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Transgenerational effects of maternal and grandmaternal age on offspring viability and performance in *Drosophila melanogaster*

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ABSTRACT

In non-social insects, fitness is determined by relative lifetime fertility. Fertility generally declines with age as a part of senescence. For females, senescence has profound effects on fitness by decreasing viability and fertility as well as those of her offspring. However, important aspects of these maternal effects, including the cause(s) of reduced offspring performance and carry-over effects of maternal age, are poorly understood. *Drosophila melanogaster* is a useful system for examining potential transgenerational effects of increasing maternal age, because of their use as a model system for studying the physiology and genetic architecture of both reproduction and senescence. To test the hypothesis that female senescence has transgenerational effects on offspring viability and development, we measured the effects of maternal age on offspring survival over two generations and under two larval densities in two laboratory strains of flies (Oregon-R and Canton-S). Transgenerational effects of maternal age influence embryonic viability and embryonic to adult viability in both strains. However, the generation causing the effects, and the magnitude and direction of those effects differed by genotype. The effects of maternal age on embryonic to adult viability when larvae are stressed was also genotype-specific. Maternal effects involve provisioning: older females produced smaller eggs and larger offspring. These results show that maternal age has profound, complex, and multigenerational consequences on several components of offspring fitness and traits. This study contributes to a body of work demonstrating that female age is an important condition affecting phenotypic variation and viability across multiple generations.

1. Introduction

Senescence is an intrinsic, stochastic, and irreversible process resulting in decreased reproductive function, decreased offspring fitness, and increased probability of death with increasing chronological age (Finch, 1990; Finch and Kirkwood, 2000; Grotewiel et al., 2005; Kern et al., 2001; Partridge, 2010). The process of senescence differs both among individuals in a population as well as within individuals over time (Bronikowski and Promislow, 2005; Finch and Kirkwood, 2000; Nussey et al., 2013). For many iteroparous insects, changes in condition as a result of senescence are underway while individuals are still reproducing thus potentially altering reproductive quality and quantity over time. Reproductive senescence has been documented in natural populations of insects from several orders (e.g., Diptera, Hymenoptera, Lepidoptera, Odonata, and Orthoptera) as well as vertebrates (reviewed in Nussey et al., 2013). Increasing awareness of complex age structures in natural populations (Nussey et al., 2013), suggests that there may be a more prevalent and varied impact of parental age on offspring performance and fitness than has been previously appreciated (Roach and Carey, 2014).

Female condition has profound impacts on offspring development and subsequent performance (Mousseau and Fox, 1998) due to female genetic, oocyte cytoplasmic, and gestational effects. Maternal age effects on offspring viability are well documented. The Lansing Effect (after Lansing, 1947), characterized as decreased offspring lifespan as a function of increased maternal age, has been observed in a variety of

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insects including an aphid (*Aphis nerii*, Zehnder et al., 2007), a beetle (*Sitophilus oryzae*, Opit and Throne, 2007), a butterfly (*Pieris brassicae*, Ducatez et al., 2012), and two species of flies (*Drosophila serrata*, Hercus and Hoffmann, 2000; *Drosophila melanogaster*, Priest et al., 2002), but is not ubiquitous (see *Nauphoeta cinerea*, Moore and Harris, 2003). Maternal age effects can also be subtle affecting traits such as; offspring development time and/or body size (*Sancassania berlesei*, Benton et al., 2008; *Pieris brassicae*, Ducatez et al., 2012; *Sitophilus oryzae*, Opit and Throne, 2007; *Hippodamia convergens*, Vargas et al., 2013; *Aphis nerii*, Zehnder et al., 2007) and diapause (reviewed in Mousseau and Dingle, 1991). The effects of maternal senescence on offspring reproductive performance are less consistent; it is negative for some insects (*Drosophila serrata*, Hercus and Hoffmann, 2000; and *Sitophilus oryzae*, Opit and Throne, 2007), and neutral in others (*Nauphoeta cinerea*, Moore and Harris, 2003; *Aphis nerii*, Zehnder et al., 2007) indicating a need for additional examination of this phenomenon. While the overall effects of maternal age on offspring fitness are relatively well documented, the taxonomic breadth of its occurrence is unknown. Furthermore, the nature and underlying developmental mechanisms of these effects are poorly understood. Maternal effects are predicted to be strongest early in development because of the central role of egg quality and maternal support during embryogenesis (Bonduriansky and Head, 2007; Mousseau and Dingle, 1991) and weaken as the zygotic genome becomes active and the juvenile begins interacting with its environment (Azevedo et al., 1997).

While one facet of maternal reproductive senescence is its impact on offspring viability and development, it can also be manifested in subsequent generations affecting grandoffspring viability and vitality (Bonduriansky and Day, 2009). Evidence of these effects is sparse, but has been reported in a fly (*Drosophila serrata*, Hercus and Hoffmann, 2000) and a beetle (*Sitophilus oryzae*, Opit and Throne, 2007). Potential mechanisms underlying these effects include epigenetic states (e.g. genomic imprinting), cytoplasmic and somatic factors (e.g., hormones, nutrients, and mRNAs), and “extra-organismal” environmental factors (e.g. oviposition site) (Bonduriansky and Day, 2009). Maternal effects can influence offspring quality, such as post-embryonic size and development time, in ways that are then transmitted to the subsequent generation (Benton et al., 2008). It is important to document these transgenerational effects for two reasons. First, they may affect the rate and extent of evolutionary change in natural populations by influencing phenotypic variation in ways that do not reflect underlying genotypes (Bonduriansky and Day, 2009; Mousseau and Dingle, 1991). Second, they are important for understanding the impact of female condition on development and its subsequent contributions to complex age demographics in natural populations (Benton et al., 2008; and reviewed in Roach and Carey, 2014).

The pomace fly, *Drosophila melanogaster*, is a valuable model for exploring mechanisms of reproductive senescence and transgenerational effects of maternal age on offspring development and fitness. Increasing female age appears to affect multiple components of reproductive success including mating behavior, facets of ejaculate transfer and female sperm use (Lüpold et al., 2011; Mack et al., 2003), fecundity (Fricke et al., 2013; Waskar et al., 2005; Zhao et al., 2008) and fertility, although the relative contributions of these aspects to overall fitness is less clear (reviewed in Miller et al., 2014). Maternal aging is also associated with decreased offspring embryonic viability (Zhao et al., 2008), embryonic to adult viability (Fricke et al., 2013), and longevity (Priest et al., 2002). These declines may be attributable to decreases in germline and ovariole somatic stem cell number and proliferation, as well as declining oocyte provisioning (Pan et al., 2007; Zhao et al., 2008). While oocytes are protected from accumulating somatic damage with increasing age, they still show signs of decreased molecular homeostasis and increased damage (Fredriksson et al., 2012). Finally, while transgenerational effects of some female conditions have been documented (e.g., immune challenge, Nystrand and Dowling, 2014; larval diet, Valtonen et al., 2012), the isolated role of female senescence on grandoffspring development and viability is not known. Evidence of cumulative, negative transgenerational effects of female age documented in a related species, *Drosophila serrata* (Hercus and Hoffmann, 2000), indicates they may also exist for *D. melanogaster*.

