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Advanced maternal age has negative multigenerational impacts during *Drosophila melanogaster* embryogenesis

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ABSTRACT

Increasing maternal age is commonly accompanied by decreased fitness in offspring. In *Drosophila melanogaster*, maternal senescence negatively affects multiple facets of offspring phenotype and fitness. These maternal effects are particularly large on embryonic viability. Identifying which embryonic stages are disrupted can indicate mechanisms of maternal effect senescence. Some maternal effects can also carry-over to subsequent generations. We examined potential multi- and transgenerational effects maternal senescence on embryonic development in two laboratory strains of *D. melanogaster*. We categorized the developmental stages of embryos from every combination of old and young mother, grandmother and great grandmother. We then modelled embryonic survival across the stages and compared these models among the multigenerational maternal age groups in order to identify which developmental processes were most sensitive to the effects of maternal effect senescence. Maternal effect senescence has negative multigenerational effects on multiple embryonic stages, indicating that maternal provisioning and, possibly epigenetics, but not mutation accumulation, contribute to decreased offspring survival. This study shows the large, early and multi-faceted nature of maternal effects senescence in an insect population.

Introduction

Maternal condition has profound effects on offspring phenotypic variation and fitness. Maternal physiological condition can vary in multiple ways including infection/immune status, levels of stress, nutritional state, and senescence. While maternal senescence can result in a direct decline in her reproductive fitness, it can also alter her offspring's fitness. Maternal effect senescence describes changes in maternal effects with advancing maternal age (Moorad and Nussey, 2016) that alters offspring fitness and traits associated with fitness such as body size and development time (Lansing, 1947; Fox and Dingle, 1994; Hercus and Hoffman, 2000; Zehnder et al., 2007; Opit and Throne, 2007; Benton et al., 2008; Ducatez et al., 2012; Priest et al., 2002; and reviewed in Nussey et al., 2013; Moorad and Nussey, 2016; Ivimey-Cook and Moorad, 2020). These effects can be particularly acute when offspring are precocious, as is the case for most insects, because there are few opportunities to compensate for poor egg or embryonic provisioning with post-embryonic maternal care (reviewed in Mousseau and Dingle, 1991; Mousseau and Fox, 1998; Bell and Hellman, 2019). Additionally, maternal effect senescence is predicted to have the greatest impact on embryonic stages prior to the maternal-zygotic transition, because of the very high maternal genetic, cellular, and gestational influence on early developmental events (Azevedo et al., 1997; Bonduriansky and Head, 2007; reviewed in Mousseau and Dingle, 1991; Lee et al., 2014; Yuan et al., 2016).[1]

Maternal effect senescence can be multi- and/or transgenerational influencing offspring over two or more generations (Opit and Throne, 2007; Benton et al., 2008). For example, in pomace flies (*Drosophila melanogaster*), offspring of older mothers have lower fertility than those of young mothers (Mossman et al., 2019). These potential generational effects have can be caused by age-related changes in egg (or gestational) provisioning (Fredriksson et al., 2012), oocyte mitochondrial condition (Kann et al., 1998; Erwin and Blumenstiel, 2019), epigenetic activity (Erwin and Blumenstiel, 2019; Yu et al., 2019), and/or genetic

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[1] Abbreviations: A - atypical embryonic development; B - blastoderm; C - cleavage; DPE - days post-eclosion; GE - early gastrulation; GL - late gastrulation; MAG – Maternal Age Group; OR - Odds Ratio; PC - pre cleavage; T - typical embryonic development.

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mutations (Garcia et al., 2010; Brengdahl et al., 2020). Maternal effects on offspring soma and germline can result in multigenerational (maternal and grandmaternal) effects, because offspring germline development occurs early during embryonic development (Mousseau and Dingle 1991) during which maternal influences through oocyte quality are still large. However, only maternal nuclear genetic effects, such as mutations and epigenetic markers, are expected to exert transgenerational (great grandmaternal) effects, because the great grandmother has no direct impact via oocyte or gestational quality (Skinner 2008). Documenting the presence and magnitude of these effects is important for understanding: 1) the impacts of maternal condition on offspring fitness in populations with complex age demographics (Benton et al., 2008; and reviewed in Roach and Carey, 2014); 2) the contribution of phenotypic plasticity to phenotypic variation within populations (Mousseau and Dingle, 1991; Bonduriansky and Day, 2009), and 3) the impact of disrupted homeostasis on subsequent sensitivity to additional environmental stressors (Barrere-Cain and Allard, 2020). Ultimately, this helps us better understand the nature of phenotypic plasticity and how selection may and may not result in evolutionary responses in natural populations.

