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Qualitative and Quantitative Fitness Consequences of Advanced Maternal Age

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Qualitative and Quantitative Fitness Consequences of Advanced Maternal Age

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Parental age can affect offspring fitness across taxa and through various mechanisms. However, the effect(s) of advanced maternal age on offspring, particularly in insects, has not been comprehensively reviewed making it difficult to draw conclusions about the effects of advanced maternal age on offspring in insects. In my first chapter, I reviewed maternal age literature and found overall negative effects of advanced maternal age on offspring fitness. However, results vary depending on which fitness measures were used, the life stages at which offspring were measured, and the experimental design of the study. In my second chapter, I conducted an experiment where I collected a suite of fitness measures, including immune response, which is often overlooked, and found that advanced maternal age has an overall positive effect on offspring fitness. My work highlights the importance of following offspring throughout all life stages and implementing various fitness measures, including immune function.

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Chapter 1: A comprehensive review of maternal age effects on offspring fitness in insects

Introduction

Aging can have widespread negative effects on individual fitness. As organisms age, individuals can experience progressive declines in physiological (e.g. metabolism, immune function, stress response, motor function, body movement), cellular (e.g. mitochondrial function, tissue regeneration), and cognitive traits (e.g. attention, memory; (Harman 1956; Semba *et al.* 2010; Linton & Dorshkind 2004; Glisky 2007; Gruver *et al.* 2007; Alt *et al.* 2012; Muller *et al.* 2013; Buzzi *et al.* 2003). The mechanisms driving these negative effects on fitness vary. For example, the mitochondrial theory of aging argues that mitochondrial DNA is highly susceptible to lesions caused by free radical oxidative damage, which accumulate over time resulting in the deterioration of an organism's physical and cognitive state with advanced age (Gavrilov *et al.* 1997; Harman 1956). Furthermore, telomeres play an important role in protecting chromosomes from oxidative stress associated with cell division but shorten in length as the organism ages, leading to cell death; individuals with shorter telomeres have been found to have shorter lifespans (Asghar *et al.* 2015; Heidinger *et al.* 2016). General cellular breakdown (or lack of upkeep) that becomes more common with age, regardless of mechanism, can lead to reduced fitness of the individual and, indirectly, of the offspring given the heritability of DNA and telomeres (Dugdale & Richardson 2018; Delgado *et al.* 2018).

Aging not only affects the fitness of individuals but may also affect the fitness of their offspring. There are two theories that predict contrasting effects of advanced maternal age on offspring: aging theory and life history theory. Aging theory predicts that as females age, their reproductive investment deteriorates, suggesting that older females may produce fewer offspring or that the offspring from older mothers could be less fit than those produced by young mothers because they can inherit both damaged mitochondrial DNA and short telomeres (Dugdale & Richardson 2018; Delgado *et al.* 2018). In contrast, a component of life history theory predicts that late in life females may invest heavily in reproduction to increase their own fitness before death (terminal investment), suggesting that the quantity or quality of the offspring from older mothers may be higher than in offspring from younger mothers (Partridge & Harvey 1988; Fessler *et al.* 2005).

Fifty-five years ago, Parsons (1964) reviewed the effects of parental age on offspring and found that the overall effects of advanced maternal age on offspring were mixed in mammals, and negative in insects and plants. For example, Parsons' (1964) review found that the occurrence of congenital abnormalities in human offspring increased with maternal age but decreased with maternal age in mice and guinea pigs. Furthermore, in *Drosophila*, eggs from old mothers hatched less frequently relative to eggs laid by young mothers and those offspring that did hatch had a lower chance of surviving to subsequent instars than offspring from young mothers (Hadorn & Zeller 1943, as cited in Parsons 1964). In his review, Parsons (1964) acknowledged the need to separate maternal and paternal age so their effects can be assessed independently, and he

emphasized the need for additional studies on a broader pool of study organisms. At the time, data were only available for five species (mice, guinea pigs, humans, *Drosophila*, and duckweed). Most notably, he emphasized the need for direct investigation of parental age effects on offspring fitness, noting that much of the data on maternal age at the time were collected as a by-product of other work (Parsons 1964). In the intervening 55 years, dozens of studies have investigated how maternal age affects a variety of offspring fitness measures in numerous study systems. To date, research on how maternal age affects offspring fitness in vertebrate systems vastly outnumbers work done with invertebrate systems (e.g. see review by Liu *et al.* 2011 on humans). Our review is the first to investigate the effects of maternal age in insects in 55 years (but see Soliman 1986, which focused on the flour beetle). We focus our review on insects because they are one of the most diverse groups of organisms and allow us to perform large scale aging projects within a relatively short period of time.

In this review, we summarize the work that followed Parsons' (1964) call to advance our understanding of parental age effects on offspring fitness. In the intervening years, maternal effects have received more widespread attention than paternal effects, particularly regarding female reproductive investment (Sinervo 1990; Mousseau & Fox 1998; Wells 2014). Although we agree with recent work suggesting paternal effects may be underappreciated (Curley *et al.* 2011), the imbalance of existing research has led us to focus maternal age effects rather than paternal age. We examine the approaches used in studies of maternal aging, the trends found in the results of this research, common pitfalls in the experimental design of the studies, and conclusions we can make about patterns in

maternal aging research. Finally, we offer suggestions for experimental design of maternal age research and future directions in the field.

Methods

To identify literature on how maternal age affects offspring fitness in insects, we searched for a set of terms in five databases: Web of Science Biological Abstracts, Web of Science Core Collection, Zoological Record Plus, Agricultural & Environmental Science (ProQuest), and Google Scholar. Our search terms were 'maternal age,' 'parental age,' 'fitness,' 'offspring,' 'progeny,' 'longevity,' 'lifespan,' 'life history,' 'development time,' 'insect,' and 'arthropod,' including different spellings of 'aging' (e.g. ageing) and every possible combination of these terms. We performed the searches between December 23, 2017 and January 26, 2018 and found a total of 253 papers. We then removed papers that did not include empirical data collected by the author(s) and studies that did not focus on insects. We also excluded papers that did not test for maternal age effects on offspring or did not isolate maternal age effects from paternal age effects on offspring if they tested parental age effects. This review narrowed the pool of 253 papers to 75 papers. However, some studies reported results for more than one organism, so we assigned a case ID for each of the experiments within a paper that used a different species. For example, if a paper performed the same study on two different species, we categorized them as two cases within the same paper (case A and case B). Thus, we analyzed 82 cases from 75 papers.

For each case, we recorded an array of details about the authors' experimental design. We determined which measurement(s) of offspring fitness were collected (See Appendix A) and whether the consequences of advanced maternal age were negative or positive on each measure of offspring fitness or if there was no effect. For example, if a case examined the size of the adult offspring and found that the offspring of old mothers had higher mass than the offspring of young mothers, we assigned the effect as 'positive' on offspring fitness. If a case examined the number of eggs laid by old and young mothers and found that old mothers laid fewer eggs, we would label this as a 'negative' effect. If there was no difference in the offspring fitness between the maternal age treatments, we assigned this as 'no effect' Finally, for cases in which the authors recorded sex ratio, we recorded whether the sex ratio was male or female biased or equal (not different from 50:50) if there was no effect. Lastly, we also recorded the features of the experimental design, which we will refer to hereafter as case attributes: whether the study investigated multiple generations of maternal effects, used inbred or laboratory-adapted populations, whether the females were virgin at mating or mated multiply, whether they used the same females for both 'young' and 'old' ages (e.g. old at first mating or mated multiply at a young and an old age), whether the authors controlled for paternal age, and the amount of time that separated 'young' and 'old' age groups (e.g. days or weeks; Appendix B).

During the coding process, we established a standardized method of categorization. We kept a concise list of fitness measures, grouping measures together when appropriate (Appendix A). For example, if a case measured 'number of adult

offspring,' we categorized it as 'survival to adulthood' or if a case measured 'larval duration,' we categorized it as 'development time.' Many cases measured different aspects of body size (e.g. mass, pronotum width), which we classified generally as 'size' for whichever life stage the organism was measured. If the case assessed the fitness consequences across a spectrum of maternal ages, we only analyzed results that compared the youngest age and the oldest age of the mother. Finally, there were four cases that reported numbers of offspring but did not specify in which life stage they were when the authors recorded this measure; we therefore included these in a fitness measure called 'total offspring' to avoid making assumptions about the authors' experimental design.

Analysis

To isolate patterns in the results and experimental designs of the 82 cases we reviewed, we created a series of contingency plots. We asked whether the proportions of the fitness effects (negative, positive, or no effect; for sex ratio: male bias, or female bias, or no effect) were all equal. We asked the same question regarding the proportion of direction of fitness effects across all case attributes (e.g. what proportion of negative effects were also in studies that used inbred or lab-adapted populations), and across all orders (e.g. what proportion of negative effects were represented in each of the taxonomic orders). Furthermore, we asked if the distribution of case attributes differed across each taxonomic order (e.g. the proportion of cases that controlled for paternal age within each taxonomic order). To determine if there were trends within each of the taxonomic orders in the distribution of the fitness effects (negative, positive, or no effect),

regardless of fitness measure, we created another contingency plot. We also performed a series of separate chi square goodness-of-fit tests to test for each fitness measure that had at least 10 cases, to determine whether negative, positive, or no effect of advanced maternal age were disproportionately represented within those cases. Lastly, we performed the same goodness-of-fit test comparing the fitness effects (negative, positive, or no effect) within each taxonomic order.

Results

In the 82 cases reviewed here, 7 orders were represented: *Hymenoptera, Diptera, Coleoptera, Hemiptera, Lepidoptera, Orthoptera, and Blattodea* (from most to least studied; Figure 1.1).

Figure 1.1.

