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Transgenerational Effects of Maternal Age on Offspring Fitness in Crickets

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Transgenerational effects of maternal age on offspring fitness in crickets

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ABSTRACT

Advanced parental age is an important aspect of parental condition that can have both positive and negative effects on offspring fitness, and thus, parental age can be considered a parental effect. As a parental effect, parental age may affect a variety of offspring traits and may cascade to influence several generations of offspring. Given the complexities of studying both paternal and maternal age, we studied the effects of maternal age only. Using the Pacific field cricket, *Teleogryllus oceanicus*, we asked 1) does maternal age have influences over several generations of offspring and 2) does maternal age influence the reproductive investment of male offspring? We found that maternal age has contrasting effects on different traits, and those effects may last a single generation or may be sustained through several generations.

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CHAPTER ONE: Transgenerational effects of maternal age on quantitative and qualitative measures of fitness in cricket offspring

Introduction

Intrinsic characteristics of parents and their experience(s) with the environment can influence offspring traits through parental effects. Parental effects, broadly defined, are any interaction between parental environment and offspring phenotype (Badyaev & Uller, 2009). Researchers are increasingly documenting parental effects that influence a broad array of fitness-related offspring traits (reviewed in Bonduriansky & Day 2009) and the characteristics of parental environment with a demonstrated link to offspring phenotypes are diverse; temperature, population density, toxins, salinity, pathogens, stress, parental age, parental condition, and predators can all affect offspring phenotypes (Burrows, Rogers, & Ott, 2011; Dantzer et al., 2019; Ernst et al., 2015; Hansen, Puvanendran, & Bangera, 2015; Kishimoto, Uno, Okabe, Nono, & Nishida, 2017; Klosin, Casas, Hidalgo-Carcedo, Vavouri, & Lehner, 2017; Lehto & Tinghitella, In Review; Sadd, Kleinlogel, Schmid-Hempel, & Schmid-Hempel, 2005). It has become clear that the experiences of generations past can have positive or negative effects on offspring fitness (reviewed in Badyaev & Uller 2009). For example, when mothers are exposed to predators in threespine sticklebacks, their offspring exhibit tighter shoaling behavior as

juveniles (an adaptive antipredator behavior) but are not as likely to orient towards predators as adults, which decreases survival (Giesing, Suski, Warner, & Bell, 2011; Mcghee, Pintor, Suhr, & Bell, 2012). Through parental effects, offspring traits can change quickly (within in a single generation), making this an important mechanism for rapid intergenerational responses. When parental effects are underlain by changes to the epigenome (Jablonka & Raz, 2009), they also have the potential to exert continuous, stable influences across several generations of offspring (Jablonka, 2017). In *Caenorhabditis elegans*, for instance, measurable differences in gene expression stemming from parental exposure to high temperatures lasted up to 14 generations (Klosin et al., 2017). Parental effects on a single generation may have transgenerational staying power, but it can be challenging to assess the impacts of parental effects across multiple generations in many study organisms, so we know less about grandparental effects (and beyond) than we do parental effects alone. Here, we investigate the grandparental effect and the transgenerational dynamics of one important intrinsic characteristic of parents, their age.

As organisms age, cellular upkeep and repair mechanisms deteriorate, making parental age an important component of parents that may affect offspring traits through parental effects. Two bodies of literature, life history theory and aging theory, consider how advanced parental age will affect offspring traits and fitness. Life history theory's terminal investment hypothesis predicts that as parents age, they should invest more in offspring as compared with their younger conspecifics because they are released from the need to invest in future survival and reproduction (Charlesworth & Leon, 1976;

Creighton, Heflin, & Belk, 2009; Partridge & Harvey, 1988; Trivers, 1974; Williams, 1966). Support for this prediction has been found in arthropods, fish, and birds (Asghar, 2012; Asghar, Bensch, Tarka, Hansson, & Hasselquist, 2014; Clark, Garbutt, McNally, & Little, 2017; Creighton et al., 2009; Hansen et al., 2015). In contrast to the terminal investment hypothesis, aging theory predicts that older parents are prevented from making a large investment in offspring because of the negative effects of age on cellular upkeep and repair mechanisms; support for aging theory comes from studies in insects and mammals, including humans (Arslan et al., 2017; Bloch Qazi et al., 2017; Descamps, Boutin, Berteaux, Gaillard, & Boutin, 2008; Kern, Ackermann, Stearns, & Kawecki, 2001; Muller et al., 2017). Broadly, aging research has focused on the effects of maternal age on offspring, and due to the complexity of testing both paternal and maternal age in a single study, we focus here on maternal age in our experiment. Maternal age can have effects on both a mother herself and also on her offspring, and these effects have been studied widely (Hallagan, Tinghitella, & Murphy, In Preparation). However, the effects of grandmaternal age have not been thoroughly studied (but see Bloch Qazi et al. 2017).

Maternal age could have effects solely in the immediately subsequent generation of offspring or could have effects that last multiple generations. If maternal age has effects on offspring that tend to 'wash-in' and 'wash-out' quickly, we would expect maternal age to induce detectable effects in offspring, but not grandoffspring. Therefore, maternal age would take precedence in determining effects on offspring traits, and we call this hypothesis the "Precedence" hypothesis. An alternative hypothesis is that any effects of aging could wash-in over several generations of old or young parental age

(Burggren, 2015; Jablonka, 2017). In this model, any differences between offspring of young and old parents would 'compound,' or grow larger with subsequent generations (Burggren, 2015), and we call this hypothesis the 'Compounding' hypothesis. A major limitation preventing investigation of these ideas is the lifespan of the study organism; addressing transgenerational dynamics, particularly multigenerational effects, requires experiments that span more than a single generation and can be particularly time- and cost-prohibitive if the study organism lives for even as long as a year. Additionally, many short-lived model systems (such as *Drosophila melanogaster*) have been adapted to a laboratory environment for so many generations that the effects of aging may not reflect those operating in wild populations (Hallagan, Tinghitella, & Murphy, In Preparation). An ideal study organism, then, would be relatively short lived, outbred and not lab adapted, but amenable to rearing in the lab.

We studied the transgenerational dynamics of aging on offspring fitness in the Pacific field cricket, *Teleogryllus oceanicus*, which is an ideal study system to study aging effects over multiple generations because *T. oceanicus* is relatively long lived for an insect, but has a short enough lifespan to rear multiple generations in a single year. We designed a fully factorial experiment in which mothers and grandmothers were either old or young at the time of mating, allowing us to investigate the consequences of two generations of advanced maternal age (Figure 1.1). We measured a comprehensive suite of fitness measures in the offspring and grandoffspring, including standard fitness measures such as body size and survival to adulthood, and less traditional measures like immunocompetency. In our previous research, we found that after a single generation of

advanced maternal age, old mothers laid smaller eggs that hatched at lower rates compared with young mothers (Hallagan, Murphy, Smilanich, Wilson, & Tinghitella, In Preparation). Yet, notably, young and old mothers had the same number of offspring survive to adulthood and offspring of old mothers had higher rates of immunocompetency (Hallagan, Murphy, Smilanich, Wilson, & Tinghitella, In Preparation). However, from our previous work, we only know about effects after a single generation of differential maternal age, and we do not know the transgenerational effects of maternal age on offspring.