In this study, we investigated transgenerational maternal and grandmaternal age effects on offspring viability and development. First, using old and young females over two generations, we examined the presence and nature of transgenerational (P_0 and F_1) maternal age effects on offspring viability and development. Because maternal age effects appear to “cross” the soma-gamete barrier and have been documented in other insects (Hercus and Hoffmann, 2000; Opit and Throne, 2007), we predicted there would be transgenerational effects of increasing maternal age on offspring and grandoffspring. We also compared the relative impacts of maternal age on embryonic to adult viability and embryonic viability only in two common lab strains. Given the importance of maternal provisioning on embryonic development (Mousseau and Dingle, 1991) and the failure of protein homeostatic mechanisms in oocytes of aging females (Fredriksson et al., 2012), we predicted a larger effect of maternal age on embryonic viability than post-embryonic viability. Second, we measured the effect of larval density on embryonic to adult viability, because condition-dependent effects can be manifested differently in sub-optimal environments. We predicted that maternal age effects would increase offspring sensitivity to larval stress from crowding. Third, we quantified maternal age-related effects on oocyte provisioning and its relationship with offspring survival. With decreased provisioning by older females (Zhao et al., 2008), we predicted smaller egg sizes as a consequence of increasing maternal age. Exploring multigenerational consequences of female reproductive senescence is valuable for understanding life history evolution, testing predictions of evolutionary theories of senescence, and informing management decisions for natural and captive populations (Roach and Carey, 2014).

2. Materials and methods

2.1. Flies

Wild-type pomace flies, *Drosophila melanogaster* (Meigen) from Oregon-R and Canton-S strains (hereafter OR and CS, respectively) were maintained on Lewis medium (Lewis, 1960) at 25 °C and a 12 h : 12 h light : dark cycle. Both cultures have been maintained in this lab for at least five years under similar culture conditions. New cultures were initiated at two-week intervals, with 40–80 males and females that were 1–5 days post-eclosion (hereafter d pe), in pint bottles containing 75 mL of Lewis medium. Experimental females were collected within 6 h of eclosion to ensure virginity and stored in groups of 10 females on 5–6 ml of Lewis medium with a small amount (~10 grains) of live yeast. They were transferred without anesthesia to fresh medium weekly. Dead females were counted and replaced with same-aged flies to maintain a consistent density of virgins in vials until they reached a pre-determined age.

In order to identify appropriate maternal ages to detect potential effects on both embryonic viability and embryonic to adult viability, before maternal death accelerated, under our laboratory conditions, four OR female age cohorts were examined: 5 d pe, 18 d pe, 30–32 d pe, and 43–47 d pe. These cohorts were separated in age by approximately two weeks and were evenly dispersed across the adult ages when female mortality remained low. Subsequent experiments limited the female ages to two cohorts that differed in maternal effects on offspring viability: 2–5 d pe and 30–33 d pe.

To generate experimental flies, source populations of 20 females of a particular age cohort were mass mated with 20 males from the same strain, and ranging in age from 2 d pe to 6 d pe, on 35 mL Lewis medium for 24 h. The 40 flies were transferred *en mass* to bottles containing scored grape (oviposition) plates with a small amount of yeast for ~12 h to oviposit. Eggs were collected from the oviposition

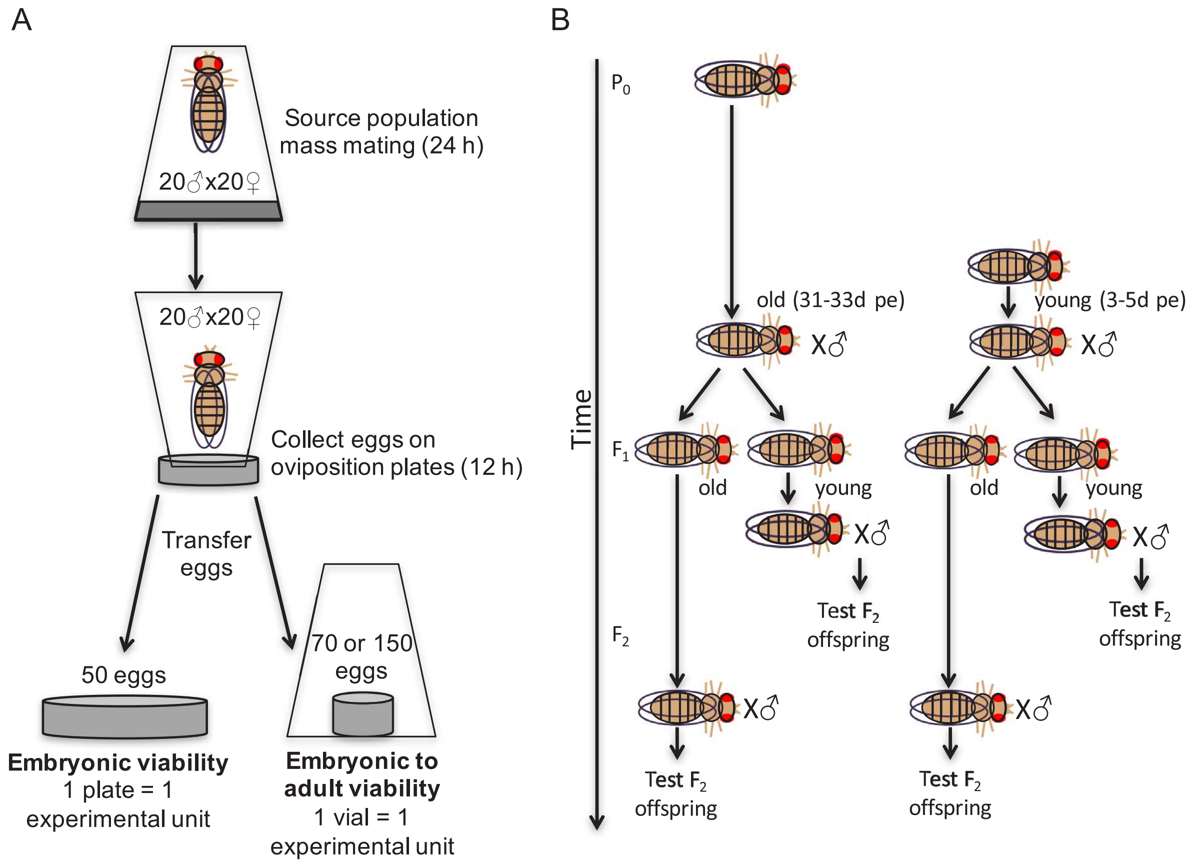

Fig. 1. Experimental design showing: (A) process of collecting eggs and offspring for use in embryonic viability, embryonic to adult viability, and offspring phenotype analysis; and (B) generation and temporal sequence of experimental transgenerational populations.

plates and transferred to either grape plates, to examine embryonic viability, or vials of Lewis medium, to examine embryonic to adult viability and other offspring traits, as described below (Fig. 1A).