The pomace fly, *D. melanogaster*, offers a powerful model system to examine potential trans- and multigenerational impacts of maternal effect senescence during offspring embryogenesis. There is extensive study of *D. melanogaster* reproduction (ovulation and sperm storage, Bloch Qazi et al., 2003; sperm storage and sperm precedence, Schnakenberg et al., 2012; oogenesis, McLaughlin and Bratu, 2015; fertilization, Loppin et al., 2015; reproductive tract development, Carmel et al., 2016; spermatogenesis, Fuller 2016; seminal fluid proteins, Wolfner, 2007) and senescence (insulin/Igf-TOR pathway, Partridge et al., 2011; reviewed in Grotewiel et al., 2005; Piper and Partridge, 2018) resulting in an emerging understanding of the effects and mechanisms of reproductive senescence in females (e.g., germline stem cell decline and decreased egg quality; reviewed in Miller et al., 2014) and males (e.g., decreasing sperm quality and declining seminal fluid protein effects; Fricke and Koppik, 2019). Female reproductive senescence is attributed to declining fecundity (Zhao et al., 2008; Fricke et al., 2013) caused by decreasing: germline stem cell number and activity (Pan et al., 2007; Waskar et al., 2009; Ishibashi et al., 2020); egg chamber development (Carlson and Harshman, 1999); and sensitivity to signaling molecules (Fricke et al., 2013) (reviewed in Miller et al., 2014). Older females' eggs are also less likely to hatch than eggs of young females (Zhao et al., 2008; Bloch Qazi et al., 2017). This may be due to higher documented levels of carbonylated proteins (Fredriksson et al., 2012), which reflects decreased homeostasis and a potentially lower quality developmental environment. However, which embryonic stages that are most sensitive to these age-related alterations is currently unknown.

In *D. melanogaster*, extensive maternal provisioning and rapid embryonic development establish favorable conditions for maternal multigenerational (P_0-F_2) and, potentially, transgenerational (P_0-F_3) age effects. While a large negative effect of increasing maternal and grandmaternal age were identified on egg hatchability, effects over specific developmental episodes during embryogenesis were not explored (Bloch Qazi et al., 2017). Well-established descriptions of embryogenesis as well as methods to collect, fix, and visualize embryogenesis allows a more precise examination of embryonic development than has previously been conducted.

In this study, we extend our previous examination of multigenerational maternal reproductive senescence by examining at what embryonic stages maternal effect senescence has the largest impact(s). We predict that if maternal (F_2) senescence affects oocyte provisioning, then early developmental stages preceding gastrulation will be impacted the most because maternal genetic and provisional influences are particularly large (Table 1; Lee et al., 2014; Yuan et al., 2016). We also extended the detection of potential transgenerational influence of maternal age effects by examining three maternal generations (P_0 - F_2) on offspring (F_3) embryogenesis. This enabled us to resolve relative roles of maternal provisioning, epigenesis, and mutation accumulation underlying observed maternal effect senescence (Table 1). We predict that if maternal effects are due to altered maternal provisioning or epigenetics, then the maternal (F_2) generation will largely determine developmental outcomes and young maternal age will compensate for old maternal age in preceding generations (i.e., P_0 and F_1). Additionally, if maternal effects are due to mutation accumulation, then negative effects of maternal age will be detected from the great grandmaternal (P_0) generation and/or accumulate across generations, and young mothers in subsequent generations will not "reset" or compensate for these negative effects. Finally, to explore potential effects of genetic background on the manifestation of maternal effect senescence, we conducted the experiments in two genetically distinct strains of flies, Canton-S and Oregon-R, that have been maintained under identical conditions in the laboratory for several hundred generations.

Describing trans- and/or multigenerational impacts of maternal effect senescence (Moorad and Nussey, 2016) offers a more accurate understanding of sources of phenotypic variation in populations with complex age structures. Similarities between different genetic lines (strains) indicate that negative maternal effects due to old age are likely a fundamental feature of this species' biology, while strain differences in trans- and/or multigenerational effects reflect a role for variation in genetic backgrounds and potential gene x environment effects.

Materials & methods

Culture conditions

Two strains of *Drosophila melanogaster*, Canton-S and Oregon-R, were cultured in parallel and on Lewis Medium (Lewis, 1960) at 25 °C and 12:12 light cycle. Development from egg to adult under these laboratory conditions takes 10–12 days (Sullivan et al., 2000) and new laboratory cultures were generally started every 14 d from the parental culture. This can result in selection for early reproduction and rapid development. To relax selection for early reproduction, before beginning these experiments cultures were initiated every 3 weeks with 20 males and 20 females in pint bottles containing 75 mL of Lewis Medium for four generations. To reduce the possibility of environmental differences impacting comparisons between the two lines, maternal generations and embryonic collections (described in Sections 2.2.-2.4.) were conducted at the same time in both Canton-S and Oregon-R flies.

Generation of experimental populations

Initiation of the great grandmaternal (P_0) generation began with the collection of at least 320 newly-eclosed females: 120 females were allocated to the young P_0 group (3–5 days post-eclosion, hereafter dpe) and at least 200 females were allocated to the old P_0 group (n = 220 Canton-S, 30–32 dpe; n = 200 Oregon-R). Young females (3–5 dpe) are sexually mature and show high fecundity and fertility while old females (30–32 dpe) show decreased fecundity and fertility, but do not yet exhibit high rates of mortality (Bloch Qazi et al., 2017). Females were maintained in single-sex groups of 20 flies in 8 dram vials containing 6

Table 1
Potential experimental outcomes supporting different mechanisms underlying maternal effect senescence. Cell symbols reflect the nature of the impact: 0 = no effect; + = a small to moderate effect; and ++ = a moderate to large effect.