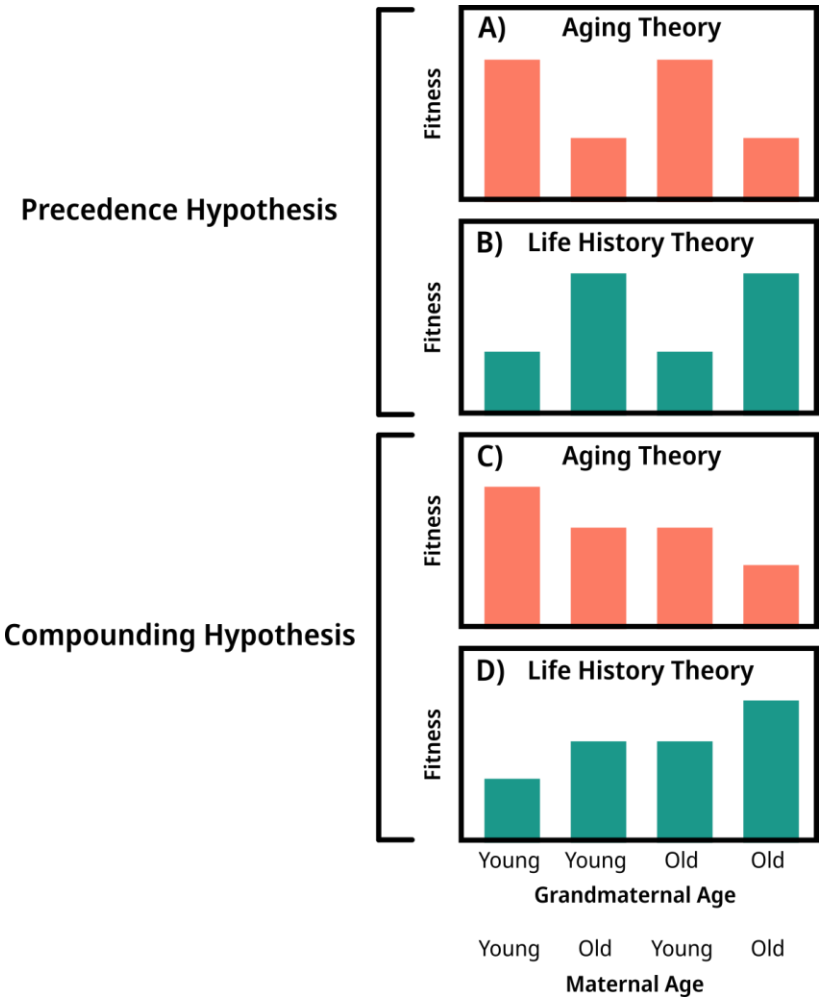

Figure 1.1: Our hypotheses consider the contrasting predictions of life history theory and aging theory, as well as effects that affect only a single generation of offspring or those that compound over several generations. A) Under the precedence hypothesis and aging theory, treatments with a young mother would have the highest measures of fitness independent of grandmaternal age, B) while under the precedence hypothesis and life history theory, treatments with an old mother would have the highest measures of fitness independent of grandmaternal age. C) Under the compounding hypothesis and aging theory, we would predict the highest measures of fitness in individuals from the treatment with both grandmothers and mothers mated at a young age, the lowest measures of fitness in individuals from the treatment with both grandmothers and mothers mated at an old age, and intermediate measures in the other two treatments. D) Under the compounding hypothesis and life history theory, we would expect the highest measures of fitness in individuals in the treatment with both grandmothers and mothers mated at an old age, the lowest measures of fitness in individuals in the treatment with both grandmothers and mothers mated at a young age, and intermediate measures of fitness in the Old/Young and Young/Old treatments.

Here, we test two major hypotheses (Figure 1.1) that are also informed by aging theory and life history theory. First, given that most parental age research has addressed impacts of only a single generation of advanced parental age, and effects of parental age on offspring are often found (Badyaev & Uller, 2009; Hallagan, Tinghitella, et al., In Preparation), we predicted that under the precedence hypothesis, we would only find significant differences in fitness that depend on the age of the mother, but no fitness differences between grandmaternal age treatments. On the other hand, maternal age may have effects across several generations, like some documented parental effects (Shama & Wegner, 2014). So, the second hypothesis, the compounding hypothesis, predicts that effects of aging wash-in slowly, so we should find the most extreme measures of offspring fitness after two subsequent generations of either young or old maternal age. With the precedence hypothesis and aging theory, we would predict any offspring of young mothers to have the highest fitness (Figure 1A). Under the precedence hypothesis and life history theory, we would predict any offspring of old mothers to have the highest fitness (Figure 1B). Under the compounding hypothesis and aging theory, we would predict offspring from young grandmothers and young mothers to have the highest fitness and offspring of old grandmothers and old mothers to have the lowest fitness with the other two treatments intermediate to those extremes (Figure 1C). Finally, under the compounding hypothesis and aging theory, we would predict offspring from old grandmothers and old mothers to have the highest fitness and offspring from young grandmothers and young mothers to have the lowest fitness, with the other treatments once again intermediate to those extremes (Figure 1D).

Methods

Study System

To examine the effect of parental age on offspring fitness across multiple generations, we use the Pacific field cricket, *T. oceanicus.* This study system allowed us to test multigenerational parental effects on offspring fitness because the cricket has a ~2-month generation time, which is a relatively long lifespan for an insect, but short enough to complete three generations of breeding in a year. Additionally, methods to test immunocompetency in *T. oceanicus* are well established (Hallagan, Murphy, et al.,In Preparation; Leigh W. Simmons, 2012; Leigh W. Simmons, Tinghitella, & Zuk, 2010) and hundreds of individuals are easy to rear in a laboratory setting. Wild populations of *T. oceanicus* are susceptible to various pathogens and parasitoids (Reinganum, O'Loughlin, & Hogan, 1970; Zuk et al., 1993), making immune response a particularly important fitness component. Finally, females mate and lay eggs throughout life, so advanced maternal age could influence offspring fitness in natural populations.

Experimental Design

We designed a fully-factorial experiment that crossed both maternal and grandmaternal age by mating females in each generation at either a young or old age (Figure 1.2). We created ten maternal lines by mating ten founding females from a colony of approximately 100 breeding adults established using individuals collected at University of California's Gump Field Station on the Polynesian island of Mo'orea in 2014. We mated all founding females at the same age, 7 days post-eclosion (DPE) to

produce the F1 generation. Roughly half of the F1 females were then mated at a young age (7 DPE) and half were mated at an old age (25 DPE). These females produced the F2 generation, and the F2 females were mated in the same manner (at young or old age) to produce the F3 generation. Thus, in our experiment, F1 females are the grandmothers and F2 females the mothers of the F3 generation. We mated all females with unrelated virgin colony males of the same age (5-10 DPE) to control for paternal age. To produce offspring in each generation, we allowed females the opportunity to mate in no-choice conditions by placing one male and one female in a 0.5L deli cup for a 4-hour period each day in a temperature and light-controlled environment for multiple consecutive days (7 days for the founding females and 3 days for both the F1 and F2 females; see Hallagan et al. In Preparation b for detailed methods). We measured fitness in the F3 generation. In the F3 generation, we had 17-46 families in each of 2 maternal age treatments crossed with 2 grandmaternal age treatments, for a total of 4 treatments: 1) young grandmother/young mother (Young/Young), 2) young grandmother/old mother (Young/Old), 3) old grandmother/young mother (Old/Young), and 4) old grandmother/old mother (Old/Old) (Figure 1.1). We did not mate any F3 females.