2.2. Transgenerational effects of maternal age

Young and old females (P_0, grandmothers; OR and CS strains) were collected, stored, and aged (as described in the previous section). Source populations were composed of young (3–5 d pe, n = 20) or old (31–33 d pe, n = 20) females each mass-mated to males (2–6 d pe, n = 20) in bottles with 35 mL of Lewis medium for 24 h. Three to 7 source populations were established for experiments. Source population flies were then transferred to oviposition plates (Fig. 1A). Eggs (n = 70) were collected from several plates and transferred to vials containing 6 mL of Lewis medium. From 12 d to 17 d after egg collection, recently-eclosed female offspring (F_1, mothers) of old and young grandmothers were collected and placed, in groups of 10 flies, within vials containing 6 mL of medium. This was done at the same time and under the same environmental conditions (Fig 1B). Vials of F_1 mothers were alternately sorted into two groups; old mothers and young mothers (Fig 1B). At 2 d to 5 d pe, groups of 30–40 young mothers (F_1) (pooled from 3 or 4 vials of virgin females) of old or young grandmothers (P_0) were combined in each of 3–5 source bottles and mass mated with males of the same strain. Eggs were collected at the same time and culture conditions. After an additional 27 d to 28 d, groups of 30–40 old mothers (F_1, 29–33 d pe), from young or old grandmothers (P_0) were pooled from 3 or 4 vials of virgin females and mass-mated with males in each of 2–7 source bottles. Eggs were collected on oviposition plates (Fig. 1B). For both young and old mothers, collected eggs were used in assays for embryonic viability and embryonic to adult viability. Males were 2–6 d pe when used and were generated from distinct cultures to avoid sibling matings. To measure egg and offspring size, eggs from OR females were collected from a single source population from each of the four maternal age combinations and either measured (egg size) or transferred in groups of 70 eggs to medium to develop into adults (adult size) (Fig. 1A and described in following sections).

To characterize the aging population, we tracked deaths and fertility for a group of females from each genotype in parallel. At 1–4 d pe, 100% of OR females and 87% of the CS females were alive (n = 82 and 116 living virgins at 6 h pe, respectively). By 30 d–33 d pe, 81.7% of OR females and 76.7% of CS females remained alive. These females were mass mated as described previously. After mating, 50 females from each genotype were selected haphazardly and aspirated into individual vials with 6 mL of Lewis medium with live yeast. After 42 h, we determined female fertility by observing vials for the presence of larvae. If larvae were absent, we dissected the female and examined her seminal receptacle and spermathecae for the presence of sperm to confirm mating had occurred. Of the 47 surviving females in each genotype, 66.0% (n = 31) and 87.2% (n = 41) of the OR and CS females, respectively, showed evidence of having mated (produced embryos that hatched and/or stored sperm).

2.3. Offspring viability

Offspring viability, measured as the proportion of offspring surviving to a given developmental stage, was estimated for two overlapping developmental episodes: embryonic only and embryonic to adult (Fig. 1A). Embryonic viability, measured as the proportion of eggs hatching, was quantified by carefully transferring 50 OR or CS eggs from source plates to the surface of a fresh, un-scored grape plate (Fig.1A). Thirty-six hours later, we counted the number of unhatched eggs using a dissecting scope at 20× magnification. Embryonic

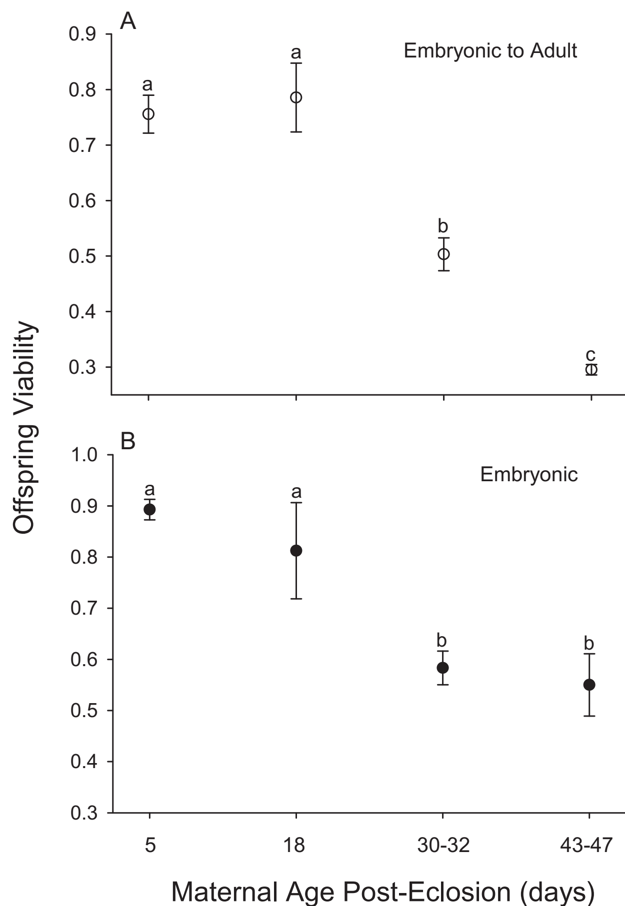

Fig. 2. Effect of maternal age on offspring viability, measured as the proportion of surviving offspring across two developmental stages in Oregon-R flies: (A) embryonic to adult viability by mothers 5 d (n = 10 groups of 70 eggs), 18 d (n = 6 groups), 30–32 d (n = 9 groups), and 43–47 d (n = 3 groups) post-eclosion; and (B) embryonic viability by mothers 5 d (n = 7 groups of 50 eggs), 18 d (n = 4 groups), 30–32 d (n = 6 groups), and 43–47 d (n = 5 groups) post-eclosion. Symbols represent group means (± 1 SEM). Different lower case letters above the error bars identify statistically significant differences ($p \leq 0.05$) among groups.

viability was calculated as: (50 – # unhatched eggs)/50. Each group of 50 eggs was an experimental unit (e.g. n = 60 represents 60 groups of 50 eggs). Embryonic to adult viability was measured by transferring either 70 or 150 eggs (low and moderate densities, respectively) from source plates to the lightly scored surfaces of vials containing 6 mL of Lewis medium (Fig. 1A). Emerging adults were collected from the cultures and counted every 12 h from the first day of eclosion up to 17 d post-transfer. Embryo to adult viability was calculated as the proportion of eggs developing to adults: # adults/# eggs. Each group of 70 or 150 eggs was an experimental unit (e.g. n = 30 represents 30 groups of either 70 or 150 eggs). To decrease potential effects of individual source populations, experimental plate and vial units were composed of eggs combined from several oviposition (i.e. source) plates.

