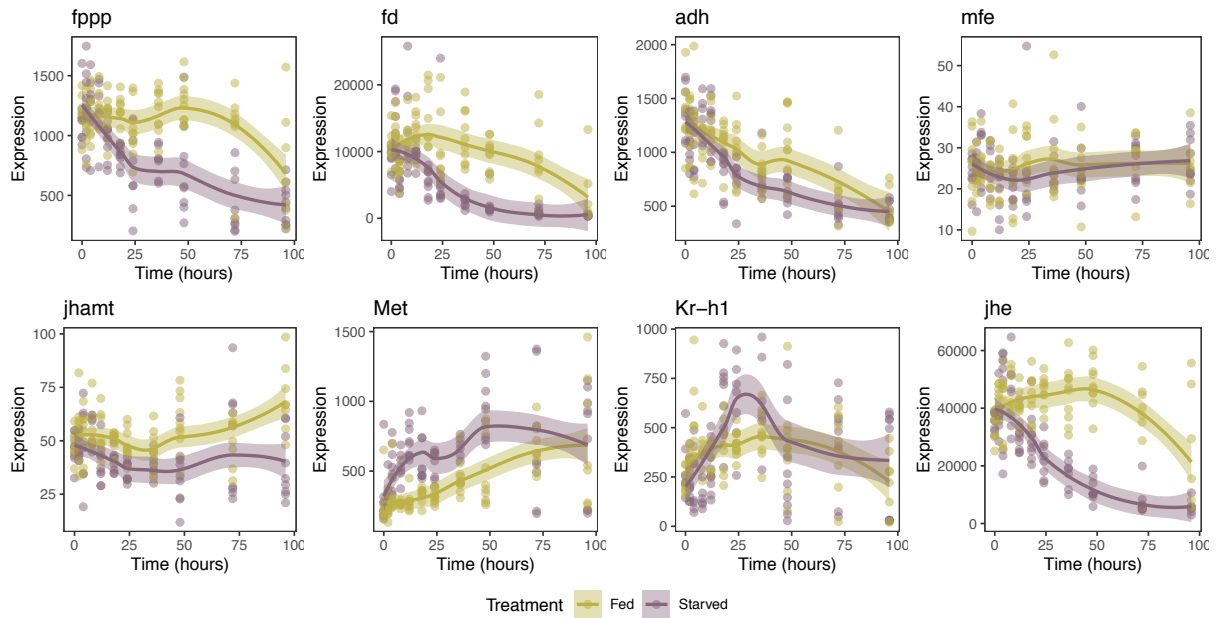


Fig. 17. Genes involved in JH synthesis and response

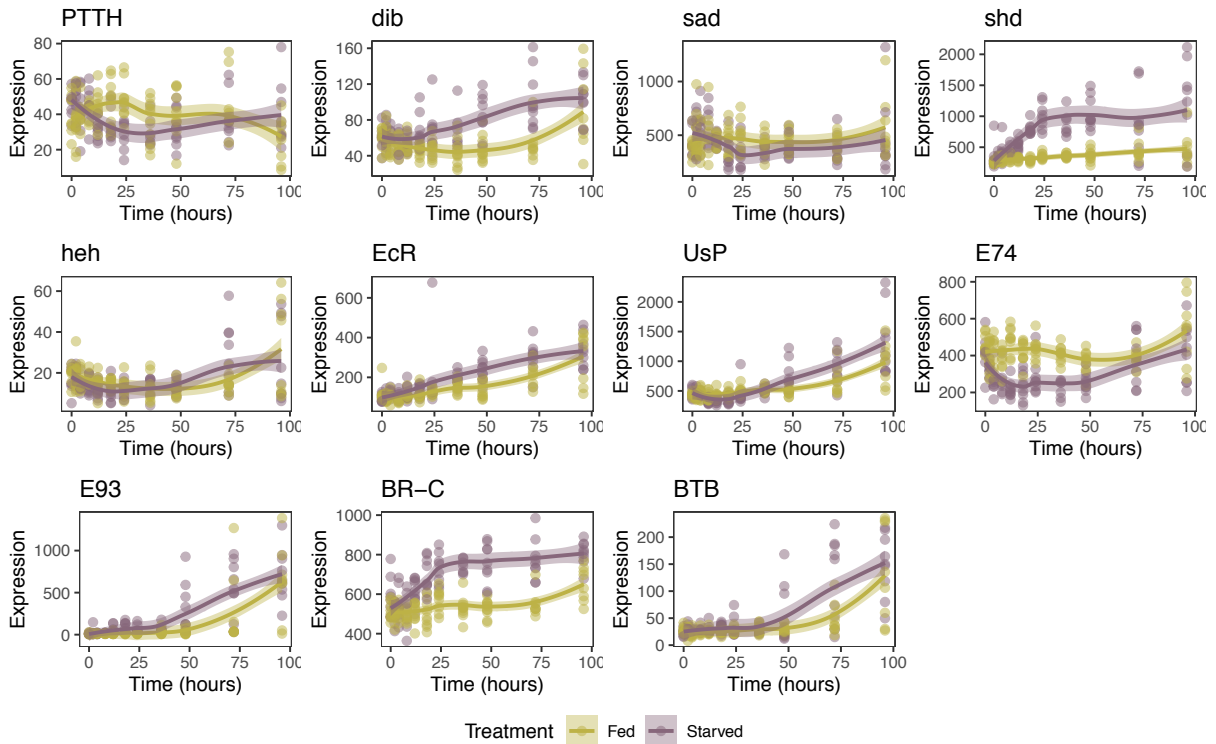


Fig. 18. Genes involved in Ecdysone synthesis and response

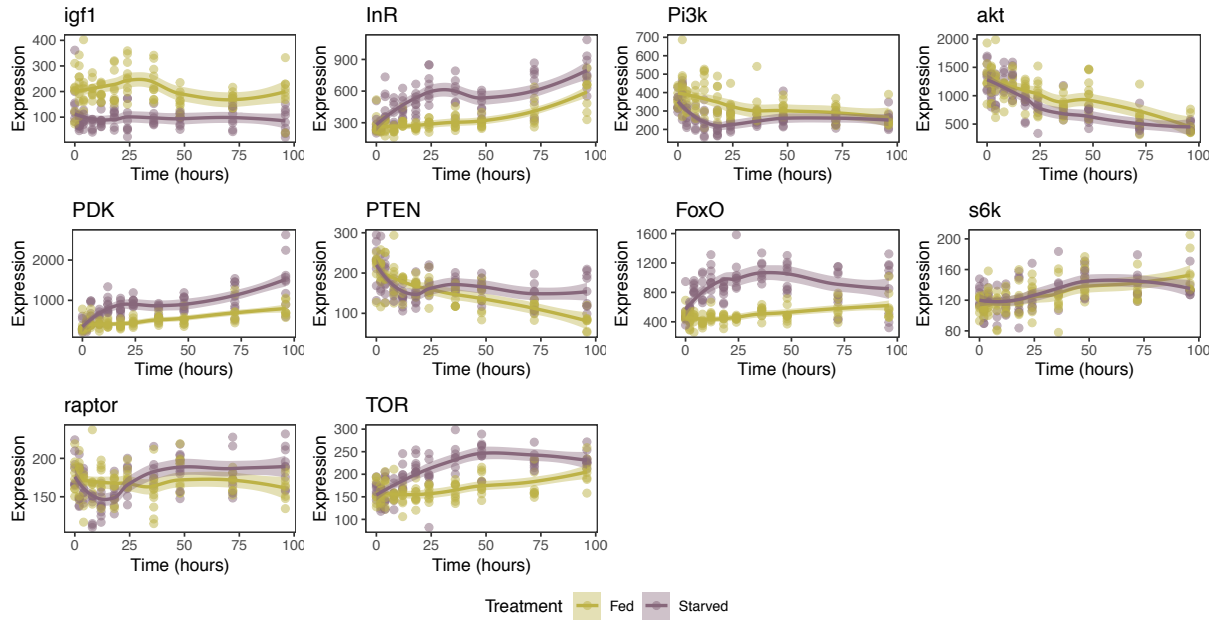


Fig. 19. Genes involved in the ISS/TOR pathway

Table 6. Gene expression data from NanoString analysis

Pathway	Gene Name	Model term	P value	Upregulation
Ecdysone	BRC	Hours	<0.001	Increase
		Treatment	<0.001	Starved
		Interaction	<0.001	***
	BTB	Hours+	<0.001	Increase
		Treatment	0.324	NS
		Interaction	0.014	**
	Dib	Hours+	<0.001	Increase
		Treatment	<0.001	Starved
		Interaction	0.0255	**
	E74	Hours+	<0.001	Increase
		Treatment	<0.001	Fed
		Interaction	0.099	
	E93	Hours+	<0.001	Increase
		Treatment	0.089	NS
		Interaction	0.069	
	Ecr	Hours	<0.001	Increase
		Treatment	0.195	NS
		Interaction	0.023	**
	Heh	Hours+	<0.001	Increase
		Treatment	0.385	NS
		Interaction	0.691	
	PTTH	Hours	0.138	NS
		Treatment	0.223	NS
		Interaction	0.699	
Sad	Hours+	0.016	Decrease	
	Treatment	0.105	NS	
	Interaction	0.104		
Shd	Hours+	0.052	NS	
	Treatment	<0.001	Starved	
	Interaction	<0.001	***	
Usp	Hours+	<0.001	Increase	
	Treatment	0.246	NS	
	Interaction	<0.001	***	
JH	adh	Hours+	0.036	Decrease
		Treatment	0.0215	Fed
		Interaction	0.815	
	Fd	Hours	<0.001	Decrease
		Treatment	0.001	Fed
		Interaction	0.020	**
	Fppp	Hours	<0.001	Decrease
		Treatment	0.090	NS
		Interaction	<0.001	***
	Jhamt	Hours+	0.020	Increase
		Treatment	<0.001	Fed
		Interaction	0.003	***
	Jhe	Hours	<0.001	Decrease
		Treatment	0.001	Fed
		Interaction	<0.001	***
	Krh1	Hours+	<0.001	Decrease
		Treatment	0.535	NS
		Interaction	0.225	
	Met	Hours	<0.001	Increase
		Treatment	<0.001	Starved
		Interaction	0.076	
	Mfe	Hours	0.291	NS
		Treatment	0.549	NS
		Interaction	0.948	

Table 6. Gene expression data from NanoString analysis (continued)

Pathway	Gene Name	Model term	P value	Upregulation
Insulin/TOR	Akt	Hours+	<0.001	Decrease Fed
		Treatment	<0.001	
		Interaction	0.132	
	FOXO	Hours	<0.001	Increase Starved
		Treatment	<0.001	
		Interaction	0.256	
	Igf1	Hours	0.120	NS Fed
		Treatment	<0.001	
		Interaction	0.440	
	Inr	Hours	<0.001	Increase Starved ***
		Treatment	<0.001	
		Interaction	0.0295	