	Mutation Accumulation	Epigenetic Effect	Provisioning
Negative effects $P_0 \geq F_1 \geq F_2$	++	+	0
Effect during early embryogenesis	+	+	++
F_2 compensates for negative F_1/P_0 effects	0	+	++

mL of Lewis Medium seeded with 10–20 grains of live yeast. To avoid reproductive quiescence and approximate more natural culture conditions, females in the old P_0 groups were housed with an equal number of young (3–5 dpe) males from different source cultures for three days, then removed, each week until the week of egg collection. Preliminary experiments indicated that this pattern of mating did not have a significant effect on embryonic viability (see Supplemental Materials). To maintain consistent culture density, old P_0 females that died were replaced weekly with same-aged females from cultures of similar densities as the experimental cultures. This resulted in 10–15 % replacement of female flies over the 30–32 dpe aging period.

Establishment of transgenerational populations

To create subsequent generations of old and young mothers for each strain, three replicate groups of 60 young P_0 females and old P_0 females were mated with 60 young males of the same strain. To avoid inbreeding, males came from different natal cultures. Males and females were removed from the culture after 3 dpe to control larval density and prevent overlapping generations. After 10 d, at least 320 eclosing F_1 generation female flies (and an equal number of male mates) were collected every 12 h for 24–48 h and immediately separated into same-sex groups of 20 flies for 3–5 dpe (young F_1 females) and 30–32 dpe (old F_1 females). This resulted in four age-groups of females: 1) old P_0, old F_1; 2) old P_0, young F_1; 3) young P_0, old F_1; and 4) young P_0, young F_1. This process was repeated for two more generations (Fig. 1). In each subsequent generation, young females were mated when 3–5 dpe and old females were mated when 30–32 dpe.

Embryo collection

After three generations, there were eight maternal treatment groups of every permutation of P_0, F_1, and F_2 maternal age for Canton-S and Oregon-R strains (Fig. 1). To collect embryos for analysis, females (n = 50) from each of the eight groups were mated with young males (3–5 dpe) in lightly yeasted vials with 2 mL of fresh Lewis Medium (n = 6 vials per maternal age group). Older females dump (lay large numbers of lower-quality) eggs within 24 h of mating (personal observations; Mossman et al., 2019) and young daughters of old mothers also dump eggs early after mating (Mossman et al., 2019). In order to more accurately compare embryogenesis between old and young F_2 mothers, we began collecting embryos from mated F_2 females 24 h after they were introduced to males by transferring the flies to yeasted oviposition grape plates (after Sullivan et al., 2000). 18 h after F_2 females were exposed to the oviposition plates, F_3 generation embryos were collected, methanol-fixed, and stored in methanol at −20 °C (after Rothwell and Sullivan, 2000). This process was repeated with two additional unique groups of females for a total of three embryo collections for each of the eight maternal treatment groups (24 total collections per genetic strain).

Embryo staging

The developmental stage of approximately 150 eggs/embryos per maternal F_2 group (50 eggs/embryos x 3 oviposition plates; exact sample sizes provided in Table S2) were determined by observing nuclei quantity, form, and organization. Stored eggs/embryos were rinsed and rehydrated (after Rothwell and Sullivan, 2000). They were transferred to a glass slide and nuclei stained with Fluoroshield Mounting Medium with DAPI (Abcam Inc.). Embryo nuclei abundance, form, and organization was visualized using an epifluorescence microscope (Leica DM LP) at 20x objective with a 360/40 nm excitation range and a 460/50 nm emission range. Images of the embryos were collected using a Canon EOS Rebel T5i DSLR camera with a microscope adapter.

Each observed egg/embryo was categorized by developmental stage and status. Five stages were distinguished: Pre-cleavage (PC), Cleavage (C), Blastoderm (B), Early Gastrulation (GE), and Late Gastrulation (GL) (Table 2). Status was designated as apparently typical (T) when the nuclei number, organization and distribution was consistent with published descriptions (Bownes, 1975; Rothwell and Sullivan, 2000; Campos-Ortega and Hartenstein, 2013); or atypical (A) because nuclear organization deviated from canonical descriptions of embryogenesis (Table 2; Fig. 2). We do not know if atypical embryos would have failed to develop further, but suspect they were less viable because some phenotypes categorized as atypical resemble non-viable mutant phenotypes (Pushpavalli et al., 2013). Additionally, levels of atypical development were similar to levels of unhatched eggs in a previous,