2.4. Egg & offspring size

To measure egg size, OR eggs were gently washed off of the surface of grape plates with egg wash solution (Sullivan et al., 2000), transferred to a hemocytometer, and their images captured at 4× magnification. Egg surface areas were measured using the freeform polygon tool in the Motic system (Motic Image Plus 2.0ML, 2007, Richmond, British Columbia, Canada). The calculated volume of oocytes with this method of measurement (mean volumes ranged from 7.47×10^{-3} mm^3 to 10.93×10^{-3} mm^3) was similar to measurements from other published studies (Azevedo et al., 1996, 1997; Markow et al., 2009). The relative amount of variation (i.e. coefficient of variation, CV) among 22 eggs was 9.7× higher than the mean relative variation from repeated measurement (n = 5) of 10 eggs (mean CV = 0.45%). Therefore, estimated variation in egg size was largely attributable to differences among eggs rather than experimenter measurement error. To estimate adult offspring size in OR, the Motic Image Plus 2.0 ML line tool was used to measure thorax length from the anterior edge of the scutum to the posterior tip of the scutellum (at 2.0× magnification) for approximately 10 female and 10 male offspring per maternal and grandmaternal age group.

2.5. Analyses

The effects of maternal ages (n = 4 ages) on embryonic viability and embryonic to adult viability in OR flies were examined using analysis of variance (ANOVA). Tukey HSD *post hoc* tests were performed to determine statistically significant changes in offspring viability among pairs of maternal age cohorts. Effects of maternal and grandmaternal age on embryonic viability were explored with a linear mixed-model ANOVA with maternal and grandmaternal ages as fixed factors and experimental day (for OR and CS, n = 2 and 7 days, respectively) as a random factor. Day was designated a random factor, because the day eggs were collected was not of interest in itself for examining the possible existence of transgenerational maternal effects, and it enabled us to statistically control for experimental day as a potential source of variation in the results (Searle et al., 1992). Effects of maternal age, grandmaternal age, and larval density on embryonic to adult viability were explored with a linear mixed-model ANOVA with maternal age, grandmaternal age, and larval density as fixed factors and experimental day (for OR and CS, n = 3 days) as a random factor. Effects of maternal and grandmaternal age on egg size (surface area) were examined with a two-way ANOVA. Finally, effects of maternal age, grandmaternal age, and offspring sex on offspring size, measured as thorax length, were examined with a three-way ANOVA. We tested interactions up to the second order and used *post hoc* tests, with Sidak adjustment, to identify significant pair-wise differences. Assumptions of statistical tests were examined and generally satisfied. However, the assumption of homogeneity of variances was violated with egg surface area. In this instance, Mann-Whitney tests were also performed to test the effect of maternal or grandmaternal age on the variable of interest. Because the results were consistent with the parametric tests, the parametric test statistic is reported. Means and standard errors of the mean (SEM) are presented in all figures. Statistical analyses were performed using SPSS Statistics version 22.0 (IBM, Armonk, NY, USA).

3. Results

3.1. Maternal age effects on embryonic viability and embryonic to adult viability

Increasing female age had multiple negative effects on offspring viability. As OR females aged, embryonic to adult viability, measured as the proportion of eggs developing into adults, decreased (Fig. 2A; $F_{3,24} = 22.10$, $p < 0.0005$). The deleterious effect of maternal age on embryonic to adult viability became apparent after 18 d pe (Tukey HSD *post hoc* test; mean difference between 5 d and 18 d pe = 0.033, p = 0.798) with a 36.0% decrease between 18 d pe and 30–32 d pe (mean difference = 2.49, $p < 0.0005$) followed by an additional 41.3% decrease by 43–47 d pe (mean difference = 0.175, p = 0.002). Increasing maternal age also negatively affected embryonic viability, measured as the proportion off eggs hatching (Fig. 2B; $F_{3,11} = 17.91$, $p < 0.0005$) with a significant decline (28.2%) between 18 d and 30 d pe (Tukey HSD *post hoc* test; mean difference = −0.229, p = 0.009), but not between 5 d and 18 d pe (mean difference = −0.084, p = 0.498) or 30–32 d and 43–47 d pe (mean difference = −0.0333, p = 0.923).

Table 1
results from mixed-model ANOVAs of embryonic to adult viability, measured as the proportion of eggs developing into adults, as a function of maternal and grandmaternal age in low and moderate density larval environments.

	Oregon-R (OR)			Canton-S (CS)		
	df (n, d)	*F*	*P*	*df (n, d)*	F	P
Maternal	1, 69.97	106.34	**< 0.0005**	1, 110.07	551.24	**< 0.0005**
Grandmaternal	1, 67.93	12.86	**0.001**	1, 110.07	25.30	**< 0.0005**
Density	1, 68.53	4.27	**0.043**	1, 110.07	24.87	**< 0.0005**
Maternal × Grandmaternal	1, 68.38	4.68	**0.034**	1, 110.07	1.38	0.243
Maternal × Density	1, 68.46	< 0.0005	0.997	1, 110.07	6.89	**0.010**
Grandmaternal × Density	1, 68.21	0.36	0.552	1, 110.07	6.13	**0.015**
Maternal × Grandmaternal × Density	1, 68.21	0.49	0.486	1, 110.07	1.25	0.267

P < 0.05 are presented in bold. df refers to degrees of freedom: n is the numerator df and d is the denominator df.

Effects of maternal age on post-embryonic viability were estimated by comparing embryonic to adult viability with embryonic viability (i.e. embryonic to adult viability – embryonic viability = estimate of post-embryonic viability). In the initial characterization of maternal age effects in OR, embryonic to adult viability declined significantly between 30–32 and 43–47 d pe, but embryonic viability did not (Fig. 2).