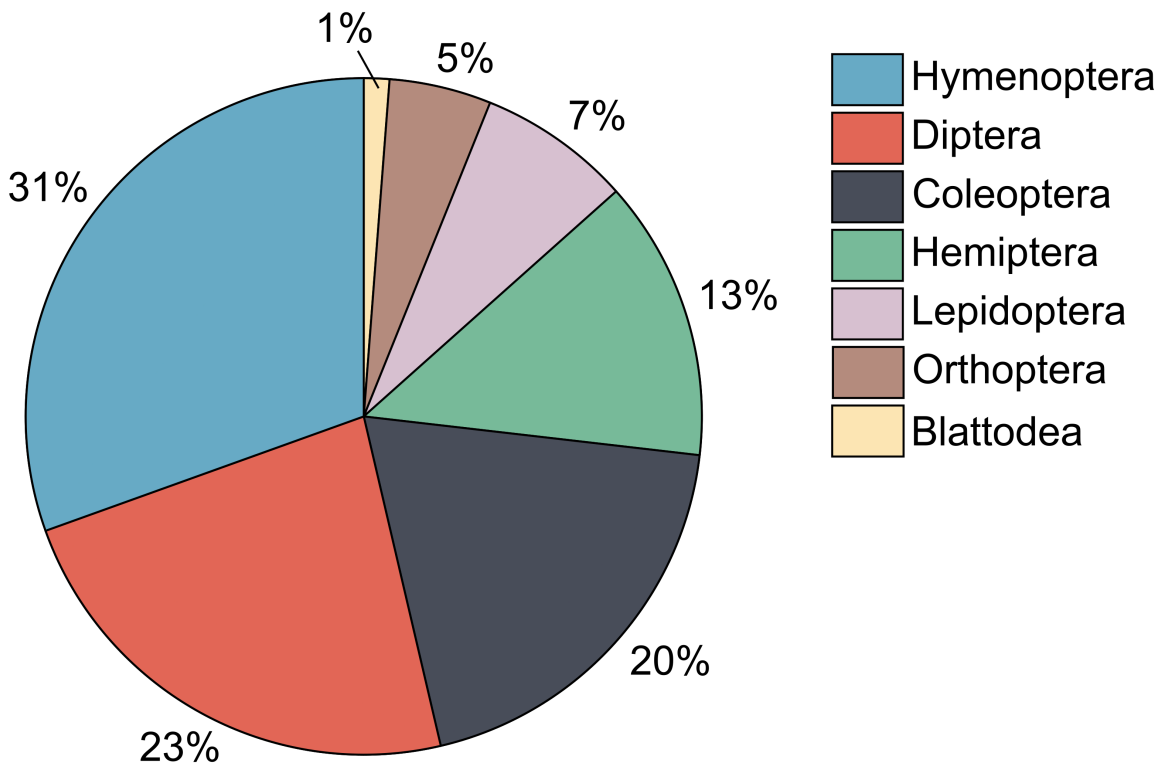

Figure 1.1. The relative proportion of each of the 7 insect orders included in the 82 cases reported in this review. The color for each order is the same as in Table 1.1.

Within each and across all orders, we found that authors report fitness measures on the egg and adult life stages of the offspring more often than larval or pupal life stages (Figure 1.2).

Figure 1.2.

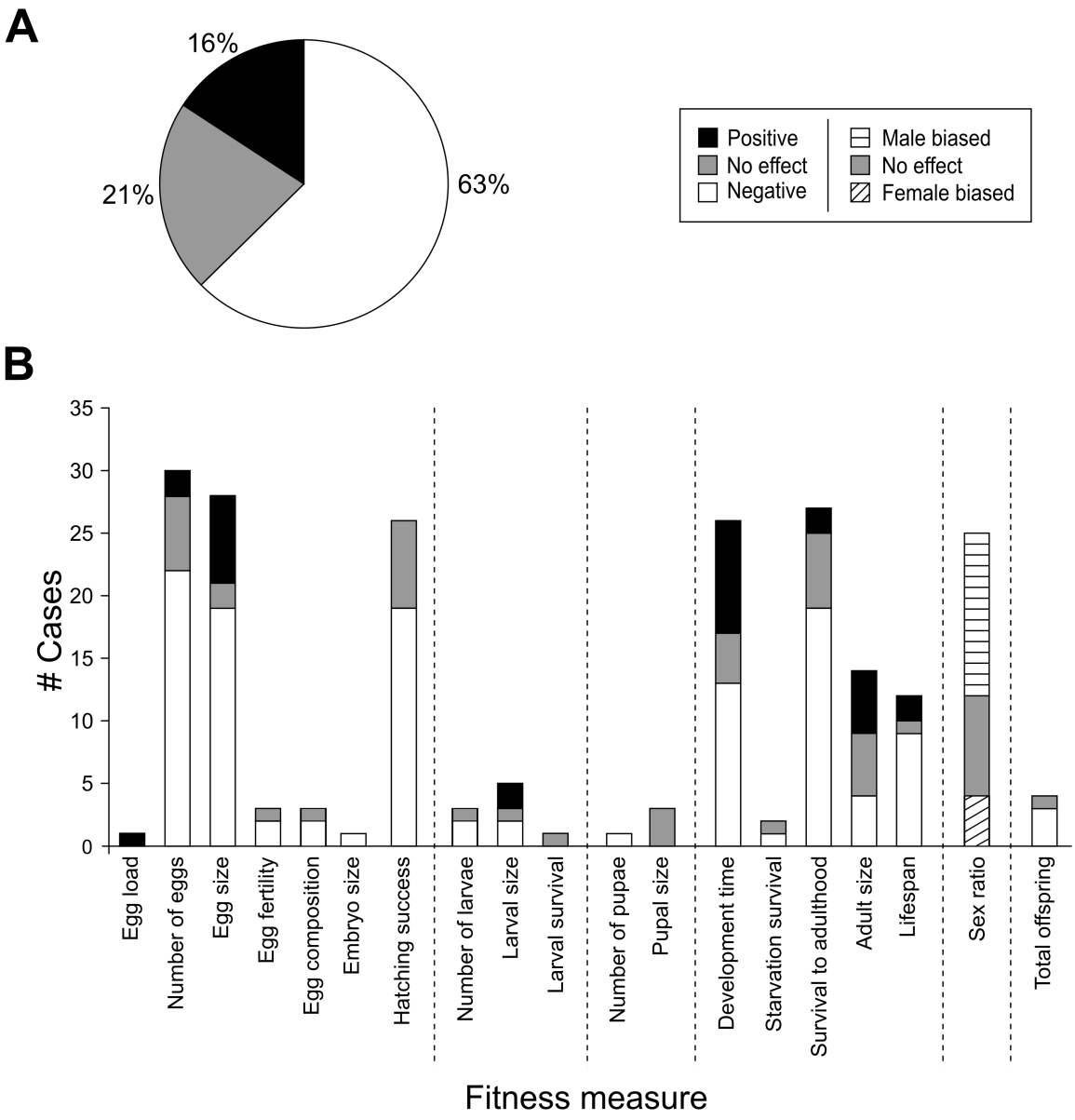

Figure 1.2. A. The relative proportion of overall fitness effects of advanced maternal age across all 82 cases reported in this review. **B.** The number of cases in this review that address each of the 19 fitness measurements used to assess the effects of advanced maternal age on offspring. The dashed vertical lines subdivide the measurement into categories of various offspring life stages during which the measurements were recorded by the authors. Total offspring is considered a separate category here because the term was used in four cases that did not specify from which life stage the fitness measures were recorded. The colors of the bars indicate the number of cases that found negative,

positive, or no effect of advanced maternal age on offspring. For example, if an old mother produced smaller offspring than a young mother, this would be a negative effect on fitness (empty bars). If there was no difference in the size of the offspring between young and old mothers, this would be a considered 'no effect' on fitness (grey bars). If an old mother produced larger offspring than a young mother, this would be a positive effect on fitness (black bars). The sex ratio of the offspring from old mothers is also subdivided to avoid associating male-bias (horizontal dashed bars) or female-bias (diagonal dashed bars) in adult offspring as either positive or negative. Sex ratios that did not differ from 50:50 are also represented as grey bars, indicating no effect of advanced maternal age.

Of the 82 cases we reviewed, we found that 11% performed multigenerational studies. Authors used inbred/lab-adapted populations of organisms in 55% of the cases whereas 34% did not and 11% of cases did not report this information. In 35% of cases, mothers were mated as virgins, 16% were non-virgin (mated multiply), and in 49% of the cases this information was not provided. In 54% of cases, researchers used the same female for old and young mating, 36% used different females for the two age groups, and in 10% of the cases did not provide this information. Finally, 8% of cases controlled for paternal age, 37% did not, and in 55% of the cases this information was not provided.

When we consider all of the fitness measures for which we could record negative, positive, and no effect results for (i.e. all variables except sex ratio), we found that 63% of all results reported negative effects of advanced maternal age while 21% found no effect and only 16% reported a positive effect (Figure 1.2A). Within each taxonomic order, we found that, 4 taxonomic orders had been studied in 10 or more cases, allowing us to perform chi square goodness-of-fit tests. We found that 82% of *Hymenoptera* cases, 64% of *Coleoptera*, 58% of *Diptera*, and 55% of *Hemiptera* cases resulted in negative effects of advanced maternal age, regardless of fitness measure. The proportion of

negative effects also differed across taxonomic orders with 28% of all negative fitness effects found in cases that examined *Coleoptera*, 23% found in *Hymenoptera*, 18% found in *Diptera*, and 15% found in *Hemiptera*. These results suggest that negative fitness effects on offspring may be confounded with how often each order was studied to answer maternal aging questions.

On average, we found that cases assessed 3 measures of fitness; the most frequently reported measurement was the number of eggs laid by mothers of young and old ages, followed by egg size (Table 1.1). However, some measures across orders were severely underrepresented (e.g. egg load was measured by just one case compared to the number of eggs laid, which was measured by 30 cases). Finally, we found that the life stages of the offspring at which they were assessed for the fitness measures were not equally represented; more cases measured fitness in the egg-related and adult-related life stages than pupal or larval life stages.

Of the 19 fitness measures reported in the dataset, we found that 8 were measured at least 10 times, allowing us to assess whether the proportions of negative, positive, or no effect fitness results equally represented for these fitness measures (Figure 1.2B). For measures related to eggs, we found that authors most often reported egg size (n=28 cases) number of eggs laid (n=30), and hatching success (n=26). For measures related to adult offspring, authors most often reported adult size (n=14), development time (n=26), lifespan (n=12), sex ratio (n=24), and survival to adulthood (n=27).

Egg measures

In the egg-related life stage, authors collected 7 types of measurements (Table 1.1, Figure 1.2).

Table 1.1. A heat map showing the fitness measures assessed in each of the 7 insect orders represented in this review. In each box, the percentage reported indicates what proportion of the overall fitness measures studied in all orders is represented by studies reporting a particular fitness measure in that particular order. For example, 0.5% of all cases measured the number of eggs laid in *Blattodea* and 4% of all cases measured the number of eggs laid in *Coleoptera*. Each order is represented in a different color, and the degree of shading indicates the frequency of the fitness measure, with darker colors representing higher frequency, both within the order and overall for all fitness measures and orders. For example, in *Hymenoptera*, more cases measured sex ratio than the number of eggs, whereas in *Blattodea*, all fitness measures were equally represented. Similarly, more cases studied development time in *Hemiptera* than studied the number of eggs in *Orthoptera*. The fitness measures are listed from top to bottom in order of stage of development (egg, larva, pupa, adult), with stages of development divided by dashed lines. The right-most column shows the proportion of all cases (out of the 82 studied across all orders) that addressed each fitness measure. Please see Appendix A for descriptions of fitness measures.

