Consequences of advanced maternal age on reproductive investment by male offspring

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Abstract

Maternal age can have contrasting effects on a variety of offspring fitness traits. While the effects of maternal age on offspring traits that are not sex-specific, such as body size and growth rate, as well as on traits specific to females, have been well researched, traits that are specific to male offspring have been understudied. Across taxa, male reproductive investment is a particularly salient component of fitness, especially when females mate with several males. We tested whether maternal age affects the reproductive traits of their male offspring by comparing the investment made by male field crickets, *Teleogryllus oceanicus*, from 'young' and 'old' maternal age treatments. Female *T. oceanicus* mate with several males, and sperm competition is a fair lottery, so male reproductive investment is important for fitness in this system. After two generations of mating young and old females, we measured the testes mass, spermatophore mold mass, and sperm viability of their male offspring. Despite differences in maternal and grand-maternal age and the demonstrated effects of advanced maternal age on egg number and offspring immunocompetency in this system, the male offspring of young and old females did not differ in reproductive tissues and sperm viability. This study is one of the first to examine the effect of maternal age on fitness-related traits specific to male offspring, and we encourage future research that tests the effects of maternal age on male offspring in other species.

Keywords

aging, aging theory, life history theory, male fitness, maternal effect, sperm viability

Introduction

Intrinsic characteristics of parents and their experiences through life can have profound effects on offspring traits through parental effects (reviewed in Badyaev and 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 et al. 2004, Hansen et al. 2015), mammals (Descamps et al. 2008), and birds (Asghar et al. 2014). However, the effects of parental age on the traits of the offspring are not consistent across studies and can be positive, negative, or neutral. The 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 at later stages 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 (Trivers 1974, Partridge and Harvey 1988); terminal investment, then, has the potential to increase the fitness of aging parents (with all else equal). Aging theory predicts that older parents are unable to make reproductive investments late in life or that such investments are poor due to the detrimental effects of senescence (Nussey et al. 2013, Lemaitre and Gaillard 2017); if so, offspring born to old females may be less fit than offspring born to young females. Even if females of advanced age invest heavily in their offspring (terminal investment), consistent with life-history theory, limited resources late in life may mean that that investment is lower than investments made at younger ages. An alternative, of course, is that there may simply be no change in maternal investment with maternal age. Both the terminal investment literature and aging literature have traditionally focused on the effects of advanced parental age on traits that are relevant to both sexes (such as body size or growth rate) or investigated fitness effects only for female offspring.

Male fitness is often determined, to some extent, by the investment made in postcopulatory reproductive traits (Harcourt et al. 1981, Taborsky 2002, Parker 2015). Though males invest less in individual gametes than females, males are limited in the amount of sperm they can allocate to each reproductive opportunity (Wedell et al. 2002). Males can optimize investment in reproductive bouts by adjusting the size or contents of their ejaculate to match perceived levels of mate availability and sperm competition (Simmons 2003, Reinhardt et al. 2011, Vahed et al. 2011). Little work has linked maternal age to investment in traits specific to male offspring, though

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research has found that older female seed beetles had male offspring with longer sperm (Dowling et al. 2007, Gay et al. 2009); the fitness consequences of sperm length for males were, however, unclear in these studies. In other studies, no link was found between maternal age and the fitness of their male offspring (Mossman et al. 2019).

In crickets, females mate with multiple males and store sperm in a round spermatheca, leading to a fair 'lottery' in determining which sperm fertilize available eggs (Larson et al. 2012). Therefore, in crickets, male investment in reproductive traits, such as sperm volume and sperm viability, are particularly important determinants of paternity, more so than other factors like mating order (Sakaluk and Eggert 1996, Simmons 2003, Bretman et al. 2009). We studied the Pacific field cricket, *Teleogryllus oceanicus*. In this species, 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 and 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 et al. 2010, Gray and Simmons 2013). We tested if maternal age influences male reproductive traits, given that 1) male investment in reproductive traits can be adjusted through plasticity, 2) male investment in reproductive traits is important for male fitness, 3) quality-related traits of offspring are sometimes dependent on parental age (e.g., Bloch Qazi et al. 2017), and 4) maternal age has been found to have an impact on daughters' traits in this species (unpublished results).