3.2. Transgenerational maternal age effects

3.2.1. Embryonic to adult viability

Maternal age affected offspring fitness and performance across two generations in ways that differed by genotype. In OR, maternal and grandmaternal ages interacted to influence embryonic to adult (F_2) viability (measured as the proportion of eggs developing into adults). Within old mothers, embryonic to adult viability was significantly (26.0%) higher for offspring of old grandmothers than young grandmothers (mean diff. = −0.120, p < 0.0005), but no difference existed within young OR mothers (mean diff. = −0.029, p = 0.323) (Table 1; Fig. 3A). However, old mothers (F_1) had 37.0% lower embryonic to adult viability than young mothers (Table 1; Fig. 3A), a difference larger in magnitude and opposite in direction to the grandmaternal effect. The negative effect of moderate larval density on embryonic to adult viability was statistically significant, yet small (effect size, r = 0.094). While this warrants further exploration, we limit discussion of it here.

In CS, both maternal and grandmaternal age influenced offspring response to larval stress, but did not interact with each other to influence embryonic to adult viability (Table 1). Moderate larval densities had a significant negative effect on embryonic to adult viability for young mothers (15.7% lower viability, mean diff. = 0.122, p < 0.0005), but not old mothers (mean diff. = 0.038, p = 0.098). Additionally, embryonic to adult viability was 56.2% lower for old mothers than young mothers (Table 1; Fig. 3B). Grandmaternal (P_0) age effects on offspring viability differed from those on maternal age effects: among old grandmothers, embryonic to adult viability in moderate larval density conditions was significantly lower (22.0%) than under low larval density conditions (mean diff. = 0.120, p < 0.0005), but not when young (mean diff. = 0.040, p = 0.079; Table 1; Fig. 3C). This effect was not detected for young grandmothers.

3.2.2. Embryonic viability

Embryonic viability, measured as the proportion of F_2 eggs hatching, decreased 14.7% and 64.0% for older mothers (F_1) in OR and CS (Fig. 4A and B, respectively; Table 2). In OR, embryonic viability was 5.5% lower for old grandmothers than young grandmothers. However, no grandmaternal effects was detected in CS (Table 2; Fig. 4A and B). There were no statistically significant interactions between maternal and grandmaternal age in OR or CS (Table 2).

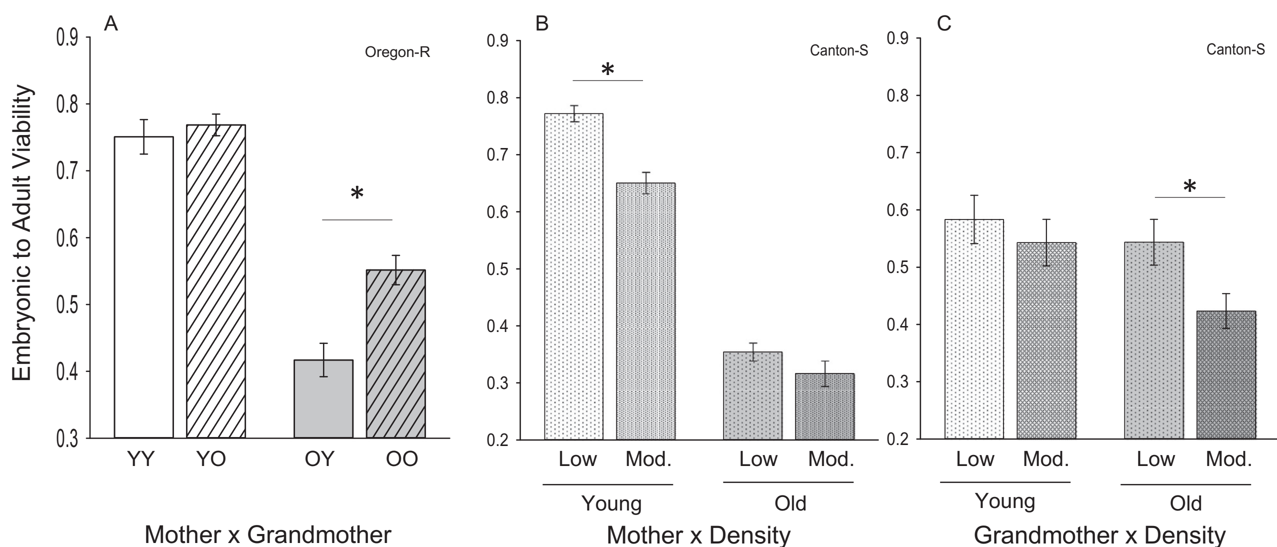

Fig. 3. Embryonic to adult viability, measured as the proportion of eggs developing into adults, as a function of: (A) maternal and grandmaternal age in Oregon-R flies (n = 20 groups of 70 or 150 eggs from **y**oung mothers of **y**oung grandmothers; n = 20 groups from **y**oung mothers of **o**ld grandmothers; n = 19 groups from **o**ld mothers of **y**oung grandmothers; and n = 19 groups from **o**ld mothers of **o**ld grandmothers); (B) maternal age under two larval density conditions in Canton-S flies (n = 30 groups of 70 or 150 eggs for each maternal x density group); and (C) grandmaternal age under two larval density conditions in Canton-S flies (n = 30 groups of 70 or 150 eggs for each grandmaternal x density group). Bars represent group means (± 1 SEM). Asterisks denote a statistically significant difference between groups.