Table 1.1

	Blattodea	*Coleoptera*	*Diptera*	*Hemiptera*	*Hymenoptera*	*Lepidoptera*	*Orthoptera*	Total
Egg load	—	—	—	—	0.5%	—	—	0.5%
Number of eggs	0.5%	4%	2.5%	3%	3%	1%	0.5%	14.5%
Egg size	0.5%	4%	2.5%	3%	1%	2%	1%	14.0%
Egg fertility	—	—	—	—	0.5%	1%	—	1.5%
Egg composition	—	—	0.5%	—	0.5%	0.5%	—	1.5%
Embryo size	—	—	—	—	0.5%	—	—	0.5%
Hatching success	0.5%	5%	2.5%	1%	1%	1%	—	11.0%
Number of larvae	—	—	—	—	1%	—	—	1.0%
Larval survival	—	—	—	—	—	0.5%	—	0.5%
Larval size	—	—	0.5%	0.5%	0.5%	0.5%	0.5%	2.5%
Number of pupae	—	—	0.5%	—	—	—	—	0.5%
Pupal size	—	—	1%	—	—	0.5%	—	1.5%
Development time	0.5%	5%	2%	3%	0.5%	1%	1%	13.0%
Starvation survival	—	—	—	—	0.5%	0.5%	—	1.0%
Survival to adulthood	—	3.5%	2%	2%	4%	1%	—	12.5%
Adult size	—	2.5%	2.5%	2%	—	—	—	7.0%
Total offspring	—	0.5%	—	—	1%	—	—	1.5%
Lifespan	—	1%	2.5%	1%	—	0.5%	—	5.0%
Sex ratio	—	1%	1%	1%	7%	—	0.5%	10.5%

Overall, 71% of the results showed a negative effect of advanced maternal age, 11% were positive, and 18% found no effect (Figure 1.2B). We found that the fitness effects of advanced maternal age (negative, positive, or no effect) differed from the null expectation of equal representation for egg size (χ^2=16.36, df=2, p<0.001), number of eggs laid (χ^2=22.4, df=2, p<0.001), and hatching success (χ^2=21.31, df=2, p<0.001) with negative effects more common than positive or no effect. Notably, all cases measuring hatching success found either negative or no effect of advanced maternal age; no cases reported a positive effect of advanced maternal age on hatching success.

Larval and pupal measures

In the larval life stage, authors collected only 3 types of measurements (Figure 1.2B). Overall, 45% of the results showed a negative effect of advanced maternal age, 22% were positive, and 33% found no effect. In the pupal life stage, authors collected only 2 types of measurements, and 25% of results found negative effects of advanced maternal age and 75% found no effect (Figure 1.2B).

Adult measures

Authors collected seven types of fitness measures in the adult life stage (Figure 1.2B). For the fitness measures on adult offspring (excepting sex ratio), we found that, overall, 58% of results were negative, 21% were positive, and 21% found no effect. However, while overall fitness effects were mainly negative, we highlight that this is fitness measure-dependent. Within the five adult measures that had a sufficient number of cases that allowed us to compare the distribution of fitness effects (negative, positive, or no effect) using chi square goodness-of-fit tests, only two showed a non-equal

distribution (i.e. a non-equal proportion of fitness effect results): offspring lifespan (χ^2=9.5, df=2, p<0.01 and survival of offspring to adulthood (χ^2=17.56, df=2, p<0.001). However, we did not find a difference in the direction of fitness effects of the size of adult offspring from old mothers (χ^2=0.14, df=2, p>0.9) or the development time (χ^2=4.69, df=2, p>0.1). In cases that assessed sex ratio as a consequence of advanced maternal age (n=24), 17% of cases were female-biased, 54% of cases were male-biased, and 29% showed no difference in sex ratio of the offspring from old and young mothers (Figure 1.2B). We did not find a difference in the direction of fitness effects of the sex ratio of the adult offspring from old mothers (χ^2=5.25, df=2, p>0.1).

Case attributes

We performed multiple contingency plots to determine the proportion of fitness effects (negative, positive, or no effect) across each fitness measure. Most notably, 100% of cases that resulted in female-biased sex ratios as a result of advanced maternal age were from studies of inbred or lab-adapted populations. Most cases did not report whether the authors had controlled for paternal age. However, of the cases that reported female-biased sex ratios, 100% of them did control for paternal age while only 25% of all male-biased results were from studies that controlled for paternal age. The results of the contingency plots on the other case attributes are more variable and reveal no clear pattern on how fitness effects may be affected by case attributes. Of all negative fitness effects, 51% were from inbred or lab-adapted populations while 57% of no-effect results and 69% of all positive fitness effects were also from inbred or lab-adapted populations. Furthermore, 38% of all male-biased sex ratios were from populations that were inbred

and/or lab-adapted. Similarly, we found highly variable results when authors used virgin females: 50% of all female-biased sex ratios resulted from old females mated as virgins, 25% of all male-biased sex ratios came from old virgin mothers, and 42% of all negative results, 21% of all fitness measures where no effect was found, and 31% of all positive effects on offspring fitness were in studies that mated old females as virgins. None of the female-biased sex ratio results were from experimental designs where the authors used the same female for both young and old but 38% of the male-biased results did use the same female for both maternal age treatments. Further, 56% of all negative results, 43% of all results where no effect was found, and 77% of all positive effects were in studies where the same females were used as both young and old.

Discussion

Aging affects all organisms and can have an impact on future generations across taxa. While paternal age is important, maternal age effects have received more attention, most likely due to the perceived difference in maternal investment. Parsons (1964) laudably reviewed the maternal age literature and concluded that a more direct and thorough approach to the examination of maternal age effects was needed to find patterns across and within taxa. In the subsequent 55 years, impacts of maternal aging on offspring fitness have received much more attention, especially for insects. Here, we present findings and conclusions from our comprehensive review covering 55 years of research on the effects of maternal aging on insect offspring.

The results of the 82 cases (from 75 papers) included in our review highlight the incredible variability in fitness effects of advanced maternal age on insect offspring (Figure 1.2B). When viewed across all fitness measures investigated and all taxa, the overall trend (63%) in maternal aging research in insects is that having an older mother negatively affects offspring (Figure 1.2A). However, we show that the direction of fitness effects on offspring depends to some extent on which taxa are investigated, the fitness measures studied, and important attributes of experimental design (e.g. using the same female for both 'young' and 'old'). As such, we urge researchers to consider this overall conclusion with caution for reasons outlined below, and we emphasize the need for expansion and diversification in all aspects of authors' approach to future aging research. We found that, on average, researchers include only three measures of fitness in each case within this review and the types of fitness measures and study system(s) used seem to influence whether authors find that advanced maternal age affects offspring fitness positively, negatively, or not at all. For example, in this review we found that 100% of cases that investigated the effects of advanced maternal age on hatching success (i.e. a quantitative measure) resulted in either negative effects or no effect; none of these studies reported a positive effect. Due to the lack of variety in types of measurements (i.e. both quantity and quality), this trend may or may not be indicative of true overall effects of maternal age on offspring fitness if researchers do not employ a broad-spectrum approach in assessing offspring fitness. Although we found equal representation of measures of quality and quantity across all cases in this review, with only three fitness measures per case (on average), it is clear that some cases were more balanced in their approach than

others in this regard. We suggest that researchers collect data on a greater variety of fitness measures to gain a comprehensive assessment of the impacts of advanced maternal age on offspring fitness (see Hallagan *et al. In Prep*; Wilson *et al. In Prep*).

The life stages at which offspring are measured by the authors of the cases presented here are not equally represented; egg-related and adult-related measurements were disproportionately recorded over larval- and pupal-related measures. We acknowledge that not every insect species has a larval or pupal stage, but juvenile stages were severely underrepresented from all cases within this review and we therefore encourage authors to record an array of measurements across all life stages of the offspring. Using the same example as above, 100% of the hatching success results were either negative or showed no effect of advanced maternal age but due to the lack of variety in types of measurements (i.e. quality and quantity), this trend may or may not be indicative of true overall effects of maternal age on offspring fitness when viewed in the context of measurements made across the organisms' lifespan; viewed in isolation, this trend could be misleading.

Finally, the taxonomic orders represented in this review were not equally represented, nor were the fitness effects of advanced maternal age within each order (Figure 1.1; Figure 1.2B). For example, we found that 31% of our 82 cases used *Hymenoptera* while only 1% used *Blattodea* as the study system used for maternal aging research. The imbalance in representation of taxa may influence the overall negative results found in this review, due to trends in taxonomic orders or simply due to a difference in sample size. For instance, the cases that studied *Hemiptera* used inbred or

lab-adapted populations 73% of the time while only 25% of *Orthoptera* cases were inbred/lab-adapted. However, the sample size of cases that studied *Hemiptera* was 11 compared to *Orthoptera* which were only used in 4 cases out of 82. Therefore, any offspring fitness effects as a result of advanced maternal age may be a reflection of one or many various factors: there may be certain fitness measures that are typically used within *Hemiptera*, depending on the motivation behind the research and which fitness measures are deemed more important than others, that give rise to similar consistent results, the results found in cases (like in this comparison) are not related to the orders but instead to the negative effects of inbreeding, the difference in sample size between the two taxonomic orders is too great for the patterns to be comparable, or perhaps the life stages at which these orders typically measure offspring fitness. Notably, 82% of cases that used *Hymenoptera* found negative results, regardless of which fitness measure(s) the authors recorded. When we compare this trend to, for example, *Hemiptera* with 55% negative effects, it suggests that some taxa may be more likely to have negative results than others (e.g. studies that typically use inbred populations, like in *Drosophila*). However, we acknowledge that the trends we find in fitness effects from advanced maternal age in cases that use a limited number and type of measurement, the life stages of the study organism the authors use, or the order to which the study organism belongs may not result from just one of these factors alone but instead, they may be a culmination of some or all.

Research Challenges

The approaches and experimental designs used in aging research projects can be challenging, with many variables to consider in both the design and execution of the research projects and how best to truly measure fitness. These challenges may even have unforeseen effects on experimental results. Below, we consider trends in the results reviewed here that highlight challenging areas in the field of aging research and where future authors have opportunities to consider their approach and/or avoid common pitfalls.