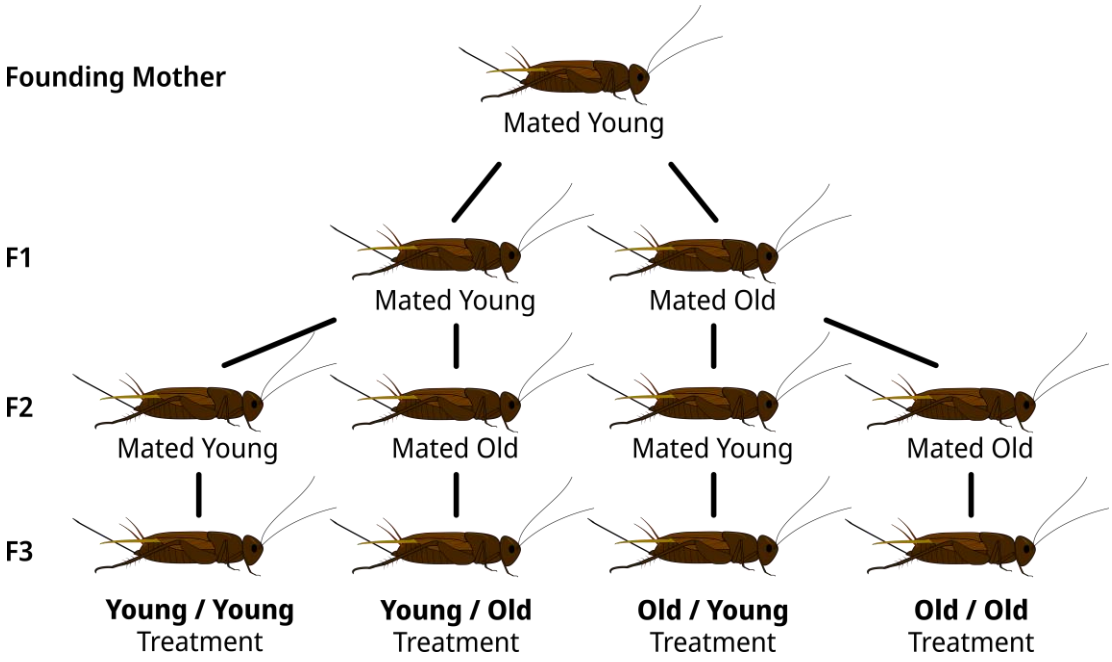

Figure 1.2: Experimental design for maternal age over two generations. In both the F1 and F2 generation, we mated females at either a young age (7 days after eclosion into adulthood) or an old age (25 days after eclosion into adulthood). Thus, F3 offspring were part of one of four treatments: young F1 grandmother/young F2 mother, young F1 grandmother/old F2 mother, old F1 grandmother/young F2 mother, and old F1 grandmother/old F2 mother. We then measured fitness of F2 mothers and F3 individuals.

Rearing and Fitness Measures

We housed all juvenile crickets by family in 0.5L deli cups provisioned with Fluker's High Calcium Cricket Chow and adult crickets in individual 0.5L deli cups provided with Kaytee's Rabbit Chow. We provided all crickets with egg carton for shelter and moist cotton for water. We reared all crickets in Percival Incubators (model I36VLC8) on a 12:12 light:dark schedule and constant temperature (27^0C).

We measured several standard measures of fitness for F3 offspring. For each F3 family, we recorded the number of eggs laid by the F2 mother, mean egg mass,

hatchability, the number of individuals that survived to adulthood, and the sex ratio of surviving adults (17-46 families per treatment; see Table S1 for sample sizes). For each F3 individual we recorded development time and body size at adulthood (see Table S2 for sample sizes). Immediately after the first day of mating, we replaced the cotton in each F2 female's individual container with a roll of moist cheesecloth. After two weeks, we removed the female, counted how many eggs she laid in the cheesecloth, recorded the average mass of a random subset of 5 eggs (mean egg mass), and then two weeks later counted the hatchlings and calculated hatchability (the proportion of eggs hatching) (following Hallagan, Murphy, et al., In Preparation). We transferred the hatchlings to a clean deli cup and housed them by family, checking each deli cup for eclosed adults daily. Development time was the time between the hatch date of the family and eclosion to adulthood of the individual and we recorded sex ratio of each family as the proportion of males in the offspring that survived to adulthood. When an individual eclosed from the juvenile to adult stage, we measured pronotum width (a standard measure of body size) to the nearest 0.01mm using Mitutoyo Absolute Digimax calipers.

Immunocompetency Measures

Immunocompetency is an organism's last line of defense once pathogens and parasites evade morphological and behavioral barriers (Schmid-Hempel & Ebert, 2003; Siva-Jothy, Moret, & Rolff, 2005). Therefore, immunocompetency is an important fitness-related trait. For up to five male and five female F3 individuals in each family, we recorded two proxies of insect immunocompetency: number of hemocytes and

encapsulation response. Hemocytes are the insect equivalent of white blood cells, and previous work has shown that higher hemocyte counts indicate a stronger immune response (Graham et al., 2011). At eclosion, we isolated all crickets used for immune testing in individual 118mL Ziploc containers with moist cotton for water for up to 48 hours before testing (Table S2 for sample sizes). We counted hemocytes by piercing the membrane beneath one hind leg and collecting 1μL of hemolymph to mix with 23μL of anticoagulant (EDTA, citric acid, and PBS regulated at pH 7.4; adapted from Adamo et al. 2001). After gentle vortexing, we placed 7μL of this mixture in a Kova Glasstic Slide System and counted the intact hemocytes in all 9 gridded squares. We noted if a bubble or clumping of cell debris made counting a square impossible and later corrected for uncounted squares by averaging only the counted squares.

The second measure of immunocompetency we used is the encapsulation response. When foreign material is introduced to the body cavity of most insects, cells in the insect's hemolymph adhere to the foreign object and melanize (darken), forming a hardened capsule. In the case of parasitoid eggs, the formation of this hard shell can suffocate the egg and kill the developing parasitoid (Beckage, 2008; Smilanich, Dyer, & Gentry, 2009). We measured the encapsulation response in our crickets by first making a hole in the pronotum of each tested cricket using a sterilized 30-gauge hypodermic needle. Then, we inserted a roughened, sterilized 3mm length of 0.2mm diameter fishing line (filament). After 24 hours, we removed the filament and stored it in a sterile 1.5mL microcentrifuge tube until imaging. We used a Leica S6 dissecting scope with a mounted iPhone SE (attached using an iDu Optics LabCam Microscope Adapter for iPhone) to

photograph the filaments and imaged all filaments at 2.25X magnification. After imaging, we processed all images using FIJI (Schindelin et al., 2009) according to established protocols to generate the percent of the surface area of each filament that was melanized (following Hallagan, Murphy, et al., In Preparation).

Statistical Analysis

We measured fitness after two generations of females being mated at young or old maternal age. Our F3 individual fitness measures were body size (recorded as pronotum width), development time, hemocyte count, and percent filament area melanized. We also measured several aspects of fitness for each F2 mother: the number of eggs she laid, mean egg mass, hatchability, survival to adulthood of her offspring, and sex ratio of her offspring. To meet assumptions of normality, we log transformed percent filament area melanized plus one, and square root transformed hemocyte counts. All other measures were normally distributed.

We used linear mixed models to analyze all individual-level fitness measures (pronotum width, development time, square root transformed hemocyte counts, and log transformed percent filament area melanized plus one). Our model included *maternal age* at mating (of the F2 mother, young or old), *grandmaternal age* at mating (of the F1 grandmother, young or old), the *interaction of maternal and grandmaternal age*, and *sex* of each F3 offspring as main effects. To account for the possible covariance of size and fitness measures, *pronotum width* of the F3 individual was a covariate in all individual-level tests except where pronotum was the response variable. We also included *maternal*

line as a random effect in all individual-level tests. The *interaction of maternal and grandmaternal age* was not significant in any of the individual-level fitness models, so we removed it from the models. Thus, our final model for all individual-level fitness measures included *maternal age*, *grandmaternal age*, *pronotum width*, and *sex* as main effects and *maternal line* as a random effect.