	Pdk	Hours	<0.001	Increase Starved ***
		Treatment	<0.001	
		Interaction	<0.001	
	pi3k	Hours+	<0.001	Decrease Fed ***
		Treatment	<0.001	
		Interaction	0.011	
	Pten	Hours+	0.005	Decrease NS ***
		Treatment	0.224	
		Interaction	<0.001	
	Raptor	Hours	<0.001	Increase Starved ***
		Treatment	0.010	
		Interaction	<0.001	
S6k	Hours	<0.001	Increase NS	
	Treatment	0.101		
	Interaction	0.144		
TOR	Hours+	0.015	Increase Starved	
	Treatment	<0.001		
	Interaction	0.176		

Genes are listed according to broad functional groups. Results from linear regression. Upregulation is referring to which treatment the corresponding gene showed higher expression. Increase, and decrease refers to the change in expression over time. NS indicates no significance

+significant quadratic model reported

*** indicates significance in the interaction term (hours*treatment)

Discussion

The critical weight and entry into metamorphosis shows different patterns among insects. The critical weight differs in the two model insects *Manduca* and *Drosophila* in three major ways. First, the timing of the critical weight during the last larval instar differs between the two organisms. *Manduca* reaches their critical weight at a later point in their final larval instar than *Drosophila* (Nijhout and Williams, 1974; Mirth et al., 2009) Second, the influence of starvation on the critical weight also differs between the two organisms. Starvation speeds up timing to metamorphosis after the critical weight has been reached in *Drosophila* and not *Manduca* (Mirth

et al., 2009). Third, the physiological response to hormones also differs between *Manduca* and *Drosophila* during the critical weight period: a decline in JH titers are the cue for metamorphosis in *Manduca*, while JH is not as important in influencing *Drosophila* metamorphic timing (Fain and Riddiford 1974, Baker et al., 1987; Colombani et al., 2005; Nijhout and Williams, 1974; Caldwell et al., 2005; Mirth et al., 2005, Mirth et al. 2004). In *Drosophila*, the cue for metamorphosis is influenced by insulin/TOR signaling in the Prothoracic Gland, which regulates ecdysone synthesis (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005; Layalle et al., 2008).