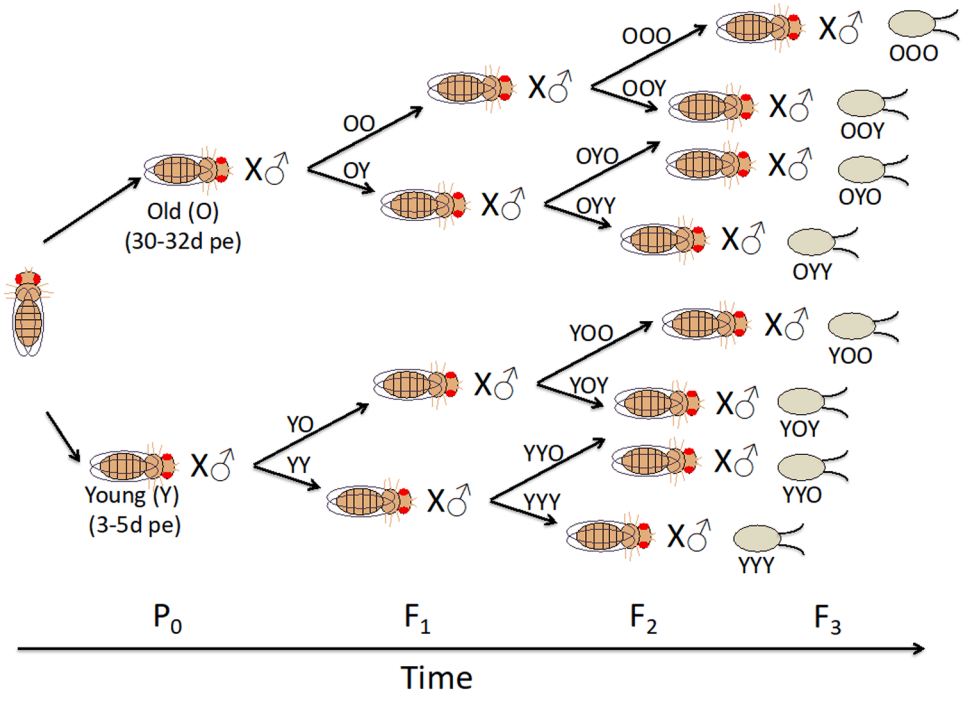

Fig. 1. Schematic of design to generate experimental maternal age groups (MAG) of every combination of old (O; 30–32 dpe, n = 200 each generation) and young (Y; 3–5 dpe, n = 120 each generation) mothers over three generations (n = 8 MAGs) for both Oregon-R and Canton-S strains. Embryos (F_3) were collected and staged (n = 150 embryos per MAG) from each of the eight MAGs (n = 3 groups of 50 females per MAG). The three letters utilized to label the offspring represent great-grandmaternal (P_0), grandmaternal (F_1), and maternal (F_2) age treatments, respectively. For example, OYO refers to embryos collected from old great-grandmothers, young grandmothers, and old mothers.

Table 2
Embryonic features used to categorize embryos by developmental stage and characterize development as typical or atypical.

	Typical development (T)	Atypical development (A)
Pre-cleavage (PC)	No staining or one stained nucleus. Diffuse, even DAPI staining on A-P poles (potential binding with mRNAs).	Uneven DAPI staining throughout the oocyte.
Cleavage (C)	≥ 2 nuclei, nuclei are small and round, staining is discrete. Nuclei are evenly dispersed centrally or through-out the oocyte.	Nuclear staining is discrete with heterogeneously-sized aggregations of nuclear material. Staining is limited in presence (less nuclear material than usual). Nuclei are unevenly distributed throughout the cytoplasm.
Blastoderm (B)	Nuclei are even, small & discrete, there are >100 nuclei. Pole cells observed at posterior end. Many nuclei are at the periphery and they are evenly distributed.	Staining is bright, but clumped. Nuclear fall-out in embryo interior (large, more diffuse staining). Nuclei are unevenly distributed around the periphery.
Early Gastrulation (GE)	Presence of ventral and/or cephalic furrows. Dorsal and anterior migration of pole cells. Head is distinct.	Events are not bilaterally symmetrical. Events are progressing in one region of the embryo, but not other regions.
Late Gastrulation (GL)	Parasegments form and are evenly spaced in the posterior region. Germ band retracts.	Events are not bilaterally symmetrical. Stage-specific events are progressing in one part of the embryo, but not other parts. Embryos are shorter.

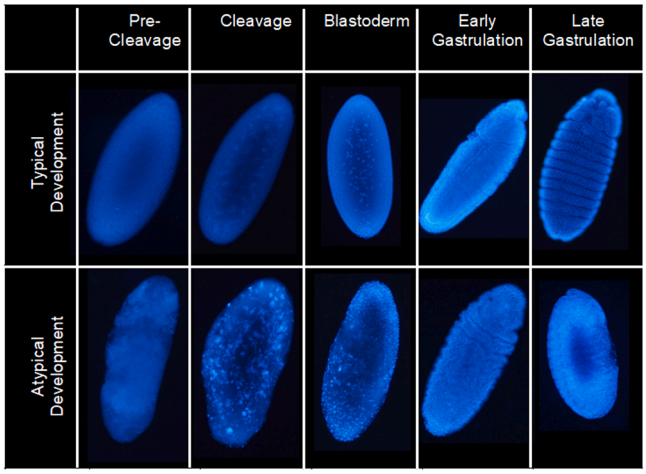

Fig. 2. Example images of embryos at each recorded developmental stage. Typical and Atypical embryos are shown. When discernible, anterior ends are positioned at the top of each cell in the Table. Embryo nuclei are stained with DAPI and visualized using an epifluorescence microscope at 20x objective. Images of the embryos were collected using a Canon EOS Rebel T5i DSLR camera.

similar experiment (see Section 4).