We also used linear mixed models to analyze all measures of fitness of F2 mothers (number of eggs laid, egg mass, hatchability, number of individuals that survived to adulthood, and sex ratio). Our models included *maternal age* at mating (of the F2 mother, young or old), *grandmaternal age* at mating (of the F1 grandmother, young or old), the *interaction of maternal and grandmaternal age*, and *maternal pronotum width* (of the F2 mother) as main effects and *maternal line* of the F2 female as a random effect. If the *interaction of maternal and grandmaternal age* was significant in any analysis, we used Tukey's HSD to determine which treatment means differed significantly.

To correct for multiple comparisons of means, we used the Benjamini-Hochberg false discovery rate (FDR) method (Benjamini & Hochberg, 1995). For both the individual fitness measures and the F2 mother fitness measures, we used a Q value of 0.05. For the F3 individual fitness measures, we ranked the p-values of *maternal age* and *grandmaternal age*. For the F2 mother fitness measures, we ranked the p-values of *maternal age*, *grandmaternal age*, and the *interaction of maternal and grandmaternal age*. Because we were only interested in the effects of maternal and grandmaternal age on fitness, we did not correct p-values for any covariates. We used JMP Pro version 13.0.0 for all statistical analysis.

Results

In our F3 individual-level analyses, we found that offspring development time followed the predictions of the compounding hypothesis and life history theory (Figure 1.1D, Figure 1.3A). Both *maternal age* and *grandmaternal age* affected the development time of F3 offspring (Figure 1.3A, Table 1.1) and these effects remained significant after FDR correction. F3 offspring with old mothers developed 9% faster than F3 offspring with young mothers, and F3 offspring with old grandmothers developed 6% faster than F3 offspring with young grandmothers (Figure 1.3A, Table 1.1). *Sex* and *pronotum width* were also associated with development time; large males took the most time to develop (Table 1.1). We found no significant effect of *maternal age* or *grandmaternal age* on pronotum width of F3 offspring, but there was a significant effect of *sex* in that males were larger than females (Table 1.1).

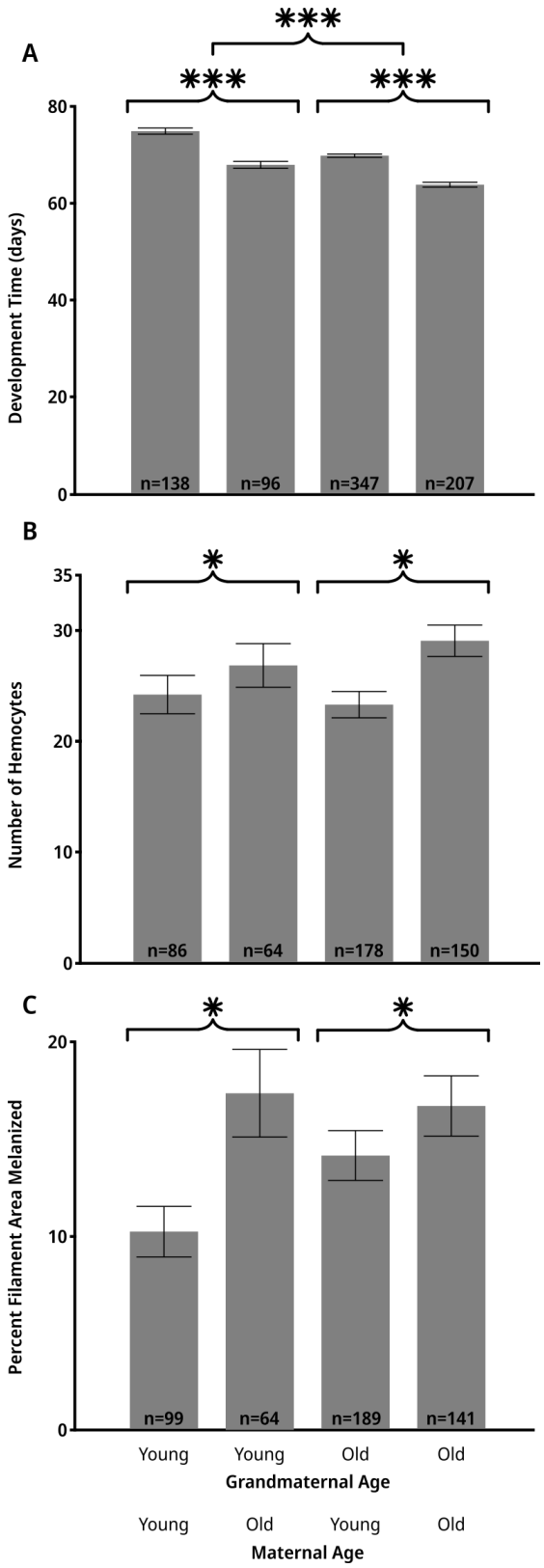

Figure 1.3: For all measured F3 individuals: A) development time from family hatch date to individual eclosion date, B) number of hemocytes in hemolymph sample, and C) percent filament area melanized. Bars indicate means ± SE, and n = number of individuals included in analysis. In A, top bracket indicates the significant effect of grandmaternal age from the model, and the bottom brackets indicate the significant effect of maternal age from the model. In B and C, brackets indicate the significant effect of maternal age from the model. Asterisks indicate significance level of the FDR adjusted p value. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Table 1.1: Effects of advanced maternal and grandmaternal age on offspring fitness (F3 individual fitness measures). Bold indicates significant effects from the model. "Adjusted p" represents the adjusted p-value using FDR correction with Q=0.05. Dashes represent the p-values that we did not adjust with FDR.

Measure	***Main Effect***	***F***	***df***	***p***	***Adjusted p***
Pronotum	Maternal age	0.05	1, 87.57	0.82	0.82
	Grandmaternal age	0.25	1, 90	0.62	0.82
	Sex	**51.54**	**1, 730.7**	**<0.0001**	**-**
Development time	**Maternal age**	**53.92**	**1, 86.73**	**<0.0001**	**<0.0001**
	Grandmaternal age	**20.66**	**1, 90.12**	**<0.0001**	**<0.0001**
	Pronotum	**150.11**	**1, 771.5**	**<0.0001**	**-**
	Sex	**27.97**	**1, 740**	**<0.0001**	**-**
sqrt(Hemocyte Number)	**Maternal age**	**7.68**	**1, 60.19**	**0.007**	**0.018**
	Grandmaternal age	0.07	1, 73.25	0.80	0.82
	Pronotum	0.48	1, 347.2	0.49	-
	Sex	0.01	1, 459.9	0.92	-
log(Filament Area Melanized+1)	**Maternal age**	**7.18**	**1, 74.44**	**0.009**	**0.018**
	Grandmaternal age	1.56	1, 83.57	0.22	0.34
	Pronotum	**7.18**	**1, 369.6**	**0.008**	**-**
	Sex	1.76	1, 480.4	0.19	-

We found significant effects of *maternal age* on both measures of immunity, hemocyte count and percent filament area melanized, and both effects followed the predictions of the precedence hypothesis and life history theory (Figure 1.1B). The F2 mothers that we mated at an old age had offspring with 22% higher hemocyte counts than offspring of F2 mothers mated at a young age (Figure 1.3B, Table 1.1) and this effect remained significant after FDR correction. *Grandmaternal age* did not affect the number of hemocytes in F3 offspring (Table 1.1). F3 offspring of F2 mothers mated at an old age

(*maternal age*) also had a higher percent filament area melanized than offspring of F2 mothers mated at a young age (Figure 1.3C, Table 1.1) and this effect remained significant after FDR correction. *Grandmaternal age* did not affect the percent filament area melanized in F3 offspring (Table 1.1). We also found that larger individuals tended to have higher percent filament area melanization (Table 1.1).