Like *Drosophila*, we found that starvation in *M. rotundata* speeds up time to growth cessation such that starved larvae initiate cocoon spinning sooner than fed larva. In *Drosophila*, a starvation period after the critical weight will speed up the time to metamorphosis (Mirth et al., 2005; Stieper et al., 2008), but in a post-critical weight *Manduca* larvae, starvation will not impact timing to metamorphosis (Nijhout and Williams 1974). Our results show that bees in the starved treatment began web spinning at 24 hours before bees in the fed treatment. We found that *M. rotundata* has a critical weight, which is indicated by a significant breakpoint in the slope in which larvae below the critical weight delay timing to metamorphosis. The slope before the critical weight in the starved treatment is -5831.9 and the slope after the breakpoint was -418.3. We see a similar pattern in the control treatment, which also had a critical weight, the slope before the critical weight was -2281.80 and the slope after the critical weight was, -504.02. It was surprising to find a critical weight in the control treatment, because food was not manipulated in that treatment. There are multiple definitions for critical weight, when it was first discovered in *Manduca*, it was defined as the weight at which starvation does not delay timing to metamorphosis. This trend does not hold true in *Drosophila* because when larvae are starved

after the critical weight, the timing to metamorphosis speeds up (Mirth et al., 2005). This shows that starvation can change timing to growth cessation, so a more general definition has been applied to the critical weight, which is the size at which an insect is physiologically committed to metamorphosis (Davidowitz 2003). We found that *M. rotundata* larvae have a critical weight around 0.0125g. The timing of the critical weight is similar to what is seen in *Drosophila* because the critical weight appears to occur fairly early in the final larval instar. In *Drosophila*, the critical weight occurs during the first 25% of the final instar (Mirth and Riddiford, 2009), while in *Manduca*, it occurs later in their final instar (Nijhout and Williams, 1974). Our results show that the critical weight occurs very early in the starved treatment we see the critical weight occurs in the first 3.2%, and the critical weight in the control treatment occurs in the first 2%. In both *Manduca* and *Drosophila*, starvation delays time to critical weight, which is not what we see in our data (Koyama et. al, 2014).

The critical weight seen in *M. rotundata* is different than what is seen in other solitary bees. *O. lignaria* does not have a critical weight. Metamorphosis is induced by food removal. Starved bees initiated metamorphosis ~24 hours (Helm et al. 2017), even when starved at or below the minimum viable weight. In *O. lignaria*, excess feeding can prolong the time to growth cessation up to 20 days. In this study, the difference in growth cessation between the fed and starved treatment was 24 hours on average. The control treatment differed from the starved treatment by two hours. Fifty percent of the bees in the starved treatment began web spinning at 24 hours, fifty percent of bees in the control treatment began web spinning at 16 hours, and in the fifty percent of bees in the fed treatment began web spinning around 48 hours. This indicates that the terminal growth period in *M. rotundata* is much shorter than *O. lignaria*. Our results indicate

a critical weight: larvae smaller than 0.0125g delayed webspinning, indicating that the removal of food was not the cue for metamorphosis.

We saw that JH titers decreased over time. JH only decreased in the fed and control treatments. The lack of correlation between JH titers and time in the starved treatment could indicate that JH titers are low in that treatment. A decline in JH titers appears to be important for morphometric commitment, and initiates metamorphosis in *O. lignaria* (Helm et al. 2017). This is similar to what is seen in response to the critical weight in *Manduca*, which show declines in JH titers appear to be the key response to the changes based on metamorphic commitment at the critical weight period in *Manduca* (Nijhout and Williams 1974; Fain and Riddiford, 1975; Baker et al., 1987), while JH does not appear to be as influential in the critical weight of *Drosophila* (Mirth et al., 2014). In *Manduca*, JH titers are high before the critical weight and plateau before JH declines after the critical weight. Our results in *M. rotundata* show that there is no plateau in JH before the critical weight, and we see a negative correlation between JH and time. This may be because JH sampling in *M. rotundata* was biased toward larger individuals in order to obtain enough hemolymph to measure hormone titers. Future work should attempt to include larvae below the critical weight, or earlier instar larvae in order to determine when the decline in JH titers occurs. This study shows that a decline in JH is important for entry into metamorphosis, although the lack of correlation of JH and time in the starved treatment complicates these results and requires more investigation.

The transition into the pupal phase relies on JH titers being low (Gilbert et al., 2000). From our results, JH titers decrease with time in *M. rotundata*, and influence the timing of metamorphosis. Our results also indicate that bees in the starved treatment are committing to metamorphosis earlier than bees in the fed treatment. The downregulation of genes involved in

JH synthesis indicate that the clearance of JH occurs earlier in the starved treatment. The genes involved in JH synthesis including *aldehyde dehydrogenase (adh)*, *farnesyl dehydrogenase (fd)*, and *juvenile hormone methyl transferase(jhamt)* are all upregulated in the fed treatment and decrease overtime, although *methyl farnesoate epoxidase / farnesoate epoxidase (mfe)* and *farnesyl diphosphate phosphatase (fppp)* were not. This indicates that JH production is decreasing with time in both treatments, and at a slower rate in the fed treatment. *Juvenile hormone esterase (jhe)* which regulates JH catabolism, appears to decrease over time and is upregulated in the fed treatment. This may indicate that fed bees are still in the process of clearing JH. *Methoprene-tolerant (Met)* is thought to be the JH receptor, and responds to levels of JH. *Met* also regulates *Krüppel-homolog 1 (Kr-h1)* and *Broad-complex (BR-C genes)*. *Kr-h1* and *Met* function to repress metamorphosis in the presence of JH. Our results show *Met* increases over time, and was upregulated in the starved treatment, which is not what we expect, but the expression of *Met* tends to fluctuate during the final larval instar, and prepupal phase (Minakuchi et al., 2008). Our results show that *Kr-h1* is not differentially expressed by treatment but declines with time after a peak around 24 hours in the starved treatment. The spike in *Kr-h1* in the starved treatment is not what we expect as it is typically associated with maintaining the larval stage, but the upregulation of *Kr-h1* has been seen in the prepupal stage in *Tribolium castaneum* (Minakuchi et al., 2008) this may be what we are seeing in *M. rotundata*. This upregulation of *Kr-h1* in the prepupal stage is associated with an upregulation in *BR-C* (Minakuchi et al., 2008). *BR-C* is upregulated in larval-pupal molts (Konopova et al., 2011), our data show *BR-C* and *brick-a-brack-tamtrack-broad (BTB)* (a component of *BR-C*) increasing with time in the, and *BR-C* is upregulated in the starved treatment. These data confirm that bees in our study are entering metamorphosis through the decline in JH, and transitioning to the prepupal phase, and it