Analysis

Embryo status was modelled as a discrete-time survival process; at each stage, an embryo was considered "failed" if it was atypical, and "survived" if it was typical up to that stage. We used a binomial (logistic) regression to model the number of atypical embryos out of the number of embryos survived to that stage (Singer and Willett, 1993). Because survival to the next stage is conditionally independent, individual embryo random effects are not needed, however we do include random effects for each culture bottle to account for any shared environmental condition which may affect odds of typical development. These models were fit as a generalized linear mixed model using the glmer function from the lme4 (v1.1–30) package (Bates et al., 2015 and b) in R version 4.2.1 (R Core Team, 2022). The number of embryos surviving to each developmental stage are provided in Table S2.

Genotype (strain-level) differences on maternal effect senescence were explored by comparing the survival trajectories across all stages of Canton-S and Oregon-R strain flies using a likelihood ratio Chi-squared test of a model interaction of survival by strain (Section 3.1). Because we found significant differences between strains, all further analyses were conducted separately. Further model comparisons using likelihood ratio Chi-squared tests investigated whether differences between strains were more pronounced for embryos of old or young mothers at the maternal generation.

Next, we investigated maternal age effects on embryogenesis at each stage (Section 3.2). First, we tested whether all stages were affected similarly by maternal age (interaction of maternal age and embryonic developmental stage). Odds ratios (OR) comparing young and old maternal generation odds of atypical development for each stage were reported with associated p-values. We also reported the p-values adjusted with the Holm adjustment for multiple comparisons (Holm, 1979).

We further compared the effect of old mothers at the maternal (F_2), grandmaternal (F_1), and great-grandmaternal (P_0) generation to embryos with young mothers to determine whether effects persist across generations. For this analysis, we fit a binomial regression model with random effects (separate models for each strain) including all eight treatment groups (YYY, YYO, etc.) and tested for differences between relevant pairs of treatments (e.g. YOY and YYY to test for grandmaternal age effect). Specific, *a priori* hypotheses tested are discussed further in Section 3.3; all tests are likelihood ratio Chi-squared comparisons of nested binomial regression models with Holm adjustment for multiple testing. Please refer to Supplementary Materials for further explanation of particular statistical methods used.

Results

Strain differences in embryogenesis

Significant differences in embryonic development trajectory between strains existed (Fig. 3). While there is no evidence of a strain effect when comparing the probability of atypical development of embryos of young maternal (F_2) generation ($\chi^2 = 5.84$, df = 5, $p = 0.3226$), there is evidence of a strain effect on the probability of atypical development for embryos with old mothers at maternal (F_2) generation ($\chi^2 = 59.74$, df = 5, $p < 0.001$). Within these old mothers, the most notable differences between strains are at the pre- cleavage ($z = -2.511$, $p = 0.012$) and cleavage ($z = 5.10$, $p < 0.0001$) stages (Fig. 3). While Canton-S flies had very high rates of atypical development at the pre-cleavage stage (18 %) and low atypical development at the cleavage stage (1 %), Oregon-R flies had high levels of atypical development at both the pre-cleavage (12 %) and cleavage (13 %) stages. Interestingly, the cumulative probability of atypical development through the two stages were very similar for the old Canton-S flies (19 %) and old Oregon-R flies (23 %). However, because of the significant differences in trajectory, subsequent analyses were conducted separately for each genetic strain.

Maternal age effects on embryogenesis

Advanced maternal age disrupted embryogenesis in both fly strains (Canton-S: $\chi^2 = 56.39$, df = 5, $p < 0.0001$; Oregon-R: $\chi^2 = 25.51$, df = 5,