In our family-level analysis, we found no effects that were significant after FDR correction. Before FDR correction, we found that there was a significant *interaction of grandmaternal age and maternal age* such that F2 females in the Old/Young treatment laid significantly more eggs than F2 females in the Old/Old treatment, but did not differ from the number of eggs laid by females in the other two treatments (Young/Young and Young/Old) (Table 1.2). Before FDR correction, *maternal age* (age of the F2 female) also affected the number of eggs laid by F2 females (Table 1.2), with females mated at a young age laying more eggs than females mated at an old age. There was no significant effect of *grandmaternal age* (age of the F1 mother to each F2 female) on the number of eggs laid by F2 females (Table 1.2). Before FDR correction, we found that *grandmaternal age*, but not *maternal age*, impacted the number of offspring surviving to adulthood; F1 grandmothers mated at an old age had on average 60% more F3 surviving grandoffspring than F1 grandmothers mated at a young age (Table 1.2). We found no significant effects of *maternal age* or *grandmaternal age* at mating on mean egg mass, hatchability, or sex ratio of F3 offspring (Table 1.2).

Table 1.2: Effects of advanced maternal and grandmaternal age on offspring fitness (F2 mother fitness measures). F2 Age*F1 Age is the interaction between the maternal age and grandmaternal age. We measured sex ratio as the proportion of male offspring. Bold indicates significant effects from the model. "Adjusted p" represents the adjusted p-value using FDR correction with Q=0.05. Dashes represent the p-values that we did not adjust with FDR. No family-level measures were significant after FDR correction.

Measure	***Main Effect***	***F***	***df***	***p***	***Adjusted p***
Egg Number	**Maternal age**	**5.34**	**1, 118.8**	**0.0226**	**0.11**
	Grandmaternal age	0.14	1, 23.61	0.71	0.82
	F2 Age*F1 Age	**8.17**	**1, 118.4**	**0.005**	**0.07**
	Pronotum	3.58	1, 127.8	0.06	-
Egg Mass	Maternal age	0.46	1, 117.7	0.50	0.75
	Grandmaternal age	0.16	1, 23.91	0.69	0.82
	F2 Age*F1 Age	1.51	1, 117.4	0.22	0.48
	Pronotum	1.94	1, 129.3	0.17	-
Hatchability	Maternal age	0.34	1, 125.7	0.56	0.77
	Grandmaternal age	0.01	1, 23.24	0.94	0.94
	F2 Age*F1 Age	0.81	1, 125.2	0.37	0.62
	Pronotum	0.48	1, 118	0.49	-
Number Survived to Adulthood	Maternal age	1.50	1, 95.77	0.22	0.48
	Grandmaternal age	**8.08**	**1, 20.52**	**0.0099**	**0.07**
	F2 Age*F1 Age	0.01	1, 95.79	0.92	0.94
	Pronotum	2.82	1, 103.6	0.10	-
Sex Ratio	Maternal age	2.85	1, 90.95	0.09	0.35
	Grandmaternal age	2.11	1, 22.6	0.16	0.48
	F2 Age*F1 Age	1.27	1, 91	0.26	0.49
	Pronotum	0.00	1, 70.72	0.99	-

Discussion

Parental and grandparental aging can have complex effects on offspring fitness. Grandoffspring of old grandmothers developed more quickly than grandoffspring of young grandmothers. Offspring of old mothers also developed more quickly than offspring of young mothers. Additionally, offspring of old mothers had higher measures of both immunocompetency measures (hemocyte abundance and percent filament area melanized) and also developed faster than offspring of young mothers. We found support for both the precedence hypothesis and the compounding hypothesis, but with different traits.

Our two-generation experimental design allowed us to test two hypotheses: the precedence hypothesis and the compounding hypothesis (Figure 1.1). The precedence hypothesis predicted that because parental effects on a single generation of offspring are often found (Badyaev & Uller, 2009; Mousseau & Fox, 1998), maternal age will be the only factor in our experiment determining effects on offspring fitness traits. Contrastingly, the compounding hypothesis predicts that maternal age would have additive effects in the same direction across generations, so both maternal and grandmaternal age would influence offspring traits in the same direction. The patterns we found in both measures of immunocompetency supported the predictions of the precedence hypothesis and life history theory; only maternal age had a significant effect on measures of hemocyte abundance and percent filament area melanized, and offspring of old mothers had higher hemocyte abundance and percent filament area melanized than offspring of young mothers (Figure 1.1B). Our results for development time supported

the predictions of the compounding hypothesis and life history theory. There was a significant positive effect of advanced grandmaternal age at mating, and that effect was in the same direction as the positive effect of maternal age at mating (Figure 1.1D).

Broadly, our results supported the predictions of the terminal investment hypothesis of life history theory. The terminal investment hypothesis predicts that as organisms age, they will invest more in reproduction because of a reduced future cost of reproduction (Charlesworth & Leon, 1976; Williams, 1966). Our results showed that older mothers had offspring with higher measures of immunocompetency and faster development (Figure 1.1B and 1.1D). An alternative hypothesis that would predict the same outcome as the terminal investment hypothesis of life history theory is that there is differential survival of high quality individuals to the age that we consider "advanced maternal age" (25 days after eclosion to adulthood), and low quality individuals are likely to only survive to the young maternal age. However, we chose 25 days for our advanced mating age because it is relatively old compared to the young treatment, but still allowed females to live at least enough after mating to lay eggs for two weeks and not be in severely poor health by the time egg laying was over. Therefore, senescence could have had time to take an effect by 25 days, but not to the extent that only the highest quality individuals would be able to survive to that age.

We aimed to test the transgenerational effects of maternal age on offspring, but a large question remains: what mechanisms could drive transgenerational effects of aging? Characteristics intrinsic to parents and their experience of the environment can be indirectly communicated to offspring in a variety of ways, including hormones (Díaz &

Esponda, 2004), nutrients (Fox & Czesak, 2000), and the multiple mechanisms of epigenetics (reviewed in Jablonka & Raz 2009). To narrow these possibilities, further work must be done in the broad field of insect epigenetics. While some insects have wash-in of epigenetic effects over multiple generations (Burrows et al., 2011; Ernst et al., 2015), we do not know how or if DNA methylation patterns are conserved through several generations. In mammals, methylation is reprogrammed multiple times in an organism's early life and some methylation patterns are conserved only because underlying genetic signals encode for those methylation patterns (Kazachenka et al., 2018). In insects, we do not know if methylation is similarly reprogrammed during early life, or if some of these seemingly non-genetic inheritance patterns are, like in mammals, actually genetic. If insect patterns of methylation are reprogrammed early in life, it would lead to results similar to those predicted by our precedence hypothesis, where only the age of an individual's mother has a significant effect. On the other hand, if methylation patterns are not reprogrammed early in an insects' life, we would expect results similar to those predicted by the compounding hypothesis. Our study helps lay a foundation for future research into the transgenerational effects of aging and there is ample opportunity for researchers to examine the underlying mechanisms at work across aging effects.