appears that this is occurring faster and earlier in the starved treatment compared to the fed treatment.

Genes that are upregulated in ecdysone synthesis and response are associated with the release of ecdysone in preparation for a metamorphic molt (Riddiford, 1993). Genes involved in the synthesis of ecdysone from cholesterol including *disembodied (dib)*, *shadow (sad)*, and *shade (shd)* showed different patterns of expression in relation to time and treatment. *Dib* expression increased with time, *sad* expression decreased with time, and *shd* expression did not change with relation to time. Both *dib*, and *shd* were upregulated in the starved treatment, but *sad* was not. *Sad* is a byproduct of *dib*, so it is unclear why it is not similarly expressed. The activation of *shd* occurs after *sad*, and it is *shd* which is the final step in the conversion to ecdysone (20E). Ecdysone binds to the *EcR-UsP* complex, which is the ecdysone receptor. We saw the *ecdysone receptor (EcR)* and *Ultraspiracle (UsP)* increasing with time there no difference in expression by treatment. Genes involved in ecdysone response (*E93* and *E74*) increased with time. *E93* does not show differential expression by treatment, and *E74* was upregulated in the fed treatment. *E93* and *E74* are expressed throughout the lifespan in *Drosophila*, but are also involved in metamorphosis, prepupal and pupal development (Mou et al., 2012 Thummel et al., 1990 Urness and Thummel, 1990, Fletcher et al., 1995). We see genes involved in ecdysone synthesis increasing with time. This indicates that bees are beginning a metamorphic molt.

Metamorphosis begins when JH titers are low which allows the release of Prothoracicotropic hormone (PTTH) and stimulates the release of ecdysone. In our study, PTTH was not differentially expressed by treatment and expression did not change over time. PTTH expression was relatively low during our experimental timepoints, indicating that the release of PTTH has not occurred. *M. rotundata* diapause as prepupae so the absence of a pulse of PTTH

could be due to a diapause commitment in these individuals. The overall pattern demonstrated that ecdysone synthesis is increasing over time and is upregulated in the starved treatment, which corresponds to the process of molting to the pupal stage. These prepupae are preparing for a molt to pupae, but the actual molt may be paused until after the overwintering diapause.

The ISS/TOR pathway is important for insect growth and development, and it also plays a key role in influencing timing to metamorphosis by interacting with ecdysone production. (Koyama et al., 2014; Mirth et al., 2005; Ohhara et al., 2017). IIS controls ecdysone titers during the larval stage (Layalle et al., 2008), but it has also been demonstrated that genes involved in the ISS pathway are also important for metamorphic molts. When insects are feeding, the insulin signaling pathway responds to carbohydrate levels. Consumption of carbohydrates will initiate the release of insulin-like peptides. In this study we measured gene expression in larvae which were starved, and therefore not feeding, and larvae that were provided an *ad libitum* amount of food. Upregulation of genes in the ISS/TOR pathway in starved larva are likely involved in metamorphic commitment, instead larval growth. Genes upregulated in the fed treatment might be responding to nutrition. In both cases, it is likely that upregulation of genes after 48 hours show response to metamorphic commitment because majority of the larvae in both treatments had reached growth cessation at that time. The insulin signaling pathway regulates growth rates in insects. *Insulin-like growth factor* (IGF1) did not change with time and was upregulated in the fed treatment indicating a response to feeding. The activation of the *insulin-like receptor* (InR) will initiate a cascade of events that activates *phosphoinositide-3-kinase* (Pi3k), *3-phosphoinositide-dependent protein kinase 1* (PDK) and *Serine-threonine protein kinase* (Akt). Our results show *InR* and *PDK* increase over time and are upregulated in the starved treatment. Because of their upregulation in the starved treatment, it is likely that these genes are involved in

the metamorphic molt. In *Drosophila*, Mutations to the *InR* cause a delay in metamorphosis, demonstrating ISS is partially responsible for the regulation of timing of metamorphosis (Shingleton et al. 2005), which may be the case in our study. In *Bombyx mori*, *InR*, *PiK3*, and *PDK*, *Phosphatase and tensin homolog* (*PTEN*) are upregulated in response to starvation during molting and pupation when ecdysone levels were high (Liu et al., 2010). Although our results show that *PTEN* and *Pi3k* are not upregulated in the starved treatment. *PTEN*, a negative regulator of the insulin pathway, showed no difference between the treatments, and *Pi3k* was upregulated in the fed treatment. In *Drosophila*, *Pi3k* plays a role in increasing ecdysone levels during the larval stage and influences growth (Colombani et al. 2005 Caldwell et al., 2005; Mirth et al. 2005). Our study shows levels *Pi3K* expression decreasing over time and upregulated in the fed treatment, the role of *Pi3k* regulating ecdysone production in larval molts may be on the decline in the fed treatment in *M. rotundata*.

During development *Serine-threonine protein kinase* (*Akt*) phosphorylates *Forkhead box protein O* (*FOXO*). *FOXO* is a negative regulator of ISS plays an important role in the metamorphic molt from larva to pupae (Cai et al., 2016; Hossain et al., 2013). The silencing of *FOXO* delays or stops pupation (Cai et al., 2016; Lin et al. 2018). We found *FOXO* is upregulated in the starved treatment and is increasing with time. It has a peak in the starved treatment between 24 and 48 hours. This up-regulation of *FOXO* is consistent with the initiation of a metamorphic molt at those timepoints.