Defining age

Natural lifespans, of course, differ among insect species and there is no comprehensive definition for what constitutes 'young' or 'old' age. Instead, the two terms are relative and species-specific. For instance, what is considered old for *Drosophila* (e.g. 40 days post-eclosion into adulthood) may be young for some *Hymenoptera* species that spend years as adults (Bauer *et al.* 2004; Al-Lawati & Bienefeld 2009; Qazi *et al.* 2017). We must therefore design aging research that is tailored to the study organism's natural life history. We emphasize natural life histories here because laboratory-adapted populations may experience altered lifespans compared to their wild counterparts due to unintentional artificial selection under laboratory rearing conditions (Spencer & Promislow 2005; Linnen *et al.* 2001). Investigators should consider whether their study organism would realistically mate at their defined 'old' age in wild populations that are subject to selection pressures absent in the lab. For example, some of the selected ages of mating in *Drosophila* experiments lie outside of the natural lifespans of *Drosophila* in the

field (Bauer *et al.* 2004). We suggest that authors clearly justify the ages chosen and consider whether these would be realistic in a wild population of the species being studied. Furthermore, we implore all researchers to report the ages used for their 'young' and 'old' age treatments; we were surprised to find that some did not, and this oversight makes it difficult to assess results and to repeat or expand interesting experiments.

We also found that in the few cases where authors investigated maternal age effects by using a spectrum of ages (e.g. mothers aged at 3 (youngest), 10, 15, and 21 (oldest) days post-eclosion into adulthood) instead of binary 'young' and 'old' treatments, the results told a more detailed story regarding maternal investment. For instance, the effect of advanced maternal age on offspring fitness was sometimes initially either positive or negative but then shifted in the opposite direction. These results suggest that there may be periods of high, low, and potentially peaks in female fecundity that may affect offspring fitness (see Singh & Singh 2005) that may not be captured by only using two extremes in maternal age. We therefore recommend that although it is more difficult, researchers should employ an experimental design that includes a spectrum of maternal ages (as opposed to two binary extremes) as the best approach to identify patterns of female reproductive investment and at what age female fecundity and offspring fitness are highest.

Laboratory breeding and egg laying order

A pitfall of investigating lab-adapted populations is the potential confounding effect of inbreeding on offspring fitness in such studies. Of the 82 cases reviewed here, 55% studied organisms from inbred and/or lab-adapted populations (another 12% of

cases did not report sufficient information to make that classification and could potentially have been inbred as well). Inbreeding depression can have deleterious effects on offspring of closely-related parents (Wright 1922; Su *et al.* 1996; Saccheri *et al.* 1996; Van Eldik *et al.* 2006). Much of the groundbreaking aging research in *Drosophila* (63%) used heavily inbred, isofemale lines (genetic lines originating from a single wild-caught female) to conduct their maternal age experiments and found negative effects on offspring fitness 54% of the time. A criticism of this approach is that the negative fitness consequences experienced by offspring in these studies could, at least in part, be attributed to interactions between advanced maternal age and inbreeding depression. Laboratory settings often differ from natural settings, highlighting the importance of replicating natural breeding habits as closely as possible for the study system being used. We emphasize the use of large, genetically diverse populations and colonies to best replicate natural populations in the field and to avoid the complications associated with inbreeding depression.

Egg-laying organisms pose a unique challenge in understanding how maternal age affects offspring. We found that 55% of investigators use the age at which females oviposited, instead of the age at which the females were mated, as the basis for whether a mother is considered to be 'young' or 'old.' This practice could confound effects of advanced maternal age with egg-laying order (i.e. age at oviposition). Begon & Parker (1986) introduced a model, designed to be applied specifically to insects, which predicted that the eggs laid first (i.e. at a young maternal age) should be larger than eggs laid later (i.e. at an old maternal age) due to differences in maternal investment. This decrease in

egg size with egg laying order within clutches and across different maternal ages is a common occurrence in birds (Hong *et al.* 2007; Badyaev *et al.* 2002; Saino *et al.* 2002) but is not as commonly found in empirical work with insects, which tend to have more mixed results with some authors finding larger eggs laid earlier and some finding larger eggs laid later in the oviposition period (Wiklund & Persson 1983; Fox & Savalli 1998; Cherrill 2002). With such variable results regarding egg-laying order and its effect on offspring fitness in the insect-related literature, we propose that, instead of using age at oviposition, senescence research should use the organisms' age at mating, especially if there is a large gap of time between mating and oviposition, to fully understand the interaction between maternal investment and maternal aging, and how offspring may be affected.

Virginity versus multiple mating

Whether a female is virgin at the time of mating or has previously mated at a younger age could affect the differences we see in the offspring from mothers of different ages. If females are allowed to mate multiply (i.e. the same females are used for 'young' and 'old' ages), their reproductive investment may differ between matings and egg clutches as a consequence of multiple mating, rather than age per se. Furthermore, if females mate multiply, it becomes more difficult to separate maternal age effects from any potential paternal effects, particularly if the female mates with different males. The resources that females receive from males (e.g. nuptial gifts, ejaculate proteins, and hormones) can not only affect female fitness, but can also affect offspring fitness both directly and indirectly (García-Palomares *et al.* 2009; Curley *et al.* 2011). Therefore, the

effects of multiple female mating opportunities may be confounded with maternal age consequences (positive or negative). While virgin females allow for a straightforward analysis of maternal reproductive investment, there can also be costs associated with old virginity (e.g. shorter lifespans; Markow 2011). Of the 82 cases reviewed here, 49% did not report whether the females used in their maternal age treatments were virgin, making it difficult to isolate any patterns between maternal virginity and maternal age effects on offspring. We therefore recommend that authors make this information available for the reader in their publications. Given the complexity of virgin and multiple matings, we suggest future research opportunities investigate the interactions between maternal virginity at young and old ages and multiply mated females of the same ages.

Female condition

Female condition also contributes to offspring fitness. Frequently in maternal age research, authors use 'young' and 'old' mothers that are also introduced to a form of stress before and/or during mating and egg production; exposure to toxic metals or heat, forced mobility, and resource depletion stress are most commonly used in the literature (Augustyniak *et al.* 2009; Faurby *et al.* 2005; Ducatez *et al.* 2012; Fox 1993a; Ito 1997; Jann & Ward 1999). However, the introduction of these stressors is yet another case of confounding variables; old maternal age could be considered a stressor in and of itself. Notably, parental effects are not limited to genetic inheritance. Behavior and responsivity to stress are common non-genomic heritable parental effects (Champagne & Meaney 2001; Chahwan *et al.* 2011). For example, Lehto & Tinghitella (*In Revision*) show that, in three spine sticklebacks, the offspring from predator-exposed parents have altered mate

choice behavior that reflects the behavior that we would see in conspecifics that have been directly exposed to a predator. Considering how these parental effects that are not related to age influence offspring, if female condition is not accounted for, offspring fitness effects may not solely be the result of maternal age but rather non-genomic changes resulting from maternal experience (e.g. stress). It is imperative to isolate the effects of maternal age from other possible parental effects.

Multigenerational experimental design

We discovered that several authors attempted a multigenerational approach to understand the effects of maternal age and also grandmaternal age on offspring. These attempts are admirable and ambitious given their widescale approach to find potentially additive or diminished effects of advanced maternal age across multiple generations. However, we encourage authors to take certain measures to isolate maternal and grandparental effects as well as to take into consideration the other attributes listed in this review. To discover whether effects of advanced maternal age are cumulative through multiple generations or if offspring may recover from any negative effects, we propose that researchers should use a fully factorial design, similar to Andersen *et al.* (2005) in which maternal age effects are tested separately from grandmaternal effects (e.g. young grandmother and young mother, young grandmother and old mother, old grandmother and old mother, and old grandmother and young mother as separate treatments). However, we suggest that researchers compare the magnitude of effects between the generations. For example, isolating the effects that the grandmaternal generation have on the maternal generation while also isolating the effects the maternal generation have on

the offspring generation allows investigators to determine if maternal age effects are additive (or diminished) across the generations. Another exciting area of proposed research in the maternal aging field could investigate ultimate and proximate perspectives of how maternal age affects offspring fitness. Particularly, long-term effects and the mechanisms behind the differences we see in the quantity and quality of the offspring from mothers of various ages.

Importance of paternal effects

Maternal and paternal effects may be equally important for offspring fitness, but maternal age has received more attention than paternal age in the literature, which is why this review focuses on maternal age only. Yet, evidence exists to suggest that paternal age and experience affect offspring fitness (Curley *et al.* 2011; Crowl & JE Alexander 1989). Males have historically been overlooked citing their lack of reproductive investment (sperm is less costly to produce than female gametes; Trivers 1972). However prezygotic investment (e.g. nuptial gifts in *Hemiptera*) and postzygotic investment (e.g. guarding in *Odonates*) are indirect paternal effects mediated through maternal effects and direct paternal effects, both being considerable paternal contributions that can affect the offspring. Advanced paternal age, specifically, can have adverse effects on offspring fitness (e.g. reduced reproductive success and longevity, increased risk of disease and disorders in offspring; (García-Palomares *et al.* 2009; Curley *et al.* 2011). We therefore suggest that reports on parental age should control for the age of one parent in order to isolate which effects are caused by which parent and at which age. If paternal age is not controlled for, conclusions made about maternal age effects on offspring may not carry

the same validity and importance as if researchers do control for paternal age. Given the imbalance of maternal to paternal effects in the literature, we suggest that future research could investigate solely paternal age effects or a fully factorial design where insect parents were mated as 'young' parents, 'young' father + 'old' mother, 'old' father + 'young' mother, and 'old' parents and compare offspring fitness from each treatment. This experimental design would not only allow investigators to isolate parental age effects but also improve the shortage of paternal effects in the literature.

Conclusions and suggestions for future directions

Our review has summarized the last 55 years of research investigating maternal age effects on insect offspring. Our overall conclusion is that maternal age has a negative effect on offspring fitness. However, we urge caution when interpreting this overall conclusion considering the many challenges that accompany aging research. While the cases examined in this review have offered valuable insight, there are certainly opportunities to improve experimental design. We suggest that future researchers consider the importance of using wild-caught populations to better replicate natural conditions, collecting a suite of fitness measures that assess both the quantity and quality of the offspring throughout multiple life stages, female condition, and controlling for paternal age. With these recommendations in mind, we should find that future research will be more indicative of trends solely due to advanced maternal age and not due to a confounding of variables, seen or unforeseen.