We used a two-generation experimental design to measure the transgenerational dynamics of the effect of parental age on offspring fitness traits in a laboratory setting. We found that age can have different influences on different traits. Maternal age has effects on offspring immunocompetency that last only a single generation; grandmaternal age had no effect on either the hemocyte abundance or the percent filament area

melanized of offspring. Contrastingly, offspring development time was influenced by the mating age of both mothers and grandmothers, and the effect of the mating age of grandmothers and mothers was compounded by subsequent generations of old or young mating age. In all cases in which our results were significant, we found positive effects of advanced maternal age on offspring fitness traits. However, our study was conducted in a controlled laboratory setting, and we plan to examine the ecological context of aging across populations of *T. oceanicus* in the wild; distinct populations of *T. oceanicus* can be found across several Pacific islands and Australia, which will allow us to test how ecological context affects age structure and to determine whether the effects we found in the lab are adaptive or non-adaptive. Although the process of aging is relatively well studied, the environmental conditions that lead to advanced age in the field are not.

CHAPTER TWO: Consequences of advanced maternal age on reproductive investment of male offspring

Introduction

Intrinsic characteristics of parents and their experience can have profound effects on offspring traits through parental effects (reviewed in Badyaev & Uller 2009). Age is a particularly important component of a parent's condition that impacts offspring traits ranging from disease resistance to growth in numerous taxa including insects (Bloch Qazi et al., 2017), fish (Berkeley, Chapman, & Sogard, 2004; Hansen et al., 2015), mammals (Descamps et al., 2008), and birds (Asghar et al., 2014). However, how parental age affects offspring traits is not consistent across studies and can be positive, negative, or neutral (Hallagan, Tinghitella, et al., In Preparation). Effects of advanced parental age on offspring fitness typically support one of two major bodies of literature: life history theory or aging theory. One component of life history theory, the terminal investment hypothesis, predicts that late in life, selection will favor life histories that invest heavily in reproduction, because the need to invest in survival and future reproduction is minimal at that stage (Partridge & Harvey, 1988; Trivers, 1974); offspring of older mothers should therefore be more or at least equally as fit as offspring from younger mothers. Aging theory, in contrast, predicts that older parents are unable to make this reproductive

investment or the investment is poor due to the detrimental effects of senescence (Lemaitre & Gaillard, 2017; Nussey, Froy, Lemaitre, Gaillard, & Austad, 2013); offspring of older mothers should therefore be less fit than offspring of young mothers. Both bodies of literature, however, focus on the effects of advanced parental age on traits that are relevant to both sexes (such as body size or growth rate), or investigate fitness effects only for female offspring. The influence of parental age specifically on male offspring has been overlooked.

Male fitness is often determined to some extent by the investment made in postcopulatory reproductive traits (Harcourt, Harvey, Larson, & Short, 1981; Parker, 2015; Taborsky, 2002). Though males invest less in individual gametes than females, males are still limited in the amount of sperm they can use in each reproductive opportunity (Wedell & Gage, 2002). Males optimize investment in reproductive bouts through adjustment of the size or contents of their ejaculate to match perceived levels of mate availability and sperm competition (Reinhardt, Naylor, & Siva-Jothy, 2011; L. W. Simmons, 2003; Vahed, Parker, & Gilbert, 2011). In crickets, females mate with multiple males and store sperm in a round spermatheca, leading to a 'lottery' in determining which sperm fertilize the most eggs (Larson, Hume, Andrés, & Harrison, 2012; L. W. Simmons, 2003). Therefore, in crickets, male investment in reproductive traits such as sperm volume and sperm viability may be more important in determining paternity than other factors such as mating order (Bretman, Newcombe, & Tregenza, 2009; Hall, BussiÈre, Demont, Ward, & Brooks, 2010; Sakaluk & Eggert, 1996; L. W. Simmons, 2003). Our study system is the Pacific field cricket, *Teleogryllus oceanicus*. In this system, females

mate with multiple males, and males that invest more in postcopulatory reproductive traits (such as sperm viability) tend to father more offspring (Garcia-Gonzalez & Simmons, 2005). Male investment in reproductive somatic tissue is particularly plastic in *T. oceanicus*, changing, for instance, in response to rearing environments that mimic a high density of males (Bailey, Gray, & Zuk, 2010; Gray & Simmons, 2013) and traffic noise (Bowen, Gurule-Small, & Tinghitella, In Preparation). Given that male investment in reproductive tissues is an important component of male fitness, and is plastic, we tested if maternal age influences male reproductive investment.

We investigated the effects of advanced maternal age on male reproductive investment by measuring testes mass, accessory gland mass, and sperm viability of male offspring following two generations of mating females at either a young or old age (Figure 2.1). We had two questions 1) does maternal age affect the reproductive investment of male offspring and, if so, 2) does the effect of maternal age on male reproductive investment support the predictions of life history theory or aging theory? In the broadest sense, support for life history theory would come from male offspring of old mothers and grandmothers having greater reproductive investment as compared with male offspring of young mothers and grandmothers. Alternatively, if male offspring of old mothers and grandmothers show lower reproductive investment than male offspring of young mothers and grandmothers, this would support aging theory.

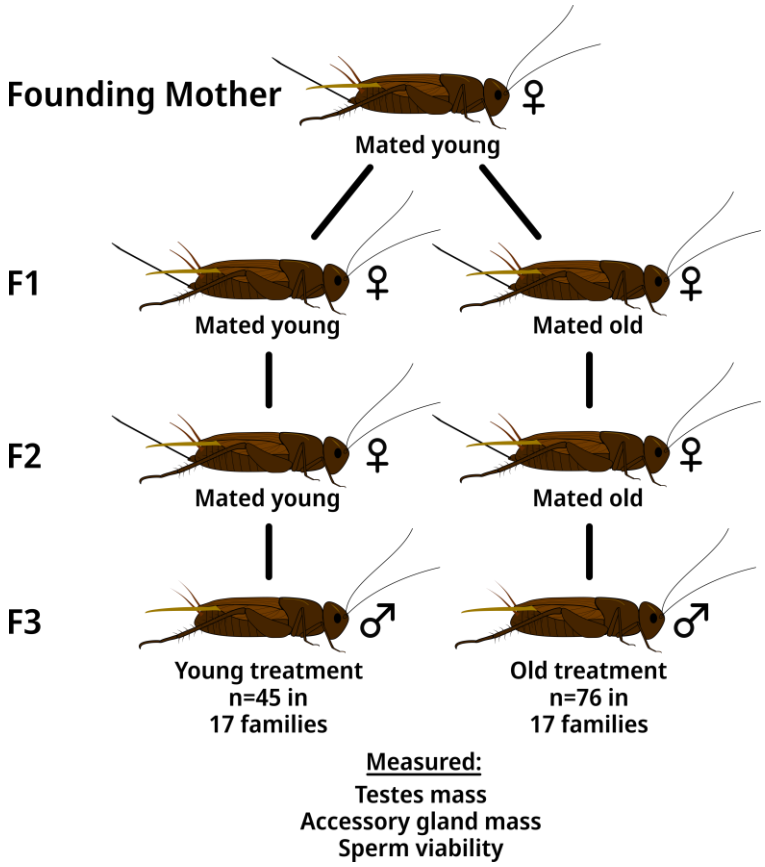

Figure 2.1: A diagram of our experimental mating design. We mated females at either a young age (7 days after eclosion to adulthood) or an old age (25 days after eclosion to adulthood) for two subsequent generations, then measured three proxies of reproductive investment in males of the F3 generation.