The Target of Rapamycin (TOR) pathway responds to amino acids nutrient signaling and activates protein synthesis. In *Drosophila*, *Target of Rapamycin* (TOR) also influences ecdysone production in the PG and is important for the ecdysone pulse during metamorphosis as demonstrated by the inactivation of TOR delaying metamorphosis (Layalle et al., 2008). In our

study, genes associated with the TOR pathway, including, the regulator-associated protein of TOR (raptor), *ribosomal protein kinase 6* (s6k), and *TOR* increased with time and were upregulated in the starved treatment with the exception of s6k which did not show a difference between the fed and starved treatments. TOR is upregulated in the starved treatment and increases over time; this trend aligns with the genes involved in ecdysone synthesis. This may indicate that TOR is influencing timing of metamorphosis, as predicted. Upregulation of genes involved in the ISS/TOR pathway do not show clear patterns based on treatment. This may indicate that the ISS/TOR pathway playing different roles based on the feeding status of the bee. While the ISS/TOR is important for controlling larval growth, we see many genes in the ISS/TOR pathway are upregulated in the starved treatment. These genes have been shown to be important regulators of metamorphic molts in other insects. Based on these data it appears that bees in the starved treatment are experiencing the start of metamorphosis earlier than the bees in the fed treatment.

The goal of this study was to determine the regulation and timing of metamorphosis in the solitary bee *Megachile rotundata*. We found the cue for metamorphosis in *M. rotundata* was a critical weight. The physiological commitment to metamorphosis appears to be influenced by both declines in JH titers, and genes in the ISS/TOR pathway. The timing of the critical weight, and effect of starvation on growth cessation in *M. rotundata* is more similar to the critical weight in *Drosophila* (Mirth and Riddiford, 2009; Mirth et al., 2005; Stieper et al., 2008), while the influence of JH for commitment to metamorphosis is more similar to *Manduca* (Browder et al. 2001, Hatem et al., 2015). We see clear patterns in our study that declines in JH are important for initiating the start of metamorphosis, while genes involved in the ISS/TOR pathway have do not show distinct patterns of upregulation indicating that these genes are involved in timing to

metamorphosis but not directly influencing this transition. Determining the critical weight in *M. rotundata* shows that there are species specific differences in the physiological control of metamorphic commitment.

References

- Anwyl, R.** (1972). The structure and properties of an abdominal stretch receptor in *Rhodnius prolixus*. *J Insect Physiol*, **18**, 2143-2153.
- Baker FC, Tsai LW, Reuter CC, Schooley DA** (1987). In vivo fluctuation of JH, JH acid, and ecdysteroid titer, and JH esterase activity, during development of fifth stadium *Manduca sexta*. *Insect Biochem* **17**, 989–996.
- Cai, M.J., Zhao, W.L., Jing, Y.P., Song, Q., Zhang, X.Q., Wang, J.X., Zhao, X.F.,** (2016). 20- hydroxyecdysone activates Forkhead box O to promote proteolysis during *Helicoverpa armigera* molting. *Development* **143**, 1005–1015.
- Caldwell PE, Walkiewicz M, Stern M** (2005). Ras activity in the *Drosophila* prothoracic gland regulates body size and developmental rate via ecdysone release. *Curr Biol* **15**, 1785–1795.
- Callier V, Nijhout HF.** (2011). Control of body size by oxygen supply reveals size-dependent and size-independent mechanisms of molting and metamorphosis. *Proc Natl Acad Sci*, **108**, 14664–9.
- Chown, S. L., & Gaston, K. J.** (2010). Body size variation in insects: a macroecological perspective. *Biol Rev*, **85**, 139-169.
- Colombani, J., L. Bianchini, S. Layalle, E. Pondeville, C. Dauphin-Villemant, C. Antoniewski, C. Carre, S. Noselli, P. Leopold,** (2005). Antagonistic actions of ecdysone and insulins determine final size in *Drosophila*, *Science* **310**, 667–670.

- Davidowitz G, D'Amico LJ, Nijhout HF** (2003). Critical weight in the development of insect body size. *Evol Dev* **5**, 188–197.
- Davies, R.B.** (1987). Hypothesis testing when a nuisance parameter is present only under the alternative. *Biometrika* **74**, 33–43.
- Davies, R.B.** (2002). Hypothesis testing when a nuisance parameter is present only under the alternative: linear model case. *Biometrika* **89**, 484–489.
- De Moed, G.H., Kruitwagen, C.L.J.J., De Jong, G., and Scharloo, W.** (1999). Critical weight for the induction of pupariation in *Drosophila melanogaster*: genetic and environmental variation. *J. Evol. Biol.* **12**, 852–858.
- Fain MJ, Riddiford LM** (1975). Juvenile hormone titers in the hemolymph during late larval development of the tobacco hornworm, *Manduca sexta* (L.). *Biol Bull* **149**, 506–521.
- Fletcher, J. C., Burtis, K. C., Hogness, D. S., & Thummel, C. S.** (1995). The *Drosophila* E74 gene is required for metamorphosis and plays a role in the polytene chromosome puffing response to ecdysone. *Development*, **121**, 1455-1465.
- Gilbert LI, Granger NA, Roe RM** (2000). The juvenile hormones: historical facts and speculations on future research directions. *Insect Biochem Mol Biol* **30**, 617–644.
- Helm, B. R., Rinehart, J. P., Yocum, G. D., Greenlee, K. J., & Bowsher, J. H.** (2017). Metamorphosis is induced by food absence rather than a critical weight in the solitary bee, *Osmia lignaria*. *P Natl A Sci*, **114**, 10924-10929.
- Hossain, M.S., Liu, Y., Zhou, S., Li, K., Tian, L., Li, S.,** (2013). 20-Hydroxyecdysone-induced transcriptional activity of FoxO upregulates brummer and acid lipase-1 and promotes lipolysis in *Bombyx* fat body. *Insect Biochem. Mol. Biol.* **43**, 829– 838.