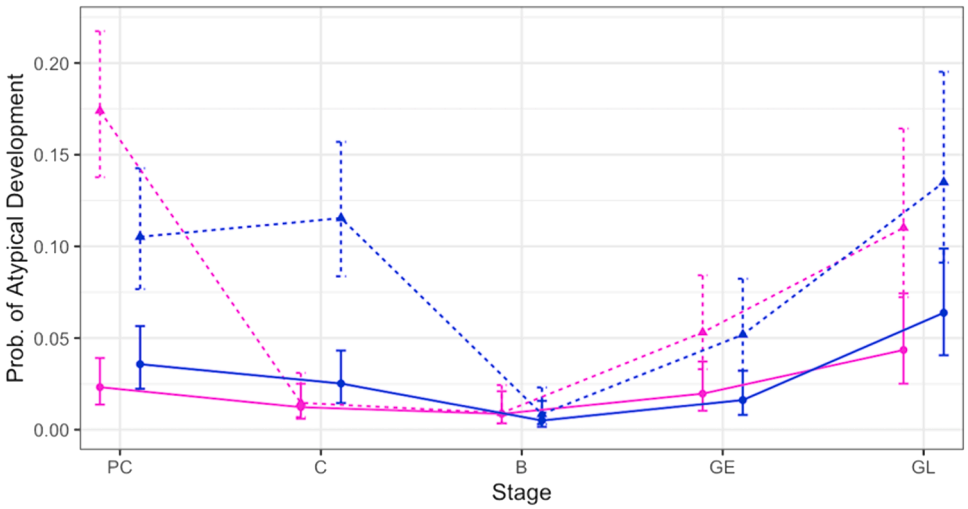

Fig. 3. Probability (+95 % CI) of atypical development at each embryonic stage for old (broken line) and young (solid line) females of the Canton-S (magenta line) and Oregon-R (blue line) strains. Stages include pre-cleavage (PC), cleavage (C), blastoderm (B), early gastrulation (GE), and late gastrulation (GL). Sample sizes are presented in Table S2. These values were used in all the calculations of risk and the p-values.

$p = 0.0001$). In Canton-S we further find that developmental stages differed in their sensitivity to maternal age (Canton-S: $\chi^2 = 20.68$, df = 4, $p = 0.0004$). In Canton-S, the largest increase in risk of atypical development for embryos of old F_2 females occurred at the pre-cleavage stage (odds ratio (OR) = 8.85, $p < 0.0001$, $p_{adj.} < 0.0001$). The atypical oocytes had uneven nuclear and/or protein staining (visualized as mottled background fluorescence; Fig. 2). The odds of atypical development were also elevated during gastrulation (early: OR = 2.79, $p = 0.014$, $p_{adj.} = 0.0421$; late: OR = 2.72, $p = 0.0074$, $p_{adj.} = 0.0295$), but not during the cleavage or blastula stages (Table 3). In Oregon-R, we find that there is not evidence that the effect of maternal age differs by stage ($\chi^2 = 4.70$, df = 4, $p = 0.3199$), and could be equally well-modelled by assuming the odds of atypical development were 3.27 times higher for old F_2 females compared to young. However, we still present the stage-specific odds ratios here for comparison to Canton-S. In Oregon-R, the risk of atypical development increased at all stages, except the blastoderm stage, with the greatest increase in risk at the cleavage (OR = 5.04, $p < 0.0001$, $p_{adj.} < 0.0001$), followed by early gastrulation (OR = 3.32, $p = 0.0059$, $p_{adj.} = 0.0178$), and pre-cleavage (OR = 3.17, $p = 0.0001$, $p_{adj.} = 0.0005$) stages. All odds-ratios and associated p-values are presented in Table 3.

Trans- and Multigenerational maternal age effects

Trans- and multigenerational effects were assessed by comparing embryonic development among P_0, F_1, and F_2 maternal groups with aged female cohorts. For example, comparison of embryos from OYY treatment with embryos from YYY treatment targets transgenerational effects of great grandmaternal (P_0) senescence. The old great grandmother (P_0; OYY) developmental trajectory was not statistically different from the corresponding young great grandmother (YYY) trajectory in Canton-S ($\chi^2 = 9.92$, df =5, $p = 0.0775$, $p_{adj.} = 0.115$) or Oregon-R ($\chi^2 = 3.53$, df = 5, $p = 0.618$, $p_{adj.} = 0.753$; Table 4, Fig. 4). Grandmaternal (F_1) age effects (YOY vs YYY) existed in Canton-S ($\chi^2 = 14.54$, df = 5, $p = 0.0125$, $p_{adj.} = 0.0375$), but not Oregon-R ($\chi^2 = 13.09$, df = 5, $p = 0.0225$, $p_{adj.} = 0.0676$). In Canton-S flies, the magnitude of the maternal (F_2) effect, measured as the increase in odds of atypical development across all stages, was nearly two-fold larger than the grandmaternal (F_1) effect (6.05x for YYO – YYY, 3.34x for YOY – YYY, respectively).

Particular patterns of maternal effects provide support for different possible mechanisms of maternal effect senescence. In addition to further support for transgenerational maternal age effects, accumulating negative generational age effects indicate the possibility of genetic mechanisms. Comparing OOO, YOO, and YYO treatment groups showed no evidence of an accumulated effect in Canton-S or Oregon-R (Canton-S: $\chi^2 = 16.68$, df = 10, $p = 0.0819$, $p_{adj.} = 0.115$; Oregon-R: $\chi^2 = 10.76$, df = 10, $p = 0.376$, $p_{adj.} = 0.753$; Fig. 5). Recovery effects, where young mothers compensate for the effects of old mothers in previous generations existed, but differed in magnitude by maternal generation. In the maternal generation (F_2), comparing OOO to OOY, embryos of young mothers had a significantly lower risk of atypical development that older mothers in Canton-S ($\chi^2 = 23.71$, df=5, $p<0.0001$, $p_{adj}=<0.001$) and Oregon-R ($\chi^2 = 30.70$, df=5, $p<0.001$, $p_{adj}<0.001$).