Chapter 2: Advanced maternal age leads to greater immune function and fitness in cricket offspring

Introduction

Aging theory predicts that reproductive investment deteriorates with age, suggesting that offspring from old mothers could be less fit than offspring from young mothers (Harman 1956; Kirkwood & Rose 1991; Gavrilov *et al.* 1997; Heidinger 2016). Mitochondrial DNA accumulates oxidative damage with age and telomeres, which protect chromosomes from oxidative stress and are directly linked with longevity, shorten over time. These two mechanisms affect an individual's fitness and, due to their heritability, also their offspring's fitness (Harman 1956; Gavrilov *et al.* 1997; Heidinger *et al.* 2016). For example, in humans, maternal age is positively correlated with risk of childhood cancers, autism, and mortality (Yip *et al.* 2006; Durkin *et al.* 2008; Johnson *et al.* 2009). Further, in some non-human organisms, mothers of advanced maternal age have fewer eggs and then their offspring have low hatching success, are smaller, and have higher rates of mortality and longer development times than offspring of young mothers (e.g. flies, mice, guinea pigs, beetles, birds; Parsons 1962; Qazi *et al.* 2017; Fox 1993b; Kern *et al.* 2001; Hercus and Hoffmann 2000; Yanagi & Miyatake 2002; Heidinger *et al.* 2016).

In contrast, the terminal investment hypothesis, which is a component of life history theory, predicts that allocation to current fitness should increase as females age

because the importance of trade-offs between current and future reproduction declines with age (Fisher 1930; Williams 1966; Partridge & Harvey 1988; Stearns 1989). Mothers of advanced age may thus reduce investment in their own survival, but invest heavily in offspring, producing more or higher quality offspring close to the time of death. This increase in investment suggests that offspring from old mothers may be equally or more fit than offspring from young mothers (Trivers 1974). In agreement with these predictions, studies that have measured fitness components including size of offspring, survival rates, offspring reproductive success, and litter size find that offspring of older mothers are more or equally as fit as offspring of young mothers (Descamps *et al.* 2008; Creighton *et al.* 2009; Hansen *et al.* 2014; Clark *et al.* 2017).

Conflicting reports of either positive or negative effects of maternal age on offspring fitness in the literature may be a consequence of the fitness measures that researchers have traditionally used. Individual studies tend to emphasize measures of either the quantity of offspring (number of eggs, hatchability, number of offspring to survive to adulthood; Fox 1993b; Yanagi and Miyatake 2002) or to a lesser extent offspring quality (egg and adult body size, starvation resistance, development time; Poykko and Manttari 2012; Ito 1997). Measuring *only* quantity or *only* quality of the offspring limits our understanding of maternal effects stemming from age and may yield conflicting reports in the literature. There may be trade-offs that would not be accounted for by measuring only one type of fitness measure (e.g. fewer but higher quality offspring as a result of maternal age).

One aspect of offspring quality that is often overlooked in the aging literature is immune function. A more inclusive view of comprehensive fitness might reveal that the fitness differences observed in offspring from old and young mothers are a result of trade-offs between the quantity and quality of offspring, including immune response (see Nystrand & Dowling 2014). Few researchers have studied the effects of maternal age in a comprehensive way that includes both traditional measures of offspring fitness and offspring immunocompetency. Aging has widespread negative effects on immune function in many different taxa; in general, older organisms do not launch as effective immune responses to an immune challenge as younger conspecifics (Linton and Dorshkind 2004; Gruver *et al.* 2007; Muller *et al.* 2014). However, offspring from old mothers have been found to be less susceptible to an introduced pathogen than offspring of young mothers in Atlantic cod (Hansen *et al.* 2014) and *Daphnia* (Clark *et al.* (2017). Therefore, maternal age can also positively affect the immunocompetency of offspring. In invertebrates, like vertebrates, offspring inherit their immunity from their mothers, who transfer immune-related proteins during egg development, establishing baseline immune function for the offspring (referred to as transgenerational priming; Sun *et al.* 1990; Huang & Song 1999; Moret & Schmid-Hempel 2001; Grindstaff *et al.* 2003; Sadd *et al.* 2005). Maternal immune function can be reflected in the immune function of the offspring throughout life (Sadd 2005; Reid 2006). Insects lack acquired immunity (the development of immune memory in response to pathogen exposure), so the immune function of an individual insect is dependent on parental immunity (Zuk and Stoehr 2002; Beckage 2008).

Much aging research is done in vertebrate study systems, predominantly humans, and the few studies that have investigated impacts of parental age on invertebrates tend to do so in heavily inbred, lab-adapted populations (Parsons 1962; Beardmore & Al-Baldawi 1975; Kern *et al.* 2001; Priest *et al.* 2002; Qazi 2017; Hallagan *et al. In Prep*). Using lab-adapted populations can be advantageous in some areas of research (e.g. artificial selection, when there is a need to control the genetic background), but the use of lab-adapted populations may also limit our ability to predict the nongenomic effects of advanced maternal age on fitness in natural populations. To gain a more complete understanding of how maternal age affects insect offspring fitness, we measured both traditional fitness proxies (e.g. body size, eggs laid, hatching success) and immunocompetence of offspring whose mothers were mated at either an old or young age. We tested the effects of maternal age on offspring fitness in a non-lab-adapted species of field cricket, *Teleogryllus oceanicus*. Trade-offs permeate our thinking about life history traits, and we may be more likely to find trade-offs between measured fitness traits when a comprehensive set of quantity and quality characteristics are measured. Generally, however, support for aging theory would come from finding that old mothers have fewer, lower quality, and/or immunocompromised offspring when compared to young mothers. Alternatively, if life history theory is supported, we expect to instead find that older mothers have either more or higher quality offspring with enhanced immune function.

Methods

Study system and experimental design

Teleogryllus oceanicus, the Pacific field cricket, is an ideal research organism to test how parental age affects offspring fitness as they are relatively long-lived insects (as compared to model organisms like *Drosophila*), mate readily in the lab and lay eggs continuously throughout their adult lives. The natural adult lifespan of *T. oceanicus* ranges from 1-2 months, allowing a complete a multigenerational experiment (maternal and offspring generations) within one year. *Teleogryllus oceanicus* is also subject to parasitism in the wild (Zuk *et al.* 2006; Tinghitella 2008), making immune response an important component of survival in parasitized populations. Methods for collecting both fitness and immune response measures are well established in this and closely related species (Adamo *et al.* 2001; Fedorka *et al.* 2004).

To test the effects of maternal age on offspring fitness and immune function, we established 10 founding maternal lines whose offspring were the F1 generation. With the F1 generation, we used a split brood design (explained in detail below) in which we assigned females to be mated at either a young or an old age (Figure 2.1).

Figure 2.1.

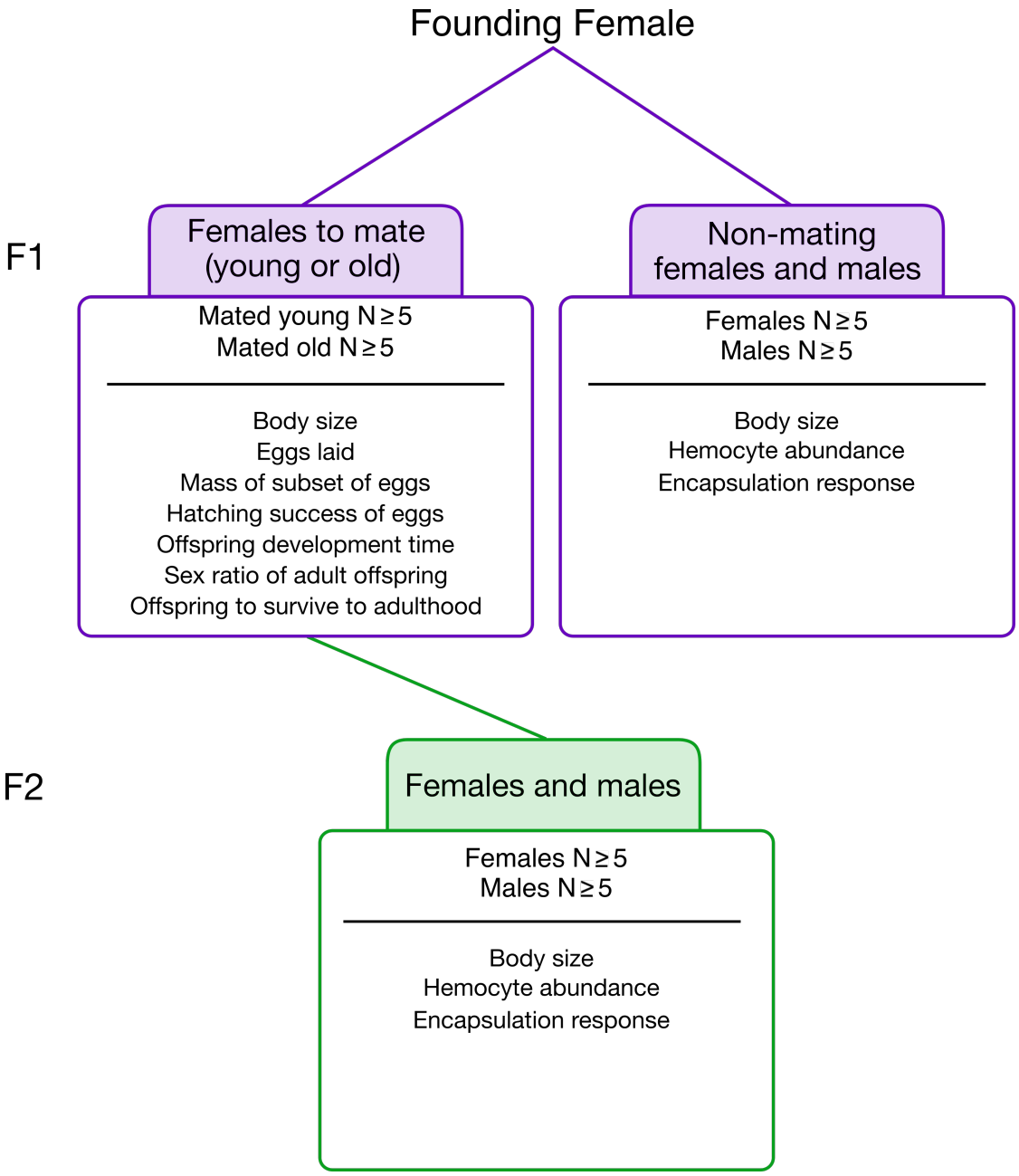

Figure 2.1. Experimental design to measure reproductive fitness and immunity for individuals in the F1 and F2 generations. We established 10 founding lines in 2016 to produce the F1 generation. We established two maternal age treatments: females mated young or mated old in the F1 generation. We aimed to mate at least five females at a young age and five females at an old age from each founding line, but often our sample size was much larger than that (Appendix D). Here, we depict the fitness measures we

recorded from offspring and grandoffspring of those founders. We measured body size for all crickets and six reproductive fitness measurements for all mated F1 generation female crickets (young and old). We could not perform immune testing on the mated crickets, so we used their full siblings instead. We measured immunocompetence for the F1 generation male and non-mating female crickets using hemocyte counts and encapsulation responses. We performed the same immunocompetency tests on the male and female F2 generation offspring of the F1 mated females.