- Kemp, W. P., & Bosch, J.** (2000). Development and emergence of the alfalfa pollinator *Megachile rotundata* (Hymenoptera: Megachilidae). *Ann Entomol Soc Am*, **93**, 904-911.
- Klowden, M. J.** (2013). *Physiological systems in insects*. Academic press.
- Konopova, B., Smykal, V., & Jindra, M.** (2011). Common and distinct roles of juvenile hormone signaling genes in metamorphosis of holometabolous and hemimetabolous insects. *PloS one*, **6**, e28728.
- Konopova B, Jindra M** (2008). Broad-Complex acts downstream of Met in juvenile hormone signaling to coordinate primitive holometabolan metamorphosis. *Development*. **135**, 559–568. doi:10.1242/dev.016097.
- Koyama, T., M.A. Rodrigues, A. Athanasiadis, A.W. Shingleton, C.K. Mirth,** (2014). Nutritional control of body size through FoxO-Ultraspiracle mediated ecdysone biosynthesis, *eLife*, **3**.
- Layalle, S., Arquier, N., & Léopold, P.** (2008). The TOR pathway couples nutrition and developmental timing in *Drosophila*. *Dev cell*, **15**, 568-577.
- Lin, X., Yu, N., & Smagghe, G.** (2018). FoxO mediates the timing of pupation through regulating ecdysteroid biosynthesis in the red flour beetle, *Tribolium castaneum*. *Gen comp endocr*, **258**, 149-156.
- Liu, Y., Zhou, S., Ma, L., Tian, L., Wang, S., Sheng, Z., Jiang, R.J., Bendena, W.G. and Li, S.,** (2010). Transcriptional regulation of the insulin signaling pathway genes by starvation and 20-hydroxyecdysone in the *Bombyx* fat body. *J insect physiol*, **56**,1436-1444.
- M.H. Browder, L.J. D'Amico, H.F. Nijhout,** (2001). The role of low levels of juvenile hormone esterase in the metamorphosis of *Manduca sexta*, *J. Insect Sci.* **1**, 11.

- Minakuchi C, Namiki T, Shinoda T** (2009). Kru¨ppel homolog 1, an early juvenile hormone-response gene downstream of Methoprene-tolerant, mediates its anti-metamorphic action in the red flour beetle *Tribolium castaneum*. *Dev Biol* **325**, 341–350.
- Mirth, C., Truman, J.W., and Riddiford, L.M.** (2005). The role of the prothoracic gland in determining critical weight for metamorphosis in *Drosophila melanogaster*. *Curr. Biol.* **15**, 1796– 1807.
- Mirth, C. K., Tang, H. Y., Makohon-Moore, S. C., Salhadar, S., Gokhale, R. H., Warner, R. D., ... & Shingleton, A. W.** (2014). Juvenile hormone regulates body size and perturbs insulin signaling in *Drosophila*. *P Natl A Sciences*, **111**, 7018-7023.
- Mirth CK, Shingleton AW** (2012). Integrating body and organ size in *Drosophila*: Recent advances and outstanding problems. *Front Endocrinol.* **3**, 49.
- Mirth CK, Truman JW, Riddiford LM** (2009). The ecdysone receptor controls the post-critical weight switch to nutrition-independent differentiation in *Drosophila* wing imaginal *Development*, **136**, 2345-2353.
- Mou, X., Duncan, D. M., Baehrecke, E. H., & Duncan, I.** (2012). Control of target gene specificity during metamorphosis by the steroid response gene E93. *P Natl A Sciences*, **109**, 2949-2954.
- Muggeo V. M. R.** (2003). Estimating regression models with unknown break-points. *Stat Med*, **22**, 3055-3071.
- Nijhout, H. F.** (1975). A threshold size for metamorphosis in the tobacco hornworm, *Manduca sexta* (L.). *Biol. Bull.* **149**, 214-225.

- Nijhout, H. F. and Williams, C. M.** (1974). Control of molting and metamorphosis in the tobacco hornworm, *Manduca sexta* (L.): growth of the last-instar larva and the decision to pupate. *J. Exp. Biol.* **61**, 481-491.
- Nijhout HF, Davidowitz G, Roff DA** (2006). A quantitative analysis of the mechanism that controls body size in *Manduca sexta*. *J Biol*, **5** 16.
- Nijhout HF, et al.** (2014). The developmental control of size in insects. *Wiley Interdiscip Rev Dev Biol* **3**, 113–134.
- Ohhara, Y., Kobayashi, S., Yamanaka, N., Kaynig, V., Longair, M., and Pietzsch, T.** (2017). Nutrient- dependent endocycling in steroidogenic tissue dictates timing of metamorphosis in *Drosophila melanogaster*. *PLoS genetics* **13** e1006583.
- P.E. Caldwell, M. Walkiewicz, M. Stern,** (2005). Ras activity in the *Drosophila* prothoracic gland regulates body size and developmental rate via ecdysone release, *Curr. Biol.* **15**, 1785–1795.
- Parthasarathy R, Tan A, Bai H, Palli SR** (2008). Transcription factor broad suppresses precocious development of adult structures during larval–pupal metamorphosis in the red flour beetle, *Tribolium castaneum*. *Mech Dev* **125**, 299–313.
- Riddiford, L.M.** (1993). Hormone receptors and the regulation of insect metamorphosis. *Am Zool*, **33**, 340-347.
- Riddiford, L.M., J.W. Truman, C.K. Mirth, Y.C. Shen,** (2010). A role for juvenile hormone in the prepupal development of *Drosophila melanogaster*, *Development* **137**, 1117–1126.
- Rulifon, E.J., Kim, S.K., Nusse, R.,** (2002). Ablation of insulin-producing neurons in flies: growth and diabetic phenotypes. *Science*, **296**. 1118–1120