Discussion

Among insects, maternal age has profound effects on offspring phenotypes and fitness (reviewed in Miller et al., 2014; Ivimey-Cook and Moorad 2020). In *Drosophila*, embryonic viability, measured as hatching success, decreases with advancing maternal age (*D. melanogaster*, Bloch Qazi et al., 2017; *D. serrata*, Hercus and Hoffman, 2000). This study

Table 3
Odds of atypical embryo development for old vs. young maternal (F_2) age by stage. Odds Ratio > 1 indicates old maternal age has increased risk of atypical development compared to young maternal age. Both unadjusted p-values and Holm adjustment p-values are shown.

	Canton-S			**Oregon-R**		
	Odds ratio	p	adj. p	Odds ratio	p	adj. p
Pre-cleavage (PC)	8.85	<0.0001	<0.0001	3.17	0.0001	0.0005
Cleavage (C)	1.20	0.738	≈1	5.04	<0.0001	<0.0001
Blastoderm (B)	1.06	0.934	≈1	1.70	0.494	0.494
Early gastrulation (GE)	2.79	0.0140	0.0421	3.32	0.0059	0.0178
Late gastrulation (GL)	2.72	0.0074	0.0295	2.289	0.0121	0.0242

Table 4
Testing for difference in trajectory of risk for atypical development for different treatment groups of old (O) and young (Y) maternal generations. Adjusted p-values use the Holm adjustment. Both unadjusted p-values and Holm adjustment p-values are shown.

	Canton-S				Oregon-R			
Null hypothesis	χ^2	df	p	adj-p	χ^2	df	p	adj-p
OYY[1] = YYY	9.92	5	0.0775	0.155	3.53	5	0.618	0.753
YOY = YYY	14.54	5	0.0125	0.0375	13.092	5	0.0225	0.0676
YYO = YYY	38.82	5	<0.001	<0.001	27.77	5	<0.001	<0.001
OOY = OOO	23.71	5	<0.001	<0.001	30.70	5	<0.001	<0.001
OOO = YOO = YYO	16.68	10	0.0819	0.155	10.76	10	0.376	0.753

1. Maternal generational ages are arranged from P_0 to F_2. For example, OYY is the treatment old great grandmother, young grandmother, and young mother.

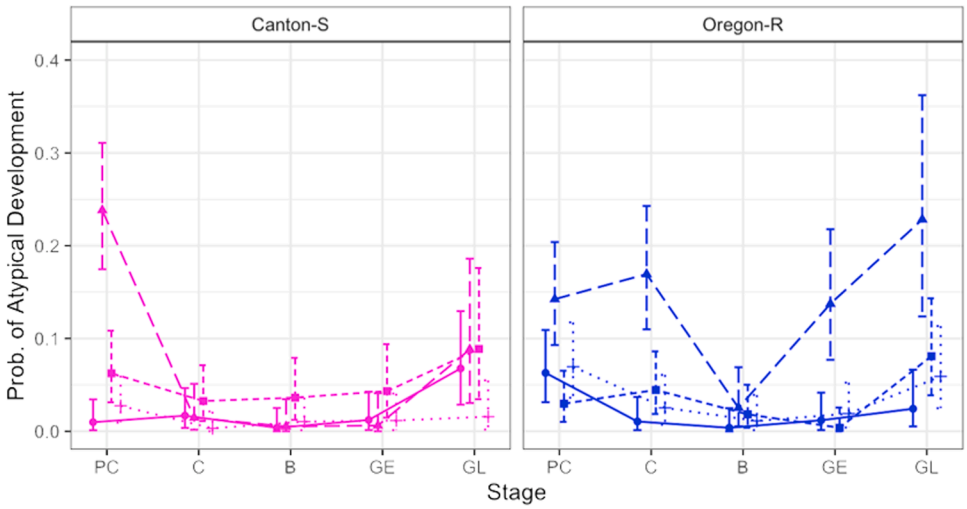

Fig. 4. Probability (+95 % CI) of atypical development at each embryonic (F_3) stage among different maternal age cohort treatment (great grandmaternal, P_0; grandmaternal, F_1; and maternal, F_2, ages). Embryos from young great grandmothers, grandmother, and mothers (YYY) are represented with a round symbol and solid line; YYO with triangle symbol and long dash line; YOY with a square symbol and short dash line; and OYY with a cross and dotted line. Relationships are shown for each genetic strain: a) Canton-S, magenta lines, and b) Oregon-R, blue lines. Stages include pre-cleavage (PC), cleavage (C), blastoderm (B), early gastrulation (GE) and late gastrulation (GL). Sample sizes are presented in Table S2. These values were used in all the calculations of risk and the p-values.