After F1 females mated and laid eggs, we reared the offspring of the mated F1 females as the F2 generation. We collected body size measurements (detailed below) for all crickets. For the F1 females that mated, we recorded 7 reproductive fitness measures (detailed below). Finally, we measured the immune responses of all offspring crickets in the F2 generation (Figure 2.1).

Rearing and mating

We used a laboratory colony of *T. oceanicus* that was established in 2014 with the offspring of field caught females collected at the Gump Field Station on the island of Mo'orea in French Polynesia. We reared early instar crickets in 1.9L plastic containers (approximately 50 hatchlings per container) and later instar crickets in 0.5L plastic containers (1-15 crickets each, depending on size and instar). All the plastic containers that we used to house crickets were held within Percival incubators (model I36VLC8) set to 27ºC and on a 12:12 light:dark schedule. We provided Fluker's High Calcium Cricket Chow (for hatchlings and juveniles), Kaytee Rabbit Chow (for adults), and water ad libitum, as well as egg carton for shelter (similar to Tinghitella 2008; Simmons *et al.* 2010; Bailey *et al.* 2017).

We reared juvenile females from the laboratory colony in isolation until 7 days after eclosing to adulthood and established 10 founding maternal lines by mating them with randomly assigned unrelated young (7-10 days after eclosing to adulthood) colony males in a low light, temperature-controlled arena or incubator (25-27ºC). To increase the probability of mating, we allowed the 10 founding females to mate inside of a 0.5L container with the same randomly assigned male up to four hours per day, once per day for one week. We isolated the males and females between each four-hour mating period to avoid aggressive behavior and to increase male reproductive effort during mating opportunities. Following the last day of mating opportunities, we isolated females in individual 0.5L containers and collected eggs from each female continuously for five weeks. We then housed the hatchlings from each founding female in isolation from other maternal lines (these hatchlings were the F1 generation). We reared F1 hatchlings in 1.9L plastic containers and, when sex could be determined, we further separated offspring by sex within each maternal line to prevent mating. When offspring eclosed from the juvenile to the adult life stage, we individually housed them in 0.5L containers until they were scheduled to mate to produce the F2 generation.

To propagate the F2 generation, we used at least ten daughters of the F1 generation from each maternal line in a split-brood design; we aimed to mate at least five of the ten daughters at a young age and five daughters at an old age (actual sample sizes can be found in Appendix D). We mated 'young' females at 7 days post-eclosion (DPE) and 'old' females at 25 DPE. These ages are realistic for the age of mating in wild populations (Simmons & Zuk 1994) and our decision to treat 25 DPE as 'old' age also

ensured that the females were sufficiently advanced in age while still allowing them time to lay eggs before death. In the lab, adult females live 1-2 months post-ecolosion. To produce the F2 generation, we allowed F1 females the opportunity to mate with a randomly assigned colony male for four hours per day for three consecutive days under the same conditions as described above. Again, we isolated mating pairs between mating opportunities. As with the founding females, we paired F1 females with unrelated males who were 7-10 DPE on the first day of mating. Thus, we controlled for male age across female age treatments.

Fitness measurements

For all F1 females that were mated, we counted the number of eggs that each female laid, measured mean egg size for a subset of eggs, and assessed the proportion of offspring that hatched (hatching success). We also measured the F2 offspring's development time, the number of offspring that survived to adulthood, and the sex ratio of the adult offspring (Fig. 1). By examining the reproductive investment of the F1 generation in the offspring (the F2 generation), we combine typically disparate methods (traditional fitness measures and immunity) and address offspring quantity and quality to better characterize the effects of maternal age on offspring fitness.

We used Mitutoyo digital calipers to measure the width of the pronotum (plating that covers the thorax) to the nearest 0.01 mm and recorded the mass of each cricket, in both F1 and F2 generations, to the nearest 1.0 mg using an Ohaus Adventurer Pro balance (model AV264). To assess egg characteristics, we provided F1 females with moistened cheesecloth as an egg-laying substrate for 14 days post-mating and counted the number

of eggs laid by each female under a dissecting microscope. To measure egg size, we randomly selected five of the counted eggs to be weighed individually and then also as a group. Hatching generally occurs 10-14 days after egg laying (personal observations), so we calculated hatching success (the proportion of eggs laid that hatched) of the eggs 14 days after we counted the eggs (28 days post-mating). For the hatched F2 offspring of young and old F1 females, we counted how many survived to adulthood, determined the sex ratio of the adult offspring, and recorded development time (time from hatching to eclosion) and size at adulthood by measuring pronotum width and wet mass (N=785; see Appendix D for all sample sizes in the maternal age treatments and fitness measures).

In addition to the traditional fitness measures above, we also assessed immunocompetency of the F2 generation (N=505) adult offspring. We performed two standard immunological tests: hemocyte abundance and encapsulation response to a foreign object (Fig. 1). Hemocytes are specialized immune response cells that aid in the encapsulation of foreign bodies in insects' bodies (Smilanich 2009; Beckage 2008). Hemocyte abundance, therefore, can be used as a measure of immune strength; increased hemocyte abundance indicates increased immune response (Graham 2011). To measure hemocyte abundance, we extracted 2uL hemolymph from each cricket and stored it in 4uL of anticoagulant (EDTA, citric acid, and PBS regulated at pH 7.4; adapted from Adamo *et al.* 2001). We dispensed the hemolymph-anticoagulant mixture in a Kova Glasstic Slide System, which we used as a substitute for a hemocytometer, and counted hemocytes in each grid under 400X magnification of a Leica compound microscope. Another method to quantify immune response is to insert a foreign object, which acts as a

proxy for parasitoid oviposition, into the body of an insect and measure the strength of the response (Zuk & Stoehr 2002; Smilanich *et al.* 2009). When an insect is immune challenged by a foreign body, like a parasitoid egg or larva, its immune cells encapsulate the foreign body with layers of hemocytes to asphyxiate the invader (Zuk & Stoehr 2002; Strand 2008; Smilanich 2009). During this process, the cells die and become melanized (dark in color) and melanization can be quantified as a measure of immune strength. To simulate a foreign body, we roughened 3mm lengths of 0.2mm gauge nylon fishing line (filaments) to provide an adhesive surface for the cells and sterilized the filaments with 90% ethanol (adapted from Rantala *et al.* 2000). We used a 30-gauge hypodermic needle to pierce into the center of the crickets' pronotum and inserted a single filament. We returned crickets to their individual containers and extracted the filaments after 24 hours using sterilized forceps. We photographed each filament on a sterilized watch glass under 40X magnification of Leica CME compound microscope using iDu Optics LabCam Microscope Adapter for iPhone and an iPhone 6. Using the image processing program Fiji (Schindelin *et al.* 2012), we quantified both the proportion and total area of a sample section of the filaments that were melanized. To avoid shadowing that occurred along the edges of the filaments, which could alter the melanization measurement, we selected a standardized section (1.5mm x 0.17mm) of the filament image and used Threshold plugin (Y=0-20; U=0-255; V=0-255; adapted from Schneider *et al.* 2012 and Tinghitella *et al.* 2017). During pilot immune testing, we found that the two methods for assessing immune function sometimes caused crickets to die shortly after the tests were performed. Thus, we performed these tests on the full siblings of the mated F1 females and not on the

females used in the mating experiments. We performed the same immunocompetency tests on both male and female offspring in the F2 generation.

Statistical Analysis

To determine whether maternal age affects maternal fitness, we tested if the fixed effect of *maternal age* (young vs. old) affected the *number of eggs laid*, *egg mass*, *egg hatching success, offspring development time*, *number offspring surviving to adulthood*, *adult offspring size*, and *sex ratio* using separate linear mixed models. We included *maternal body size* as a covariate and *founding line* as a random effect. We expected the response variables to vary with maternal body size (Berrigan 1991; Blackburn 1991; Bernardo 1996), so it was important to account for variation in maternal size to test the effect of maternal age.

To test whether offspring from young or old mothers varied in their immune response, we performed a linear mixed model, using *body size* as a covariate and *founding line* as a random effect, to compare the *mean number of hemocytes* and the *proportion of melanization of filaments* between offspring from each maternal age treatment. Given our various measures of fitness, we also performed both Bonferroni (at $\alpha<0.05$ and $\alpha<0.01$) and false discovery rate (at $q<0.1$ and $q<0.05$) adjustments to correct for type I error on the nine measures of offspring fitness (*hemocyte abundance*, *development time*, *hatching success*, *egg mass*, *filament melanization*, *number of eggs*, and *offspring mass*, *sex ratio*, and *survival to adulthood*; McDonald 2014). We used JMP Pro version 13 for all statistical analyses.

Results

We found that young mothers laid eggs that were 13% larger than eggs laid by old mothers ($F_{9.2, 172.1}$=6.32, P=0.013; Figure 2.2A) and that the hatching success of the eggs laid by young mothers was 80% higher than that of eggs laid by old mothers ($F_{8.7, 172.1}$=13.05, P=0.0004; Figure 2.2B).

Figure 2.2.

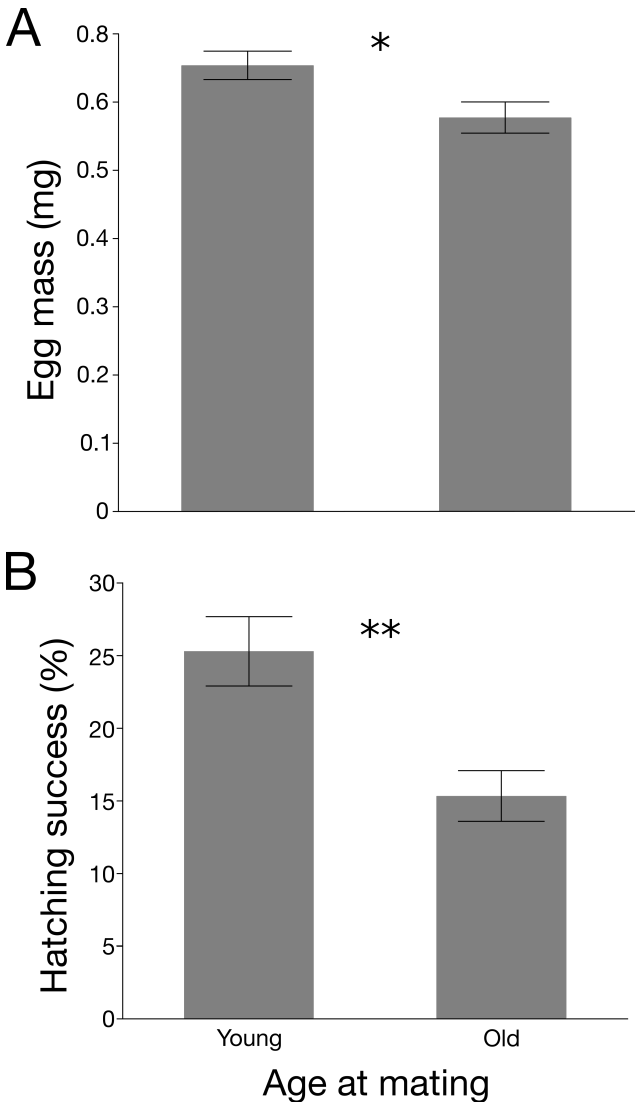

Figure 2.2. Eggs laid by young and old mothers in the F1 generation differed in their **A)** mass, and **B)** hatching success (percentage of eggs laid that hatched). Means given ± SE; * indicates P<0.05 and ** indicates P<0.01.