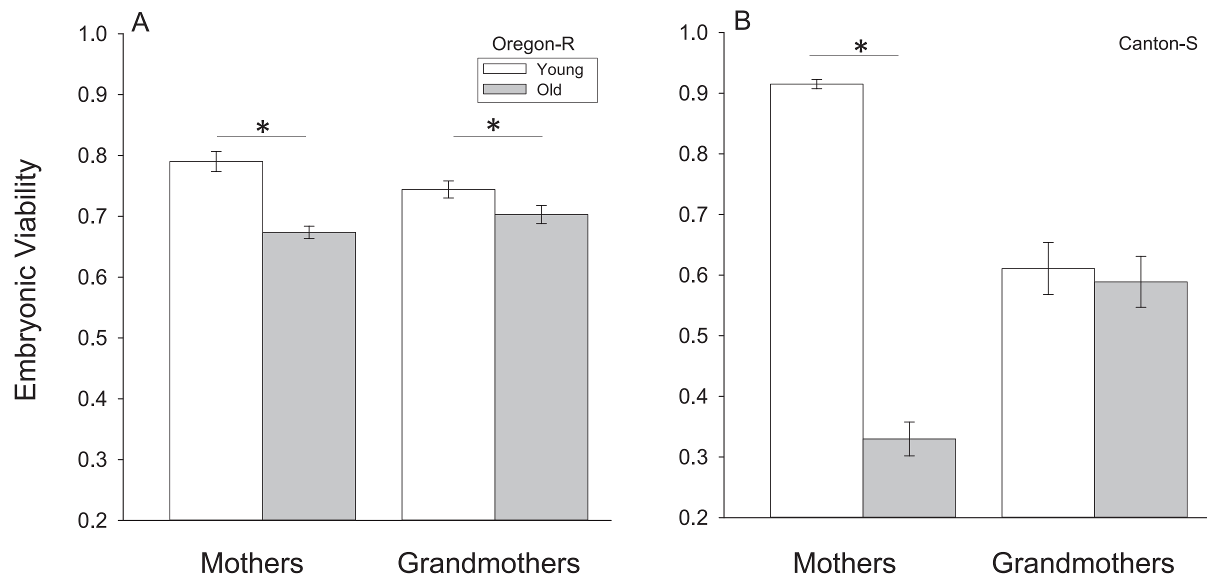

Fig. 4. Effect of maternal and grandmaternal age on embryonic viability, measured as the proportion of eggs hatching, in (A) Oregon-R (n = 60 groups of 50 eggs from young mothers, n = 80 groups from old mothers, n = 70 groups from young grandmothers, and n = 70 groups from old grandmothers), and (B) Canton-S (n = 60 groups of 50 eggs from young mothers, n = 70 groups from old mothers, n = 65 groups from young grandmothers, and n = 65 groups from old grandmothers) flies. Bars represent group means (± 1 SEM). Asterisks denote a statistically significant differences between young and old females.

3.2.3. Offspring investment & development

Older OR females (F_1) laid eggs with a surface area that was 31.0% smaller (Fig. 5, $F_{1,410}$ = 6247.35, p < 0.0005), but only slightly more variable in size ($CV_{old\ mother}$ = 4.70%; $CV_{young\ mother}$ = 4.62%) than those from young females. Eggs (F_2) from old grandmothers (P_0) were neither smaller (Fig. 5, $F_{1,410}$ = 0.322, p = 0.571) nor more variable in size ($CV_{old\ grandmother}$ = 18.54%; $CV_{young\ grandmother}$ = 19.42%) than those from young grandmothers. Body size, measured as thorax length, of adult offspring (F_2) differed by sex and maternal age. Thoraces of female offspring were 15.3% larger than those of male offspring (Table 3, Fig. 6). The thorax length of offspring of old mothers (F_1) was slightly (3.1%), yet significantly, larger than of young mothers (Table 3, Fig. 6). Grandmaternal (P_0) age did not have an effect on F_2 offspring thorax length (Table 3, Fig. 6) and there were no interactions among offspring sex, maternal age, and grandmaternal age.

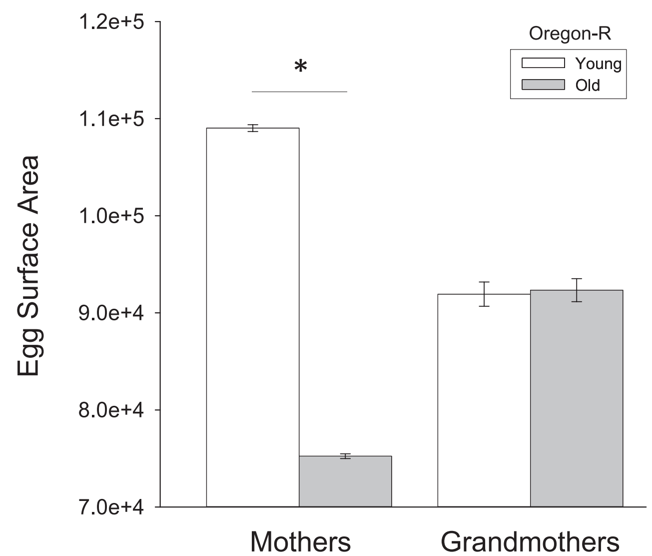

Fig. 5. Effect of grandmaternal and maternal age on (grand-)offspring egg size in Oregon-R. Bars represent group means (± 1 SEM). Sample sizes reflect the number of eggs measured with n = 207 for young mothers, n = 207 for old mothers, n = 205 for young grandmothers, and n = 209 for old grandmothers. Asterisks denote a statistically significant difference between young and old females within a generation.

4. Discussion

Reproductive senescence consists of a decline in reproductive function with increasing age. This decline affects multiple facets of reproductive function at different times and rates among individuals in a population. In this study, we show that both maternal and grandmaternal age affects offspring (F_2) viability in two different strains of *Drosophila melanogaster*. Furthermore, we provide evidence that these effects influence offspring viability at different developmental stages and in distinct ways. These results have valuable implications for understanding sources of phenotypic variation and opportunities for selection in both natural and laboratory populations.

We show that maternal age affects offspring fitness for at least two generations. Maternal (F_1) age had a substantial, negative impact on embryonic to adult viability (see also Fricke et al., 2013). Grand-

Table 2
results from mixed-model ANOVAs of embryonic viability, measured as the proportion of eggs hatching as a function of maternal and grandmaternal age.

	Oregon-R (OR)			Canton-S (CS)		
	df (n, d)	*F*	*P*	*df (n, d)*	*F*	*P*
Maternal	1, 135.03	46.77	< **0.0005**	1, 120.96	709.83	< **0.0005**
Grandmaternal	1, 135.00	6.47	**0.012**	1, 119.79	0.81	0.370
Maternal × Grandmaternal	1, 135.00	0.043	0.835	1, 119.79	0.568	0.453

P < 0.05 are presented in bold. df refers to degrees of freedom: n is the numerator df and d is the denominator df.

Table 3
results from an ANOVA of Oregon-R offspring adult size as a function of maternal and grandmaternal age.

	Oregon-R (OR)		
	df (n, d)	*F*	*P*
Maternal	1, 74	20.62	**< 0.0005**
Grandmaternal	1, 74	0.953	0.332
Sex	1, 74	579.05	**< 0.0005**
Maternal × Grandmaternal	1, 74	0.311	0.579
Maternal × Sex	1, 74	2.571	0.113
Grandmaternal × Sex	1, 74	1.04	0.311
Maternal × Grandmaternal × Sex	1, 74	2.68	0.106

$P < 0.05$ are presented in bold. df refers to degrees of freedom: n is the numerator df and d is the denominator df.