Methods

Study System and Design

To study the effects of maternal age on male reproductive investment, we used the Pacific field cricket, *T. oceanicus* because they live a relatively long time for an insect and male reproductive investment is easily measured using established methods. Female *T. oceanicus* mate throughout their life and with multiple males (L. W. Simmons, 2003), a breeding system that should lead to selection on postcopulatory reproductive traits in

males (Leigh W Simmons, 2001). Additionally, testes mass and accessory gland mass are well established measures of male reproductive investment (Bailey et al., 2010; Gray & Simmons, 2013).

The *T. oceanicus* individuals that we used in this study were from a laboratory colony established from animals collected at the University of California's Gump Field Station on the Polynesian island of Mo'orea in 2014. The colony typically contains approximately 100 breeding adults. We randomly chose 10 females from the colony to serve as our founding females in April 2017. We mated the 10 founding females at 7 days post-eclosion (DPE) and then started mating their female offspring at either a young age (Young treatment) or an old age (Old treatment) for two generations (Figure 2.1). We mated females in the Young treatment at 7 DPE and females in the Old treatment at 25 DPE, which is close to the natural adult lifespan of ~one month. Therefore, we had two treatments, one in which we mated both the grandmother and mother of our study males at a young age (Young treatment), and the other in which we mated both the grandmother and the mother at an old age (Old treatment). To mate each female, we placed her in a 0.5L deli cup with an unrelated colony male for a 4-hour period over multiple consecutive days (7 days for founding females and 3 days for both subsequent generations). To reduce possible effects of paternal age, all males that we used for matings were 5-10 DPE (for more detail on the mating procedure, see Hallagan, Murphy, et al., In Preparation).

Rearing

We kept all crickets in temperature-controlled (27^0C) Percival incubators (model I36VLC8) on a 12h:12h light:dark schedule throughout the experiment. We housed juvenile crickets in family groups inside of 0.5L deli cups and supplied them with Fluker's High Calcium Cricket Chow, egg carton for shelter, and moist cotton for water. Once the juvenile crickets eclosed, we housed all females that were to be mated individually in 0.5L deli cups provisioned with Kaytee Rabbit Chow, egg carton for shelter, and moist cheese cloth for water and egg deposition. After eclosion, we housed all male crickets in individual 118mL Ziploc containers provisioned similarly to the females.

Male Reproductive Investment

For the male crickets that we studied, we measured three aspects of male reproductive investment: testes mass, accessory gland mass, and sperm viability. We measured male reproductive investment on males that were 1-22 DPE. After collecting a fresh spermatophore from each male for sperm viability testing, we euthanized males by freezing and stored them dry in individual, sterile 1.5mL microcentrifuge tubes at -20^0C between March and April of 2018. We dissected testes and accessory glands from all males from both treatments at one of two times: July 2018 (Young treatment n=7, and Old treatment n=48) and April 2019 (Young treatment n=38, and Old treatment n=28). We therefore refer to three datasets in this manuscript; the July 2018 dataset includes the

males we dissected in July 2018, the April 2019 dataset includes the males we dissected in April 2019, and the complete dataset includes males from both dissection dates.

To test sperm viability, we used a ThermoFisher LIVE/DEAD sperm viability kit and established methods (Garcia-Gonzalez & Simmons, 2005). The ThermoFisher LIVE/DEAD kit stains live sperm green and dead sperm red. Immediately after staining, we imaged all sperm samples using a Leica M165FC scope with a EC3 camera on a computer running LAS X imaging software. We captured two images from the same view window of each sample: one image using a FITC excitation filter (for the green-stained, live sperm) and one image using a TRITC excitation filter (for the red-stained, dead sperm). After imaging, we overlaid a 36wx24h grid on both images from each sample using Inkscape (a vector graphics editing program). We counted 25% of each image by haphazardly choosing an evenly spaced subset of grid rows and counting those same rows in each image. We counted live and dead images separately and recorded any sperm cells that fell within the counted area, including those that landed on the top or bottom line. Due to the inherent difficulties of rearing two generations of crickets at different mating ages, the majority of Young treatment males were euthanized by the time we started collecting sperm viability data. Therefore, we only have sperm viability for 7 males in the Young treatment and 48 males in the Old treatment; these are the same males that we dissected in July 2018

Statistical Analysis

We used a linear mixed model to test the effect of maternal age treatment on testes mass and accessory gland mass using the complete dataset. We transformed accessory gland mass using a cube-root transformation to meet assumptions of normality and equal variance. We had two response variables: testes mass and accessory gland mass. We included *maternal age treatment* as a fixed effect and *age of the male* when euthanized and *pronotum width* (a measure of size) as covariates. We included *dissection date* as a fixed effect in the model because we noticed that tissue we dissected at the later date was generally smaller than tissue dissected at the earlier date, likely due to the extra time that the tissue spent in the freezer. We included the *maternal line* of each male as a random effect that accounted for the identity of the founding female, grandmother, and mother of each male. We also initially included the interaction between *dissection date* and *maternal age treatment*, but the interaction was not significant so we removed it from the model. Therefore, our final model included *maternal age treatment*, *age of the male*, *pronotum width*, and *dissection date* as fixed effects and *maternal line* as the random effect.

We also tested the effect of maternal age treatment on testes mass and accessory gland mass using only the individuals we dissected in April 2019 because this dataset had a more balanced sample size (Young treatment n=38, and Old treatment n=28) than the July 2018 dataset and the complete dataset. We ran the same statistical model described above for both testes mass and transformed accessory gland mass, but because these males were all from a single dissection date, we removed *dissection date* as a fixed effect.

Thus, our final model included *maternal age treatment*, *age of the male*, and *pronotum width* as fixed effects and *maternal line* as the random effect.

We used one additional linear mixed model to test the effect of maternal age on sperm viability of male offspring. We only measured sperm viability for males from the July 2018 dataset and thus our sample size was unbalanced (Young treatment n=7, and Old treatment n=48). Our statistical model included *maternal age treatment*, *age of the male*, and *pronotum width* as fixed effects and *maternal line* as the random effect.

We used post-hoc power analyses to confirm we had sufficient sample size for any non-significant results. We were not able to run power analyses on the linear mixed models described above, so we instead used models that did not include the random effect accounting for the *maternal line* of each cricket, but verified beforehand that the results of these models aligned with the results of the linear mixed models. We used JMP Pro version 13.0.0 for all analysis.

Results

We found that *maternal age treatment* did not significantly affect the reproductive investment of male offspring. In the complete dataset *maternal age treatment* did not have a significant effect on either testes mass ($F_{1,41.76}=0.11$, $p=0.74$; Figure 2.2A) or transformed accessory gland mass ($F_{1,32.5}=0.99$, $p=0.32$; Figure 2.2B). Our power analysis showed that with our means and variance, we would need 47,396 observations of testes mass and 556 observations of accessory gland mass to detect a significant difference in these variables between the maternal age treatments. *Age of the male*

($F_{1,114.8}=9.85$, $p=0.002$), *pronotum width* ($F_{1,114.5}=7.04$, $p=0.009$), and *dissection date* ($F_{1,114.7}=31.81$, $p=<0.0001$) significantly affected testes mass, such that older and smaller males dissected in April 2019 had smaller testes mass than younger and larger males that were dissected in July 2018. *Dissection date* also had a significant effect on accessory gland mass; males that we dissected during the later date (April 2019) had smaller accessory glands ($F_{1,115.9}=20.77$, $p=<0.0001$), but neither *age of the male* ($F_{1,115.3}=0.67$, $p=0.42$) nor *pronotum width* ($F_{1,110.2}=0.83$ $p=0.36$) had significant effects. In our analysis that included only the April 2019 dataset, we found no significant effect of *maternal age treatment* on testes mass ($F_{1,18.99}=0.04$, $p=0.8$) or accessory gland mass ($F_{1,29.98}=3.59$, $p=0.07$). Our power analysis showed that with our means and variance, we would need 3,539 observations of testes mass and 83 observations of accessory gland mass to detect a significant difference in these variables between maternal age treatments. In our analysis of sperm viability data we found no significant effect of *maternal age treatment* ($F_{1,29.04}=0.82$, $p=0.37$), *age of the male* ($F_{1,45.18}=0.44$, $p=0.51$), or *pronotum width* ($F_{1,47.52}=0.49$, $p=0.49$) on the sperm viability of males. Our power analysis showed that we would need 284 observations of sperm viability to detect a significant difference between maternal age treatments.