We found no significant difference in the number of eggs laid by old and young mothers ($F_{2.4, 171.1}$= 3.41, P=0.067). The offspring from old mothers that survived to adulthood developed an average of six days faster than offspring from young mothers ($F_{7, 98.3}$=13.99, P=0.0003; Figure 2.3A).

Figure 2.3.

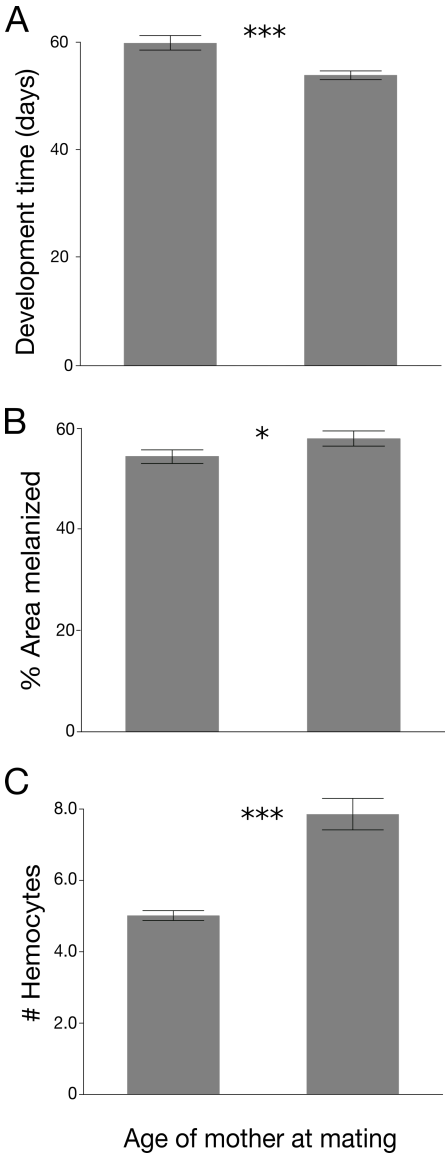

Figure 2.3. For the F2 generation offspring of young and old mothers, the **A)** development time (time from hatching to eclosing to adulthood), **B)** percent area of the filament segment that was melanized after 24 hours of insertion into the pronotum, and **C)** number of hemocytes present in the hemolymph. Means given ± SE; * indicates P<0.05, ** indicates P<0.01, and *** indicates P<0.001.

Otherwise, we did not find any additional differences in our traditional fitness measures between the two maternal age treatments: there were no differences in the number of offspring that survived to adulthood ($F_{6.8, 182.2}$=0.01, P=0.91), the size of the adult offspring (wet mass: $F_{8, 679.6}$=1.27, P=0.26; pronotum width: $F_{8.2, 767.9}$=0.48, P=0.49), or in sex ratio of adult offspring from young and old mothers ($F_{10.5, 132}$=0.75, P=0.38). We found that offspring from old mothers had a 7% higher percent area melanization than the offspring from young mothers ($F_{7.9, 499.4}$=3.95, P=0.047; Figure 2.3B). Offspring from old mothers had 56% more hemocytes in their hemolymph than offspring from young mothers ($F_{9.2, 497.5}$=50.22, P<0.0001; Figure 2.3C). Given that insects do not have an acquired immune system (the ability to build one's immunity with pathogen exposure over time), we compared the hemocyte abundance between the parental and offspring generations to ensure that any differences between the offspring of the two maternal age treatments (F2 generation) were indeed due to maternal age effects and found no difference in hemocyte abundance between the two generations ($F_{10.6, 616.3}$=0.81, P=0.37). We found no effect of maternal body size on offspring hemocyte abundance or proportion melanization (P=0.21, P=0.06 respectively).

After our Bonferroni adjustment at $\alpha<0.01$, hemocyte abundance remains significant and development time and hatching success also remain significant at $\alpha<0.05$. However, egg mass and percent area melanization of the filament are no longer significant. Similarly, when we performed the FDR at $q<0.05$, hemocyte abundance, development time, hatching success, and egg mass remained significant while only

filament melanization remained significant at $q<0.1$. A summary of all offspring fitness results can be found in Table 2.1.

Table 2.1. Offspring fitness measures from young and old mothers. The P-value reported is the original value from the statistical model used to compare the fitness of the offspring from young and old mothers.

Table 2.1.

Fitness Measure	df	F statistic	P-value
Hemocyte abundance	9.2, 497.5	50.22	0.0001**††
Development time	7, 98.3	13.99	0.0003*††
Hatching success	8.7, 172.1	13.05	0.0004*††
Egg mass	9.2, 172.1	6.32	0.013††
Filament melanization	7.9, 499.4	3.95	0.047†
Number of eggs	2.4, 171.1	3.41	0.067
Mass	8, 679.6	1.27	0.26
Sex ratio	10.5, 132	0.75	0.38
Survival to adulthood	6.8, 182.2	0.01	0.91

*Remains significant ($\alpha<0.05$) after Bonferroni adjustment to correct for type I error.
**Remains significant ($\alpha<0.01$) after Bonferroni adjustment to correct for type I error.
†Remains significant ($q<0.1$) after false discovery rate adjustment to correct for type I error.
††Remains significant ($q<0.05$) after false discovery rate adjustment to correct for type I error.

Discussion

Maternal age significantly affects offspring fitness and immune response. We found support for both life history theory (predicting positive effects of advanced maternal age in offspring) and aging theory (predicting negative effects of advanced maternal age on offspring). Notably, support for either of these theories depends on which measures of offspring quantity or quality we consider (Table 2.2).

Table 2.2. A summary of our results that support either aging theory or life history theory. We categorize support for each theory by whether or not those results were from tests assessing the quantity of offspring or the quality of offspring.

Table 2.2.

	Aging theory	Life history theory
Quantity	Fewer eggs hatch from old mothers	No difference in offspring survival between young and old mothers
		No difference in offspring sex ratio between young and old mothers
		No difference in number of eggs laid between young and old mothers
Quality	Old mothers laid smaller eggs	No difference in offspring size between young and old mothers
		Shorter development time in offspring from old mothers
		Increased immune response in offspring from old mothers

The eggs laid by young mothers were more 80% more likely to hatch than those laid by old mothers (offspring quantity) and the eggs laid by young mothers were 13% larger than those laid by old mothers (quality). These two results are consistent with decades of aging theory research that has also found that young mothers lay more eggs than old

mothers and that their eggs are more likely to hatch (Fox 1993b; Kern *et al.* 2001; Yanagi & Miyatake 2002; Qazi *et al.* 2017). However, when we consider our Bonferroni and FDR corrections, the size and hatching success of eggs laid by old and young mothers no longer remains significant under various levels of the corrections, indicating that we should interpret these data with caution when attributing them as support for aging theory.

Conversely, we also found support for life history theory in that there was no difference in the number of offspring that survived to adulthood, the sex ratio of the adult offspring between the two maternal age treatments (quantity; Table 2.2), and no difference in the size of offspring between the two maternal age treatments. We acknowledge, however, that finding no difference between the offspring from old and young mothers may be caused by other factors (e.g. pleiotropy) that are not associated with life history theory. Therefore, while we categorize many of our results as support for life history theory, we recognize that the results that show no difference between the offspring from the two maternal age treatments may not necessarily show support for life history theory, but rather a lack of support for aging theory. We also found that the offspring from old mothers had shorter development time (quality; Table 2.2), which may be advantageous for this species as they are not seasonal and do not have to time their emergence with seasonal cues. Shorter development time could reduce the amount of time individuals are exposed to predators before reaching reproductive maturity and thus increase fitness (i.e. slow-growth high-mortality hypothesis; Price 1980; Mousseau & Dingle 1991, Murphy *et al.* 2018). Short development times, even for those organisms

that spend more time as adults, can lead to higher population growth rates because they reach maturity and can reproduce sooner than individuals that take longer to develop (Roff 1980).

Further support for life history theory can be found in our results that show that offspring from old mothers have higher immune function than the offspring from young mothers (quality; Table 2.2). Offspring from old mothers had 56% more hemocytes in their hemolymph and a 7% higher encapsulation response than offspring of young mothers. However, similarly to our Bonferroni and FDR corrections on egg mass and hatching success, we found that filament melanization between the two maternal age treatments also loses statistical significance at most correction levels, though we still categorize these data as support for life history theory (or lack of support for aging theory). The difference we found between hemocyte abundance and filament melanization could be due to variation of the types of hemocytes found in insect hemolymph. The hemocytes active in the melanization process may not have been as equally represented as other types of hemocytes, accounting for the high statistical significance of overall hemocyte abundance but a lower or null difference in melanization. Our overall results suggest that the transfer of immune proteins from mother to egg may differ with age. The specific mechanism(s) involved in changes in insect maternal investment, however, remain unknown. Previous insect aging research suggests an increased provisioning of available resources by old mothers but precise mechanism(s) behind maternal investment with age have not yet been identified. Transgenerational effects are often underlaid by epigenetic effects, such as decreased

methylation with age (Maegawa et a. 2010), which may play a role in our results. Change in the non-sequencing region(s) of the genome may negatively affect the lifespan of adult offspring as well as some cellular functions (Masser *et al.* 2018). However, we did not measure any potential epigenetic effects in this study.