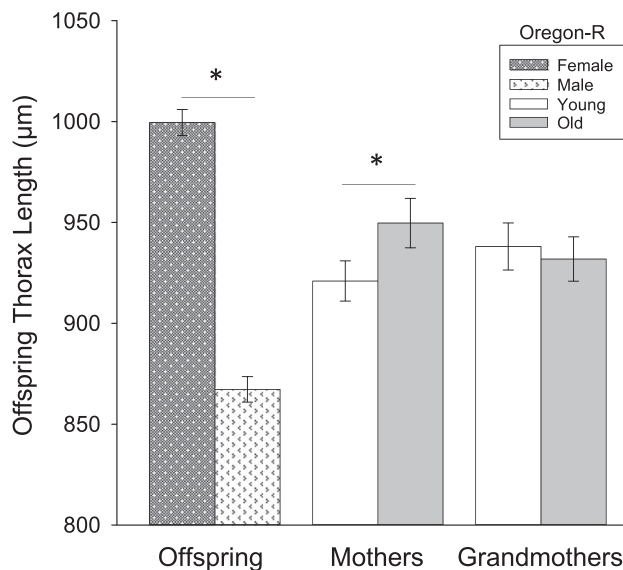

Fig. 6. Effect of offspring sex, maternal age, and grandmaternal age on offspring thorax length in Oregon-R. Bars represent group means (± 1 SEM). Sample sizes refer to the number of offspring measured; n = 42 female offspring, n = 40 male offspring, n = 42 offspring of young mothers, n = 40 offspring of old mothers, n = 41 offspring of young grandmothers, and n = 41 offspring of old grandmothers. Asterisks denote a statistically significant difference between groups within maternal age, grandmaternal age, or density.

maternal (P_0) age effects were also observed, although they were smaller in magnitude than maternal effects and differed in their nature. The difference between maternal and grandmaternal age effects shows that maternal (F_1) youth can partially recover the negative effects of grandmaternal (P_0) age on embryonic to adult viability. Changes in maternal resource allocation to eggs appears to contribute to this recovery. In the present study, this was reflected in egg size: eggs laid by younger females were larger than those laid by older females and egg size corresponded positively with subsequent embryonic to adult viability. However, since young mothers could not entirely compensate for the effects of older grandmothers on embryonic to adult viability, carry-over effects in the form of age-related germline mutations, epigenetic modifications, or oocyte-transmitted factors may have contributed to the observed transgenerational (grandmaternal) effects. Of these potential carry-over effects, our examination of egg size did not detect any quantitative changes in grandmaternal oocyte-transmitted factors. However, other studies indicate that maternally (F_1) – transmitted factors affecting egg quality, such as increased protein carbonylation (Fredriksson et al., 2012) and/or higher levels of mitochondrial DNA anomalies (Kann et al., 1998) can exist. Additionally, quantitative changes in factors not affecting egg size (e.g., proteins, hormones) could contribute to grandmaternal age effects. While our study addressed one facet of egg quality, a more complete examination among these potential causes would provide very useful information about the nature and underlying mechanisms of transgenerational grandmaternal age effects.

Maternal age effects could be influenced by the characteristics of a founding population. Trade-offs between early life reproduction and later-life longevity and/or fertility are well-documented and known to vary between populations (Chapman et al., 1995; Flatt, 2011; Hamilton, 1966; Medawar, 1952; Partridge et al., 1986; Stearns, 1989; Williams, 1957; reviewed in Miller et al., 2014). Furthermore, they are sensitive to culturing practices that affect the age structure of the parental population (Linnen et al., 2001; Promislow and Tatar, 1998; Sgro and Partridge, 2000). In our study, the laboratory conditions from which experimental flies were generated consist of populations initiated by young (1–5 d pe) founders every two weeks. These conditions select for early reproducing flies, potentially at the expense of later reproductive output and/or lifespan, and does not select against mutations with deleterious effects later in life. While trade-offs involving fecundity would not be detected in our design, potential trade-offs involving egg quality or fertility could affect our results by elevating early reproductive success at the expense of later reproductive success. Additionally, differential survival could result in an aged maternal cohort that is not representative of the young maternal population. In this case, maternal age effects could be confounded with effects from the unrepresentative aged maternal cohort. In our study, observed maternal age effects on embryonic viability do not appear to be due to the disproportionate effects of a small cohort of the original population: in addition to low female mortality with increasing female age, at least 2/3 of the females surviving to 30 d pe were fertile. To better understand how maternal age effects are manifested in natural populations, it will be useful to compare these results with those from experiments examining transgenerational effects in populations that have been maintained with a complex age demography for many generations or populations that have been recently collected from the wild.

We show that transgenerational maternal age effects are likely a general feature of laboratory-raised populations of *D. melanogaster*. However, the nature of the transgenerational effects differed between the two strains: they were larger in CS than in OR, and they were cumulative across two generations in CS, but not in OR. The very large decline in embryonic to adult viability in offspring of older CS females reflects heightened offspring sensitivity to increasing maternal age and could be part of a trade-off with higher early maternal fertility. Supporting this hypothesis, female fertility is higher for CS than OR when both young (McGraw et al., 2009). Intriguingly, within old OR mothers, offspring of older grandmothers inherited a modest advantage in terms of increased embryonic to adult viability relative to those of young grandmothers. This effect is consistent with a role for epigenetic modification and/or transmitted factors affecting oocyte quality from older grandmothers. While germ-line mutations could explain the cumulative negative effect of maternal age on embryonic to adult viability in CS, it seems less likely to account for the beneficial age effect in OR. The different strain responses we observed are likely due to genetic differences between the populations, because: 1) the populations have different origins, 2) CS and OR flies differ in several reproductive characteristics (e.g., fertility, hatchability, sperm storage; McGraw et al., 2009), and 3) strain-level genetic differences underlie maternal age effects on other aspects of offspring fitness (e.g. longevity, Priest et al., 2002). While the two strains in this study were not tested at the same time, it is less likely that environmental conditions via culturing (i.e. medium) or experimental methods contributed to observed differences. Populations of both strains have been cultured in parallel for at least 130 generations. Before and during the experiments, cultures were maintained in a growth chamber and during the experiments, medium was made on an almost daily basis making substantial differences between experimental episodes unlikely. Given

the strain-level differences in genotype and phenotype documented in other studies, we hypothesize that grandmaternal epigenetic modifications that are responsive to maternal condition have a role for the strain-specific responses observed in our study.

Maternal reproductive senescence affects more than one component of offspring fitness, but is particularly important during embryogenesis. We quantified embryonic to adult viability and embryonic viability independently, then estimated maternal age effects on post-embryonic viability by comparing the two measurements. As females from both strains aged, embryonic viability was a substantial, but not the sole, contributor to offspring survival to adulthood, and supports our original prediction that maternal age would have a larger effect on embryonic viability than post-embryonic viability. This effect was most clearly observed in the OR maternal age cohort study in which a distinct effect of maternal age on embryonic to adult viability was detected at 43-47d pe. A negative effect of increasing maternal age appeared to be larger on embryonic viability than post-embryonic viability by 18d pe. Consistent with this, in our experiments exploring maternal and grandmaternal age effects, maternal age had a significant negative effect on embryonic viability in both strains that continued to adulthood in OR, but not CS. Estimated grandmaternal age effects on post-embryonic viability may also exist, but their magnitude is difficult to ascertain due to interactions with maternal age (OR) and larval density (CS and OR).