- Sehnal, F.** (1985). Growth and life cycles. *Comprehensive Physiology, Biochemistry, and Pharmacology of Insects* **2**, 1–86.
- Sehnal T., Koyama, M.A., Rodrigues, A., Athanasiadis, A.W., Shingleton, C.K., Mirth,** (2014). Nutritional control of body size through FoxO-Ultraspiracle mediated ecdysone biosynthesis, *eLife* **3** e03091.
- Shingleton, A. W., Frankino, W. A., Flatt, T., Nijhout, H. F., & Emlen, D. J.** (2007). Size and shape: the developmental regulation of static allometry in insects. *BioEssays*, **29**, 536-548.
- Shingleton AW, Das J, Vinicius L, Stern DL S.** (2005). The temporal requirements for insulin signaling during development in *Drosophila*. *PLoS Biol* **3** e289.
- Shingleton AW** (2011). Evolution and the regulation of growth and body size. *Mechanisms of Life History Evolution*, Oxford Univ Press, New York, 43–55.
- Stieper BC, Kupershtok M, Driscoll MV, Shingleton AW** (2008). Imaginal discs regulate developmental timing in *Drosophila melanogaster*. *Dev Biol.* **321**,18–26.
- Suzuki Y, Truman JW, Riddiford LM** (2008). The role of Broad in the development of *Tribolium castaneum*: implications for the evolution of the holometabolous insect pupa. *Development* **135**. 569–577.
- Thummel, C. S., Burtis, K. C. and Hogness, D. S.** (1990). Spatial and temporal patterns of E74 transcription during *Drosophila* development. *Cell* **61**, 101-111.
- Trostle, G., & Torchio, P. F.** (1994). Comparative nesting behavior and immature development of *Megachile rotundata* (Fabricius) and *Megachile apicalis* Spinola (Hymenoptera: Megachilidae). *J Kansas Entomol Soc*, 53-72.

Urness, L. D. and Thummel, C. S. (1990). Molecular interactions within the ecdysone regulatory hierarchy: DNA binding properties of the *Drosophila* ecdysone-inducible E74A protein. *Cell* **63**, 47-61.

Warren, J.T., Y. Yerushalmi, M.J. Shimell, M.B. O'Connor, L.L. Restifo, L.I. Gilbert, (2006). Discrete pulses of molting hormone, 20-hydroxyecdysone, during late larval development of *Drosophila melanogaster*: correlations with changes in gene activity, *Dev. Dyn.* **235** 315–326.

Wickham, H., (2016). *Ggplot2: Elegant Graphics for Data Analysis*.

<https://ggplot2.tidyverse.org>

CHAPTER 5: CONCLUSION

Body size is not only important for performance and fitness on an individual scale, it is important to understand body size on a larger scale. Bee body size is in decline (Oliveira et al. 2016). A study that spanned a century and a half showed large body-sized bees are becoming smaller (Oliveira et al. 2016). Bigger bee populations are declining at a faster rate than small bee populations (Bartomeus et al. 2013). This pattern is due to anthropogenic changes in the landscape, especially the intensification of agriculture, leading to a reduction in resource availability (Renauld et al. 2016). Diminished resource availability leads to a reduced amount of food a female bee can provide to her offspring (Bosch, 2008). Maternal investment adapts to resource conditions, and when resources are scarce females provide smaller provision amounts to the developing offspring, leading to the production of “tiny” female offspring (Kim and Thorp 2001; Bosch, 2008). This is an indicator that a decrease in resource availability may lead to smaller body-sized bee populations. With concern for pollinator habitat loss, there is a need for more research to focus on the potential performance outcomes of smaller bees, as well as how these bees will respond to stress. I have found that smaller bees are not always at a disadvantage.

Body size variability is related to many aspects of performance and fitness, including flight performance (Bushwald & Dudley 2010), fecundity (Blanckenhorn 2000; O’Neill et al. 2014), foraging distance (Greenleaf et al. 2007) and overwintering survival (Bosch & Kemp, 2004). While many of these trends can be seen across species, this dissertation looked at intraspecific effects of body size. I determined how metabolic rate, and flight-related morphology scales with body size, telomere loss and aging, and the cue for metamorphosis. I found that when looked at within a species, patterns are different than what occurs across species, or in model organisms. My results show in solitary bees, there are instances in which it

is advantageous to being small. The advantages to small body size in insects often been overlooked. This dissertation demonstrates that smaller bees are not at a disadvantage and contradicts the assumption that performance will increase with body size, or bigger is better.

Chapter 2 demonstrated how body size allometry impacted metabolic and flight related morphology in *M. rotundata*. I measured resting and flight metabolic rates on a large range of body sizes in a single species. Metabolic rate was measured in bees which had a four-fold change in body size. This is a larger range in body size than has previously been measured (Bennett et al., 2013, Abdelrahman et al., 2014; Owings et al., 2014). I found a mass-specific difference in resting metabolic rate, but there was no difference in metabolic rate during flight. I found that larger bees had a higher absolute metabolic rate during flight and at rest. This difference in flight metabolic rate may be due to the allocation of resources to the thorax, as demonstrated by hyperallometric scaling. We also saw hypoallometric scaling of wings. This difference in body size allometry influenced flight performance. Larger bees had wings that grew hypoallometric to body size contributing to larger bees having less power available during flight. Smaller bees demonstrated a reduced power requirement for flight with no metabolic cost.

Chapter 3 determined the telomere dynamics in two solitary bee species, *M. rotundata*, and *O. lignaria*. Telomere dynamics may be a potential mechanism of understanding cellular aging, and telomere attrition is often associated with aging (Blackburn 2015; Haussman and Vleck 2002). This was the first study to date to measure telomere length in solitary bees. Our results show that the telomere dynamics of solitary bees differ from social bees (Korandová & Frydrychová 2016 Koubová et. al 2019 Koubová et. al 2021). I found that telomeres get longer in later developmental life stages and telomere length appears to be maintained in the adult stage in both *M. rotundata* and *O. lignaria*. These data show that telomeres remain short at early stages

in development. Telomeres appear to get longer in response to emergence from the quiescent stage. I found no effect of body size on telomere length. In solitary bees, amount of food consumed in the larval stage influences final adult body size (Klostermeyer et al. 1973). Low amounts of larval nutrition, and small body size does not appear to be stressful in solitary bees. Nutritional stress may be reflected in shorter telomeres in response to increased oxidative damage. This study demonstrates that there is not a cost to smaller body size as reflected in telomere length.

In chapter 4, I determined the cue for metamorphosis in *M. rotundata* was a critical weight which was influenced by titers of JH. Larval growth and development is important for determining final adult size. Amount of food provided to larval bees directly influences adult body size. Larval growth rate, and timing to metamorphosis also influences final adult body size (Sehnal, 1985, Nijhout et al., 2006). This chapter shows that *M. rotundata* has a critical weight which initiates the physiological commitment to metamorphosis. This was confirmed by declines in JH titers, and upregulation of genes involved in metamorphic and prepupal commitment. The critical weight in *M. rotundata* occurs at a low weight and occurs early in the final larval instar. A low critical weight may be a “bail-out mechanism” in response to low or ephemeral food availability (Hattem et. al, 2015). Solitary bee larvae rely on nutritional provisions from the mother and cannot continuously feed on their own. When resources are low the mother may not be able to adequately provide resources for the developing offspring (Bosch, 2008). Early commitment to metamorphosis ensures that the larvae can still enter metamorphosis under food stress. With increasing concern over a reduction in resource availability, it is advantageous to reach the critical weight earlier to increase chance of survival. This may indicate that small adult body size is sustainable in *M. rotundata*, and body size can adapt to resource availability.