Our research highlights a missing component in the field of aging research within natural insect populations. Much of the previous research in this field has used inbred, lab-adapted populations that may not accurately predict maternal age effects in natural insect populations. Natural populations may respond differently to immune challenges than populations that are inbred, reared from iso-female lines, or severely lab-adapted. Furthermore, laboratory populations may have different responses in traditional fitness measures in terms of both quality and quantity of offspring. For example, diminished genetic variation in heavily inbred populations may be confounded with fitness effects of offspring from old mothers, making it difficult to determine which (and to what extent) results are linked to maternal age and which are due to inbreeding. When we consider both quantity and quality of offspring, the majority of our results show that there are trade-offs associated with advanced maternal age in that old mothers may not produce more offspring than young mothers, but the offspring are of higher quality. The combination of collecting both quantity and quality measures of fitness is important in understanding the comprehensive effects of advanced maternal age on offspring fitness.

We found that offspring from old mothers were ultimately more fit than those from young mothers. More eggs may hatch from young mothers, but there is no difference in the number of offspring that survive, their body size, or their sex ratio when

compared to offspring of old mothers; furthermore, the immune systems of offspring from old mothers are stronger than those of offspring from young mothers. Our immunocompetence results suggest that maternal investment differences may be focused in the immune priming process from mother to egg. Our results emphasize the importance of measuring offspring fitness as both traditional measures (body size, survival, etc.) as well as immunocompetence for a complete understanding of the effects of maternal age. If we had only measured egg quality and quantity, as many studies do, we would have come to similar conclusions as many others in the aging field: that old mothers produce offspring of lower fitness than young mothers. In considering both a wider array of traditional fitness measures as well as immune function, however, we found that maternal age effects are far more intricate than a single strategy for measuring fitness can assess. We believe that both immunity and traditional fitness measures should be considered in all future aging research to form a complete picture of aging effects. We may need a paradigm shift in our approach to aging research; we need to consider both quantity and quality of the offspring to fully understand maternal effects.

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Appendices

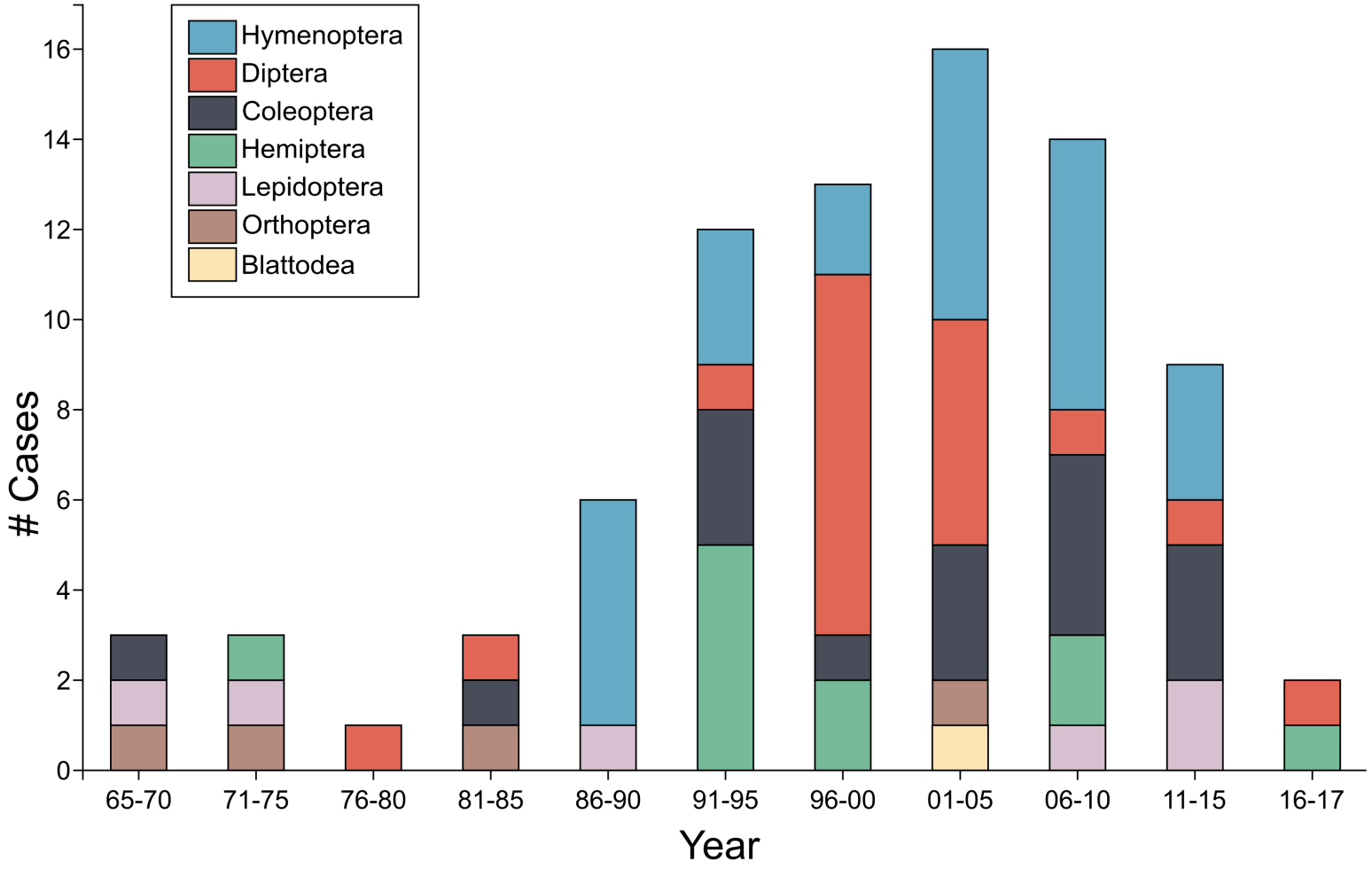
Appendix A. A list of the fitness measures used in the cases we reviewed, descriptions of each, and the number of cases in this review that recorded each fitness measure.

Fitness measure	*Description*	*Number of cases*
Egg load	The number of eggs in the female oviducts	1
Number of eggs	The number of eggs laid by the female	30
Egg size	Measures of the size of the eggs laid by the female (e.g. mass, length, width)	28
Egg fertility	Whether the eggs laid by the female were fertilized by male sperm (opposed to unfertilized eggs)	3
Egg composition	Nutrient composition of eggs (e.g. sugar, lipid, protein, carbohydrate content)	3
Embryo size	Length of the developing embryo inside the egg	1
Hatching success	Proportion of eggs laid that hatched	26
Number of larvae	For a female, the number of offspring that reached the pupal stage	3
Larval size	Measures of the size of the larvae produced by the female (e.g. mass)	5
Larval survival	The number of larvae from young and old mothers that survived on host plants that were resistant to the herbivorous insect used	1
Number of pupae	The number of pupae produced by the female	1
Pupal size	Measures of the size of the pupae (e.g. mass, length, width)	3
Development time	The time it took for the offspring to develop from the egg stage or (if there was no egg stage) the larval stage into adulthood	26
Starvation survival	The amount of time offspring could survive without feeding until death	2
Survival to adulthood	The number of offspring that survived to reach adulthood	27
Adult size	Measures of the adult offspring size (e.g. mass, length, width)	14
Total offspring	The number of offspring produced by a female, but the authors failed to provide the life stage at which the offspring were counted	4
Lifespan	The length of time the offspring survived until natural death	12
Sex ratio	The proportion of male to female offspring produced by a female	25

Appendix B. A list of case attributes and their descriptions recorded in this review.

Case attribute	*Description*
Paper number	Number attributed to each paper (1-75)
Case ID	Letter attributed to each case within each paper if the authors used more than one system or experiment
Title	Title of the paper
Author(s)	Name of author(s) of the paper
Year	Year the paper (and thus case) was published
Species common name	Common species name of the study organism in the case
Latin genus	Latin name of the genus of the study organism in the case
Latin species	Latin name of the species of the study organism in the case
Taxonomic order	Taxonomic order to which the study organism belongs
Fitness measure	Fitness measures that the author(s) collected on the offspring of young and old mothers
Direction of effect of advanced maternal age	Whether the effects (for each of the fitness measures) of advanced maternal age were negative, positive, or had no effect. For sex ratio: whether the offspring were male or female biased or if there was no effect.
Multigenerational	(0/1) Whether the author(s) examined maternal age effects beyond a single generation. “0” means they did not, “1” means they did, if unknown, we entered “NA”
Inbred or lab-adapted	(0/1) Whether the populations used for the experiment were inbred or lab-adapted. “0” means they did not, “1” means they did, if unknown, we entered “NA”
Virgin mating	(0/1) Whether the mating females used in the experiment were virgin at the ‘young’ and ‘old’ ages. “0” means they did not, “1” means they did, if unknown, we entered “NA”
Same female for ‘young’ and ‘old’	(0/1) Whether the same female was used for the ‘young’ and ‘old’ age (i.e. egg laying order). If unknown, we entered “NA”
Control for paternal age	(0/1) Whether the author(s) controlled for paternal age. “0” means they did not, “1” means they did, if unknown, we entered “NA”
Difference between ‘young’ and ‘old’ age	The numeric difference in units (see below) between ‘young’ and old’ ages of mothers
Units of difference of age treatments	The units of time used in the difference of ‘young’ and ‘old’ mothers (e.g. days, weeks)

Appendix C. The number of cases per five years, from 1965-2017, that investigate the effects of advanced maternal age in insect offspring. Each 5-year bin is subdivided to show the number of cases that were published in each of the 7 insect orders investigated (colors for the orders are the same as those used in Table 1.1 and Figure 1.1).



Appendix D. Sample size of young and old breeding F1 generation females and their offspring (F2 males and females) on which we performed immune testing from each of the ten founding maternal lines. The Founding Maternal Line (A-J) indicates the colony female that was used to establish the line for each family. The 10 founding females reported here are those that survived to produce enough offspring for the breeding experiment *and* immunocompetency testing. F1 Females Mated Young and F1 Females Mated Old refer to the number of females in the F1 generation (produced by each founding maternal line) that were mated at a young or old age (7 or 25 days post-eclosion). F1 Females Immune Tested and F1 Males Immune Tested refer to the female and male offspring of the founding females that we did not mate, but instead performed immune tests on. F2 Females Immune Tested and Males Immune Tested refer to the female and male offspring in the F2 generation, produced by the mating females in in the F1 generation, that underwent immune testing. The colors of the column headers all correspond with the colors used in Figure 2.1.

Founding Maternal Line	F1 Females Mated Young	F1 Females Mated Old	F1 Females Immune Tested	F1 Males Immune Tested	F2 Females Immune Tested	F2 Males Immune Tested
A	7	7	5	5	9	33
B	12	13	5	5	26	40
C	12	14	6	7	33	40
D	8	8	6	6	19	29
E	7	7	6	7	0	13
F	9	8	7	5	23	34
G	11	11	6	6	21	41
H	9	8	5	6	17	25
I	8	8	5	5	20	29
J	11	10	6	6	20	33