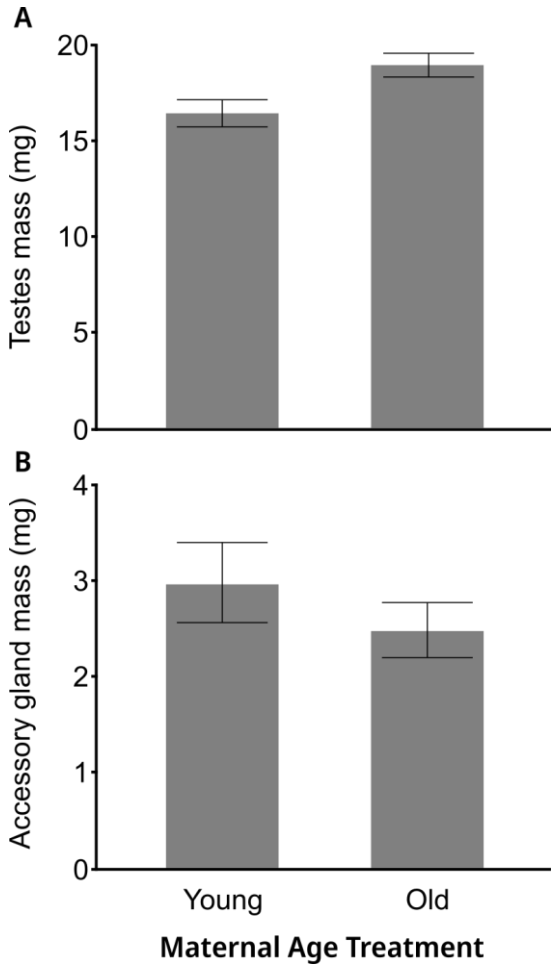

Figure 2.2: Reproductive investment of male offspring by treatment**.** For all male offspring from both maternal age treatments: **A)** Testes mass and **B)** Accessory gland mass. Bars represent least square means ± SE.

Discussion

Maternal age can have complex and contrasting influences on a number of offspring traits (Berkeley et al., 2004; Hansen et al., 2015). We asked whether advanced maternal age influenced reproductive traits of male offspring and found no influence of maternal age treatment on testes mass, accessory gland mass, or sperm viability;

however, our sperm viability results should be viewed cautiously due to our unbalanced sample size for that portion of the experiment. Given that we did not find a difference in male reproductive traits between the Young and Old treatments, we conducted power analyses that largely supported and validated our null results. For one measure, accessory gland mass in the males that we dissected later (April 2019 dataset), the power analysis suggests we may need a larger sample size.

Both life history theory and aging theory have been used to explain impacts of advanced maternal age on offspring fitness. In general, finding that offspring of older mothers are more fit than offspring of young mothers would support life history theory (Trivers, 1974; Williams, 1966) and finding the opposite would support aging theory (Lemaitre & Gaillard, 2017; Nussey et al., 2013). We do not find overwhelming support for either of these patterns in this study. There are several reasons this might be the case. First, there could simply be no link between maternal age and the reproductive investment of male offspring. We know that male postcopulatory traits (such as sperm viability and accessory gland mass) are plastic in *T. oceanicus* (Bailey et al., 2010; Bowen et al., In Preparation; Gray & Simmons, 2013), but we do not know all of the environmental factors under which that plasticity is released. Second, there may be other constraints on male reproductive investment or these traits may be pleiotropically linked with other traits. Finally, it is possible that our results may support life history theory, but without a deeper understanding of the underlying mechanisms and drivers of male reproductive investment we can only be sure that our results do not support aging theory.

Testes size is often highly variable within populations and increased size is associated with an increased risk of sperm competition (Merila & Sheldon, 1999; Leigh W Simmons, 2001), including in this study system (Bailey et al., 2010). In many taxa, this pattern is the result of selection; males in species with higher risk of sperm competition often have much larger testes than males in closely related species with lower risk of sperm competition (Merila & Sheldon, 1999). Testes size can also be plastic depending on perceived level of sperm competition during rearing (Bailey et al., 2010; Bowen et al., In Preparation; Fisher & Hook, 2018). In our analysis, we found that testes size is correlated with pronotum width, dissection date, and age of the male. We would expect testes size to covary with size of the male due to allometry, and differences from dissection date are likely a result of tissue degradation. We found that older males had smaller testes, but there is nothing in the literature suggesting that testes size might shrink with age. In many species, older males have larger testes due to sexual maturity, and there is some evidence for an increase in asymmetry between testes with age (Abdul-Rahman, Obese, & Robinson, 2018; Brown & Brown, 2003; Merila & Sheldon, 1999). Perhaps the pattern in our results of old males having smaller testes reflects a trade-off between reproduction and longevity (Austad & Hoffman, 2018); if older males have invested more resources in survival and maintenance than younger males, this investment could come at the expense of reproductive somatic tissue.

We measured the effect of advanced maternal age on traits specific to male offspring, and we suggest that researchers begin to include these male traits in studies of fitness to gain a more comprehensive view of fitness measures. Hallagan et al. (In

Preparation) measured the effects of one generation of advanced maternal age on offspring size, survival to adulthood, and immunocompetency in *T. oceanicus*, finding results that support both life history theory and aging theory depending on the fitness measure used. Young mothers had more offspring, but there was no difference between old and young mothers in number of offspring that reached adulthood and offspring of old mothers had higher measures of immunocompetency. Alongside ours, the results of Hallagan et al. (In Preparation) demonstrate that life history theory and aging theory can predict the effects of maternal age on different traits. Depending on which trait is measured, advanced maternal age may have positive, negative, or neutral effects. Our work is the first to consider effects of parental age on traits specific to male offspring, and we encourage other researchers to include male offspring fitness in a comprehensive suite of fitness measures of offspring in aging studies.

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APPENDIX

Appendix Table 1: Sample sizes for all F3 individual fitness measures from Chapter 1

Fitness Measure	Young/Young Individuals	Young/Old Individuals	Old/Young Individuals	Old/Old Individuals
Pronotum	138	96	341	207
Development Time	138	96	347	207
Hemocyte Counts	86	64	178	150
Filament Area Melanized	99	64	189	141

Appendix Table 2: Sample sizes for all F2 mother fitness measures from Chapter 1

Fitness Measure	Young/Young Families	Young/Old Families	Old/Young Families	Old/Old Families
Egg Number	32	27	46	33
Egg Mass	32	27	46	33
Hatchability	32	27	46	33
Number Survival to Adulthood	24	22	40	27
Proportion of Male Offspring	19	17	38	22