We investigated the effects of advanced maternal age on male reproductive investment by measuring the testes mass, spermatophore mold mass, and sperm viability of male offspring following two generations of mating females at either a young or old age (Fig. 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 older mothers and grandmothers having greater (or equal) reproductive investment as compared with male offspring of younger mothers and grandmothers. Alternatively, if male offspring of older mothers and grandmothers show lower reproductive investment than male offspring of younger mothers and grandmothers, this would support aging theory.

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 (Simmons 2003), a breeding system that should lead to selection on postcopulatory reproductive traits of males (Simmons 2001). Additionally, testes mass is a well-established measure of male reproductive investment in this cricket (Bailey et al. 2010, Gray and 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. A colony typically contains approximately 100 breeding adults. We randomly chose 10 females from the colony to serve as our founding females in April of 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 (Fig. 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 about one month. Thus, 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). This experiment is part of a larger fully factorial experiment in which all combinations of old and young mothers and grandmothers are included. We chose to investigate the effects of maternal age on male sexual traits in the two treatments in which we expected to see the greatest potential effect of maternal age; this means that we cannot differentiate maternal from grandmaternal effects in this experiment. To mate each female, we placed her in a 0.5 L 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 the possible effects of paternal age, all males used for matings were 5–10 DPE.

Rearing.—We kept all crickets in temperature-controlled (27°C) Percival incubators (model I36VLC8) on a 12h:12h light:dark schedule throughout the experiment. We housed juvenile crickets in family groups inside 0.5 L deli cups and supplied them with Fluker's High Calcium Cricket Chow, part of an egg carton for shelter, and moist cotton for water. We checked for eclosions daily and separated males and females immediately (<24 hours from eclosion). We housed all females that were to be mated individually in 0.5 L 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 118 mL 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, spermatophore mold 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 1.5 mL microcentrifuge tubes at -20°C between March and April of 2018. We thawed the males to dissect fully intact reproductive tissues from them: the testes (which generate sperm) and the spermatophore mold (which holds and shapes the sperm containing packet before it exits the male's body; Khalifa 1949). We were unable to dissect out the accessory glands (which are responsible for producing seminal fluid) because they had partially disintegrated while the male was frozen. We dissected the testes and the spermatophore mold from all males from both treatments at one of two times: July of 2018 (young treatment, n = 7 individuals, and old treatment, n = 48 individuals) and April of 2019 (young treatment, n = 38 individuals, and old treatment, n = 28 individuals). Hereafter, we refer to the dataset that consists of males we dissected in July 2018 as the 'early' dataset and the dataset that consists of males we dissected in April 2019 as the 'late' dataset. We refer to the dataset that includes all males as the complete dataset.

To test sperm viability, we used a ThermoFisher LIVE/DEAD sperm viability kit and established methods (Garcia-Gonzalez and 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 an EC3 camera on a computer running LAS X imaging software. We captured two images from the same view window of each sample:

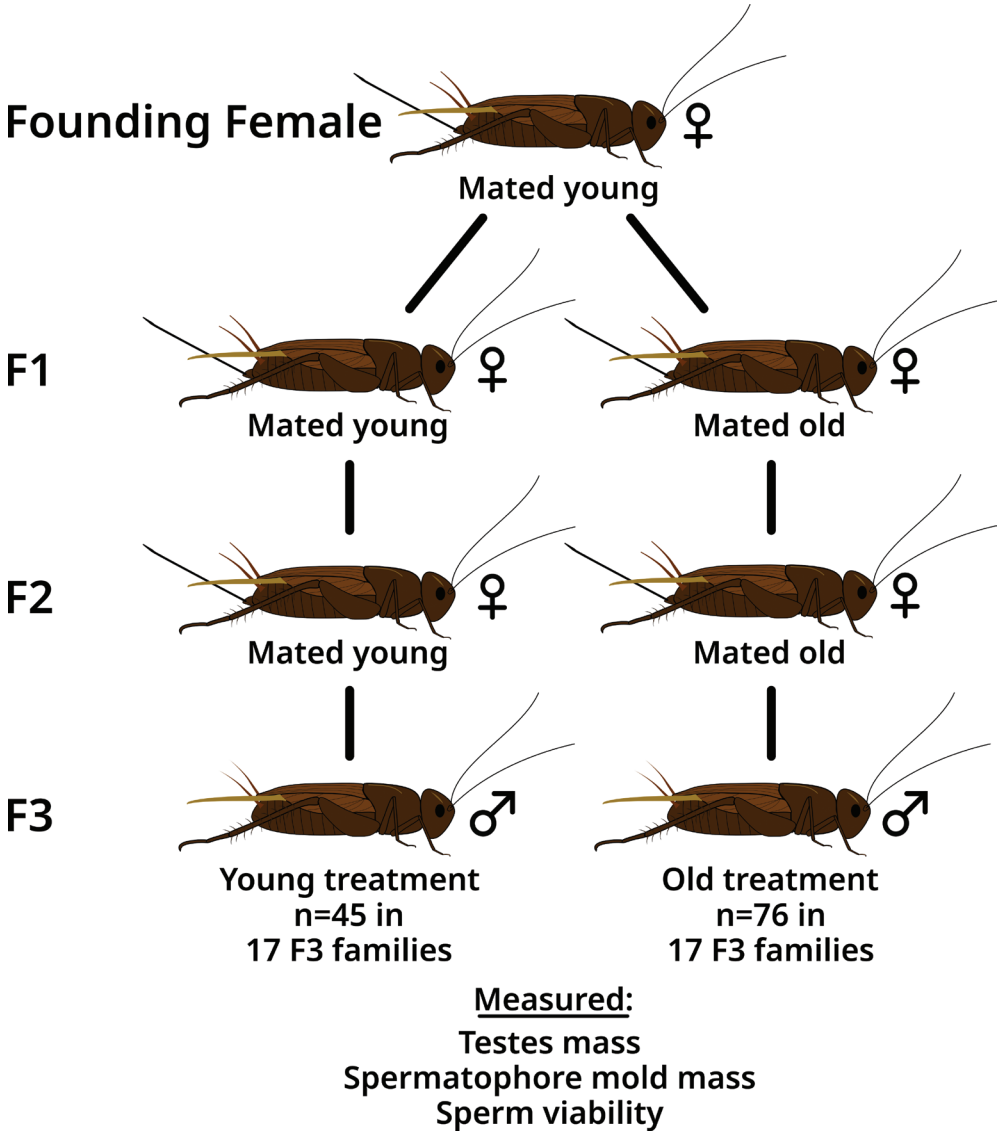

Fig. 1. A diagram of our experimental mating design. We mated females at either a young age (7 days after eclosion to adulthood) or an old age (25 days after eclosion to adulthood) for two subsequent generations, then measured three proxies of reproductive investment in males of the F3 generation. The F3 families from the Old treatment were the offspring of 8 founding females and the F3 families from the Young treatment were the offspring of 7 founding females.

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 36 × 24 grid on both images from each sample using Inkscape (a vector graphics editing program) to facilitate counting of sperm cells. We counted 25% of each image (or 216 grid squares) 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. We recorded sperm viability as the proportion of total counted sperm that were alive. 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 from the early dataset described above.

Statistical analysis.—We used a linear mixed model to test the effect of maternal age treatment on testes mass and spermatophore mold mass using the complete dataset. We transformed spermatophore mold mass using a cube-root transformation to meet assumptions of normality and equal variance. We had two response variables: testes mass and spermatophore mold 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 male age as a covariate in our models because male age impacts sperm viability in *T. oceanicus* (Garcia-Gonzalez and Simmons 2005, Dowling and Simmons 2012). We included *dissection date* as a fixed effect in the model because we noticed that tissue 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 and also any variation in rearing environments among