Consistent with its effects on offspring viability at embryonic and post-embryonic stages OR maternal age affects offspring size at two distinct developmental stages. We show that older mothers produced smaller eggs with lower (embryonic) viability. This result is similar to previous work on maternal age effects on egg size (*D. melanogaster*, Azevedo et al., 1997; reviewed in Fox and Dingle, 1994) and reflects reduced maternal provisioning (reviewed in Bonduriansky and Day, 2009; Mousseau and Dingle, 1991). The cause of this declining ability to provision eggs is unknown, but could be due to decreased ingestion of nutrients, decreased ability to direct ingested nutrients (or their products) to eggs, and/or a redirection of resources from gametic investment to somatic maintenance (Bonduriansky and Day, 2009; reviewed in Youngson and Whitelaw, 2008). Consistent with a role for decreased ingestion/direction of nutrients, Vijendravarma et al. (2010) showed that females raised on a low-nutrient diet produced smaller-sized eggs. Additionally, as adult females age, mating-induced increases in ingestion may augment resources to provision eggs. Because old females in this study remained virgins until later in life, they may have not accessed additional nutrients resulting in smaller eggs.

In addition to affecting egg size, we found that older mothers also had slightly, but significantly, larger adult offspring. While female offspring were larger than male offspring, there is no evidence that maternal age had a disproportionate effect on one sex relative to the other. Increased adult size as a result of increasing maternal age has been observed in a housefly (*Musca domestica*, McIntyre and Gooding, 2000) and a milkweed bug (*Oncopeltus fasciatus*, Phelan and Frumhoff, 1991), but see (Mousseau and Dingle, 1991). In our study, decreased embryonic viability from old OR mothers resulted in less larval crowding. This may have resulted in prolonged medium quality (more nutrients and slowed fouling) thereby favoring greater larval growth relative to environments populated by offspring of young females. The negative relationship between egg size and resulting adult size observed in our study may be indirect, because the egg is primarily a product of the female genotype (Azevedo et al., 1997) and condition (Parsons, 1962; Vijendravarma et al., 2010; present study), while adult size (weight) is determined by zygotic factors (Azevedo et al., 1997), environmental factors (Lefranc and Bundgaard, 2000; Morimoto et al., 2016) and potentially their interactions. Additionally, variation in egg size is not correlated with larval competitive ability or adult size (weight; Azevedo et al., 1997; Parsons, 1962). Therefore, the primary influences on egg and adult sizes appear to involve different physiological mechanisms. While other studies have shown that larger adult sizes offer a benefit in terms of higher reproductive success (Lefranc and Bundgaard, 2000; Morimoto et al., 2016), it seems unlikely that this fitness benefit could compensate for the aging female's decreased fertility.

In addition to the direct effects of maternal reproductive senescence on offspring viability and size, under some circumstances, maternal senescence can influence offspring sensitivity to environmental stress. We separated parental perception of the environment from larval experience by altering the larval density post-ovulation. We found that larval crowding presents a challenge to developing larvae that is sufficient to compound maternal age effects in CS and possibly reduce their survival in OR. Among CS females, young, but not old, mothers produced offspring with lower embryonic to adult viability at higher larval densities. Embryonic viability for old mothers was already so low that hatching larvae probably did not experience the intended higher density. This density effect for young mothers could be mediated through maternal effects on early larval size, development time, or feeding behavior thereby affecting offspring survival through late larval and pupal stages. The response to grandmaternal age was different: larval density affected embryonic to adult viability when grandmothers were old, but not when they were young. Here, increased grandmaternal age appears to further compromise the larvae's ability to survive in a more crowded environment and supports a largely post-embryonic effect of grandmaternal age on offspring performance. This interaction between larval density and both maternal and grandmaternal age was not observed in OR indicating that maternal and grandmaternal age effects likely influence offspring viability in ways other than by competitive ability. Together, our results do not support our prediction that increasing maternal age would increase larval sensitivity to crowding, but instead shows that grandmaternal age can influence larval responses to crowding (see also Bonduriansky and Head, 2007; Hercus and Hoffmann, 2000; Vijendravarma et al., 2010). Observed strain sensitivities may be mediated by strain-specific differences in female fertility and/or maternal effects that translate into different larval densities in routine culture. Maternal age effects may also have different impacts under even higher levels of larval stress (e.g. density) than the moderate levels used here as well as other types of environmental stress (e.g. pathogens, diet, and temperature) and deserves further study.

While observed changes in offspring viability and size are attributed to increasing female age, this design does not exclude a role for differential male investment affecting female reproductive outcomes. *Drosophila* males alter quantities of some ejaculate components (e.g. sperm, Lüpold et al., 2011; select seminal fluid proteins, Sirot et al., 2011; Wigby et al., 2016) in response to female condition (age, mating status, and size respectively) in ways that can affect female fertility, fecundity, sexual receptivity, and behavior. Alternatively, changes in female responses to male seminal fluid proteins may affect reproductive outcomes. For example, female responses to the receipt of the male seminal fluid protein Sex Peptide (measured as egg laying) decreased with increasing female age (Fricke et al., 2013). Failure of females to appropriately provision oocytes or increase the rate of oogenesis in response to male-derived signals are consistent with our observed results. Within the male age range we used (2 d to 6 d pe), male mating and fertilization success increase with age (Hollis et al., 2016; Long et al., 1980). This could contribute to variability in female reproductive output across all of the female age treatment groups. Whether or not male age effects over this range interact with female age is unknown. Further experiments will be needed to resolve potential male-contributed influences or direct female responses to male-derived signals on the observed patterns.

In conclusion, these experiments provide empirical support that reproductive senescence is distinct from increasing chronological age and emerges both independently and distinctly among multiple traits. Embryonic viability and embryonic to adult viability decreased discontinuously and distinctly with chronological age, reflecting senes-

cence's multi-faceted nature. Maternal senescence had multiple effects on progeny viability, tolerance for stress, and size that were observed to carry-over for at least two generations. These effects appeared to be mediated, in part, by maternal provisioning. They were documented in two different genetic strains reflecting that the general phenomenon of transgenerational maternal effects is common in laboratory strains of *D. melanogaster*. Different responses between strains also indicate that genetic differences may modulate responses to maternal condition and environmental stress. These experiments highlight the value of more completely: 1) characterizing reproductive senescence in a diverse array of insects, 2) exploring how female, and male, reproductive senescence affects population demography in laboratory and natural populations, and 3) documenting population/strain-level genotype sources of variation in physiology and viability.

Author contributions

Developed concepts: MBQ.
Performed experiments: MBQ, PBM, PMP, MHP, JLT, and CLM.
Data analysis: MBQ and PBM.
Manuscript preparation and editing: MBQ, PBM, MHP.

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