References

- Abdelrahman, H., Rinehart, J.P., Yocum, G.D., Greenlee, K.J., Helm, B.R., Kemp, W.P., Schulz, C.H. and Bowsher, J.H.** (2014). Extended hypoxia in the alfalfa leafcutting bee, *Megachile rotundata*, increases survival but causes sub-lethal effects. *J. of Insect Physiol.* **64**, 81-89.
- Bartomeus, I., Ascher, J.S., Gibbs, J., Danforth, B.N., Wagner, D.L., Hedtke, S.M. and Winfree, R.,** (2013). Historical changes in northeastern US bee pollinators related to shared ecological traits. *Proceedings of the National Academy of Sciences*, **110**, 4656-4660.
- Bennett, M. M., Petersen, K., Yocum, G., Rinehart, J., Kemp, W. and Greenlee, K. J.** (2013). Effects of extended prepupal storage duration on adult flight physiology of the alfalfa leafcutting bee (Hymenoptera: Megachilidae). *J. Econ. Entomol.* **106**, 1089
- Blackburn, E. H., Epel, E. S., & Lin, J.** (2015). Human telomere biology: a contributory and interactive factor in aging, disease risks, and protection. *Science*, **350**, 1193-1198.
- Blanckenhorn, W. U.** (2000). The evolution of body size: what keeps organisms small? *The Q. Rev. Biol.* **75**, 385-407
- Bosch, J.** (2008). Production of undersized offspring in a solitary bee. *Anim. Behav.* **75**, 809-816.
- Bosch, J. & Kemp, W.P.** (2004). Effect of pre-wintering and wintering temperature regimes on weight loss, survival, and emergence time in the mason bee *Osmia cornuta* (Hymenoptera: Megachilidae). *Apidologie*, **359**, 469–479.
- Buchwald, R. and Dudley, R.** (2010). Limits to vertical force and power production in bumblebees (Hymenoptera: *Bombus impatiens*). *J. Exp. Biol.* **213**, 426-432.

- Greenleaf, S. S., Williams, N. M., Winfree, R. and Kremen, C. (2007).** Bee foraging ranges and their relationship to body size. *Oecologia*, **153**, 589-596.
- Hatem, N. E., Wang, Z., Nave, K. B., Koyama, T., & Suzuki, Y. (2015).** The role of juvenile hormone and insulin/TOR signaling in the growth of *Manduca sexta*. *BMC biology*, **13**, 1-12.
- Hausmann MF, Vleck CM, (2002).** Telomere length provides a new technique for aging animals. *Oecologia* **130**, 325–328.
- Kim JY, Thorp RW. (2001).** Maternal investment and size-number trade-off in a bee, *Megachile apicalis*, in seasonal environments. *Oecol.* **126**, 451–456.
- Klostermeyer, E. C., Mech Jr, S. J., & Rasmussen, W. B. (1973).** Sex and weight of *Megachile rotundata* (Hymenoptera: Megachilidae) progeny associated with provision weights. *J Kansas Entomol Soc*, 536-548.
- Korandová, M., & Frydrychová, R. Č. (2016).** Activity of telomerase and telomeric length in *Apis mellifera*. *Chromosoma*, **125**, 405-411.
- Koubová, J., Jehlík, T., Kodrík, D., Sábová, M., Šima, P., Sehadová, H., Závodská, R. and Frydrychová, R.Č. (2019).** Telomerase activity is upregulated in the fat bodies of pre-diapause bumblebee queens (*Bombus terrestris*). *Insect biochem molec*, **115**.103241.
- Koubová, J., Sábová, M., Brejcha, M., Kodrík, D., & Frydrychová, R. Č. (2021).** Seasonality in telomerase activity in relation to cell size, DNA replication, and nutrients in the fat body of *Apis mellifera*. *Sci rep*, **11**.
- Koubová, Justina, Tomáš Jehlík, Dalibor Kodrík, Michala Sábová, Peter Šima, Hana Sehadová, Radka Závodská, and Radmila Čapková Frydrychová. (2019).**

- Telomerase activity is upregulated in the fat bodies of pre-diapause bumblebee queens (*Bombus terrestris*). *Insect biochem molec*, **115**, 103241.
- Nijhout, H. F., Davidowitz, G. & Roff, D. A.** (2006). A quantitative analysis of the mechanism that controls body size in *Manduca sexta*. *Journal Biol* **5**, 1–15.
- Oliveira, M.O., Freitas, B.M., Scheper, J. and Kleijn, D.,** (2016). Size and sex-dependent shrinkage of Dutch bees during one-and-a-half centuries of land-use change. *PLoS one*, **11**, p.e0148983.
- Owings, A.A., Yocum, G.D., Rinehart, J.P., Kemp, W.P. and Greenlee, K.J.** (2014). Changes in respiratory structure and function during post-diapause development in the alfalfa leafcutting bee, *Megachile rotundata*. *J. Insect Physiol.* **66**, 20-27.
- O’Neill, K. M., Delphia, C. M. and O’Neill, R. P.** (2014). Oocyte size, egg index, and body lipid content in relation to body size in the solitary bee *Megachile rotundata*. *PeerJ*, **2**, e314.
- Renauld, M., Hutchinson, A., Loeb, G., Poveda, K. and Connelly, H.,** (2016). Landscape simplification constrains adult size in a native ground-nesting bee. *PLoS One*, **11**, p.e0150946.
- Sehnal, F.** (1985). Growth and life cycles. In *Comprehensive Physiology, Biochemistry, and Pharmacology of Insects*, **2**, 1–86, Pergamon Press, Oxford.
- Tepedino V. J., Torchio P.F.** (1989). The influence of nest-hole selection on sex ratio and progeny size in *Osmia lignaria propinqua* (Hymenoptera: Megachilidae). *Ann. Entomol. Soc. Am.* **82**, 355-360.