families. 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 spermatophore mold mass using only the individuals from the late dataset because this dataset had a more balanced sample size (young treatment n = 38, and old treatment n = 28) than the early dataset and the complete dataset. We ran the same statistical model described above for both testes mass and transformed spermatophore mold 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 the sperm viability of male offspring. We checked the sperm viability data for equality of variance and normality before proceeding with analysis. We only measured sperm viability for males from the early 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 and to guard against making a type II error, and we compared our effect sizes to effect sizes in the literature where possible. We were not able to run power analyses on the linear mixed models described above, so we 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 affect the reproductive traits of male offspring. In the complete dataset *maternal age treatment* did not have a significant effect on either testes mass ($F_{1,41.76} = 0.11$, $p = 0.74$; Fig. 2A) or transformed spermatophore mold mass ($F_{1,32.5} = 0.99$, $p = 0.32$; Fig. 2B). Our power analysis showed that with our means and variance, we would need 47,396 observations of testes mass and 556 observations of spermatophore mold mass to detect a significant difference in these variables between the maternal age treatments. In our old treatment, males had testes that were 2% larger than the testes of young-treatment males, which is much smaller than the difference of 10% that Bailey et al. (2010) found when assessing plasticity in the reproductive organs of the same crickets in response to song heard during development. Older and smaller males from the late dataset had significantly smaller testes masses than younger and larger males from the early dataset (*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* $F_{1,114.7} = 31.81$, $p < 0.0001$). Males that we dissected from the late dataset had significantly smaller spermatophore molds (*dissection* date: $F_{1,115.9} = 20.77$, $p < 0.0001$), but there was no significant difference among males of different ages (*age of the male:* $F_{1,115.3} = 0.67$, $p = 0.42$) or of different sizes (*pronotum width:* $F_{1,110.2} = 0.83$, $p = 0.36$).

In our analysis of only the late dataset, we found no significant effect of *maternal age treatment* on testes mass ($F_{1,18.99} = 0.04$, $p = 0.8$) or spermatophore mold 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 spermatophore mold 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* ($F_{1,29.04} = 0.82$, $p = 0.37$; old treatment: 0.67 ± 0.03, young treatment: 0.76 ± 0.09). Sperm viability did not differ among males of different ages ($F_{1,45.18} = 0.44$, $p = 0.51$) or different sizes ($F_{1,47.52} = 0.49$, $p = 0.49$). Our power analysis showed that we would need 284 observations of sperm viability to detect a significant difference between maternal age treatments. Though not significant, young treatment males had 13% higher sperm viability than old treatment males; this difference is larger than the 7% difference induced by experience with song during development in Gray and Simmons (2013).

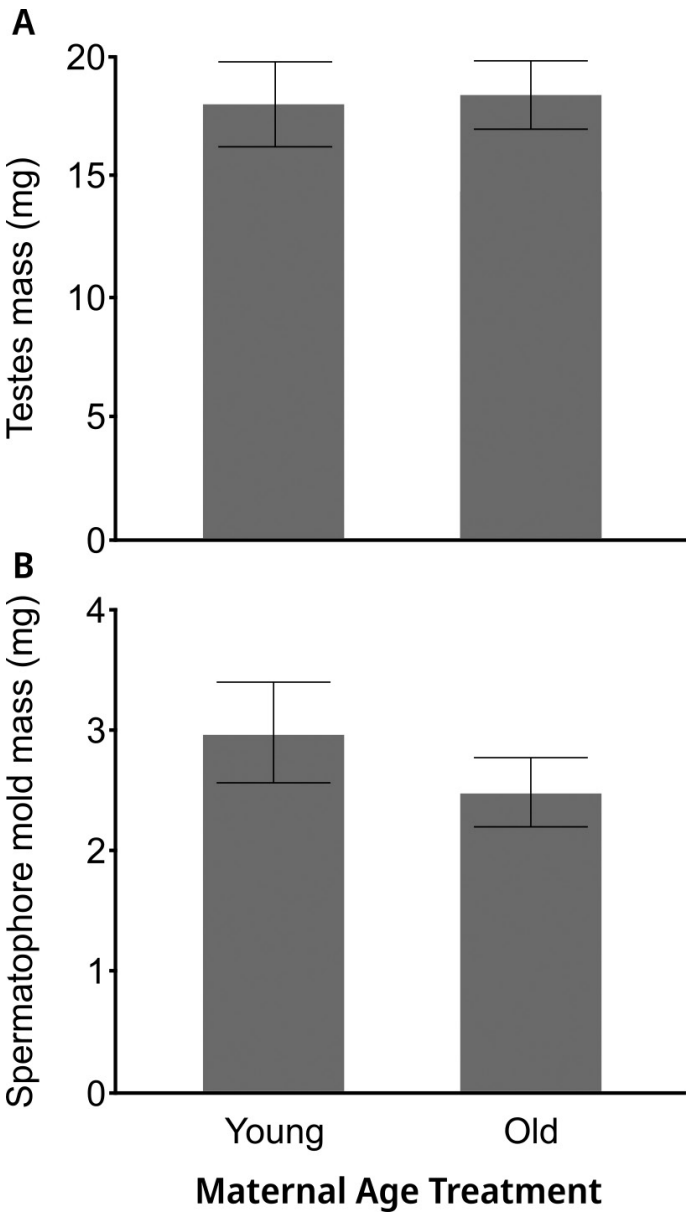

Fig. 2. Reproductive investment of male offspring by treatment. For all male offspring from both maternal age treatments: **A.** Testes mass; **B.** Spermatophore mold mass. There were no significant differences between treatments for either measure. Bars represent least square means ± SE.

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 the reproductive traits of male offspring and found no influence of maternal age treatment on testes mass, spermatophore mold 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 differences in male reproductive traits between the young and old treatments, we conducted power analyses and comparisons of effect sizes that largely supported and validated our null results. For one measure–spermatophore mold mass in the males that we dissected later (late dataset)–the power analysis suggests we may need a larger sample size to definitively conclude that there was no effect from maternal age. For the measure of sperm viability, our comparison of effect sizes showed that the difference in sperm viability between groups may warrant further exploration in future experiments.

Both life history theory and aging theory have been used to explain the impacts of advanced maternal age on offspring fitness. In the most general sense, finding that the offspring of older mothers are less fit than the offspring of younger mothers would support aging theory (Nussey et al. 2013, Lemaitre and Gaillard 2017), but mothers making terminal investments can increase offspring fitness (Williams 1966, Trivers 1974). We found no differences in the reproductive traits of males belonging to old and young mothers; 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, Gray and Simmons 2013), but we do not know all of the conditions under which that plasticity is released. Second, there may be other unmeasured constraints on male reproductive investment, or the traits measured may be pleiotropically linked with others. Third, it is possible that the pattern we found may, indeed, result from mothers of advanced maternal age making a terminal investment, but, because of the costs of aging, that terminal investment is still less than the investment made by younger mothers. Finally, it is possible that males differentially allocate resources depending on the age of their mate. If this effect counteracts age-dependent differential investment made by mothers, this could lead to no overall difference in the fitness of sons. A deeper understanding of the underlying mechanisms and drivers of both male and female reproductive investment and resource allocation would elucidate the patterns.

Testes size is often highly variable within populations and increased size is associated with an increased risk of sperm competition (Merila and Sheldon 1999, Simmons 2001), including in this species (Bailey et al. 2010). In many taxa, this pattern is the result of selection; males in species with a higher risk of sperm competition often have much larger testes than males in closely related species with a lower risk of sperm competition (Merila and Sheldon 1999). Testes size can also be plastic depending on perceived level of sperm competition during rearing (Bailey et al. 2010, Fisher and 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 the size of the male (pronotum width) due to allometry, and differences associated with dissection date are likely a result of tissue degradation. We found that older males had smaller testes, but, to our knowledge, the existing literature does not suggest that testes shrink with age. In many species, older males have larger testes because they have reached sexual maturity, and there is also some evidence for an increase in asymmetry between testes with age (Merila and Sheldon 1999, Brown and Brown 2003, Abdul-Rahman et al. 2018). Perhaps the pattern of old males having smaller testes reflects a trade-off between reproduction and longevity (Austad and 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. In an unpublished study, we 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 either life history theory or aging theory, depending on the fitness measure assessed. Notably, 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 the current results, our unpublished work demonstrates 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 among the first to consider the effects of maternal 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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