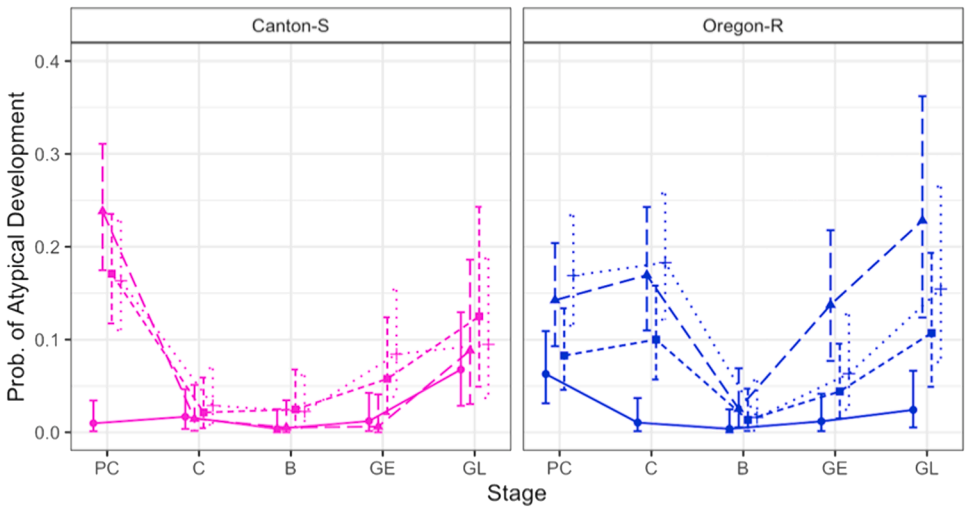

Fig. 5. Probability (+95 % CI) of atypical development at each embryonic (F_3) stage among different maternal age cohort treatment (great grandmaternal, P_0; grandmaternal, F_1; and maternal, F_2, ages). Embryos from young great grandmothers, grandmother, and mothers (YYY) are represented with a round symbol and solid line; YYO with a triangle symbol and long dash line; YOO with a square symbol and short dash line; and OOO with a cross and dotted line. Relationships are shown for each genetic strain: a) Canton-S, magenta line; and b) Oregon-R, blue line. Stages include pre-cleavage (PC), cleavage (C), blastoderm (B), early gastrulation (GE) and late gastrulation (GL). Sample sizes are presented in Table S2. These values were used in all the calculations of risk and the p-values.

identified embryonic stages most sensitive to the effects of maternal senescence and quantified the extent to which negative maternal age effects can be trans- and multigenerational. In this study, levels of embryonic disruption estimated by the incidence of atypical development (17 %–20 % young females; 33 %–42 % old females) resemble levels of hatching failure (8 %–20 % young females; 33 %–65 % old females) quantified in a previous study (Bloch Qazi et al., 2017). This supports the characterization of "typical" and "atypical" embryos as an estimate of embryonic viability.

Discrete developmental process are disrupted by advancing maternal age

Compared with young F_2 females, embryos of old F_2 females in both Canton-S and Oregon-R strains showed significant increases in atypical development at two distinct stages: pre-cleavage and gastrulation (both early and late). Therefore, multiple developmental processes appear to be disrupted by advancing maternal age. Elevated atypical development at the pre-cleavage (hereafter "oocyte") stage is attributed to disrupted oogenesis resulting in insufficiently-provisioned or constructed oocytes such as smaller yolk vesicles (Zhao et al., 2007; discussed in 4.4.1.). It is also possible that increased frequency of oocytes in samples from

senescing females reflects disrupted sperm-egg dynamics in the female resulting in unfertilized eggs. Egg dumping, the rapid ovulation of multiple unfertilized eggs, is less likely to contribute to the difference observed here, because eggs/embryos were collected more than 24 h post-mating, past the egg dumping period (Mossman et al., 2019; pers. obs.). Had "dumped" eggs been included, estimates of atypical development in older females would likely have been even greater than reported here.

Elevated atypical development during the gastrula stages (early and late, separately) implies disruption of distinct embryonic developmental processes that are largely under zygotic control. The maternal to zygotic transition consists of decreasing maternal molecular activity concurrent with two "waves'' of increasing zygotic genome activity (reviewed in Tadros and Lipshitz, 2009). The largest portion of the transition occurs during the blastoderm stage of development (Tadros and Lipshitz, 2009). In this study, the blastoderm stage was resistant to maternal effect senescence. Gastrulation is characterized by the morphogenic movements of populations of cells to establish the three germ layers as well as differentiation of cell identity within each germ layer (Gheisari et al., 2020). Gastrulation is largely under the direction of the zygotic genome, constructed with materials provided by the mother. As such, disruption of factors controlling cell movement and differentiation are predicted to contribute to these results. For example, disruption of β-catenin dynamics during gastrulation can affect subsequent activity of E-cadherin involved in morphogenic processes as well as signaling involved in mesoderm identity (Brunet et al., 2013). Multi-stage developmental effects of maternal age indicate that reproductive senescence is a complex phenomenon and will likely have variable outcomes both within and among populations.

Trans- and Multigenerational maternal effects

Maternal effect senescence had negative multi- (F_1 and F_2), but not transgenerational (P_0), impacts on embryogenesis. Maternal age negatively impacted embryogenesis in the maternal (F_2) generation in both strains (Canton-S and Oregon-R), and the grandmaternal (F_1) generation in Canton-S only. Neither strain revealed a negative transgenerational (P_0) maternal age effect, reflecting an attenuating impact of maternal effect senescence across each preceding generation. Negative multigenerational maternal age effects on offspring viability were also detected in one line of a related species, *Drosophila serrata* (Hercus and Hoffman, 2000). Together, these studies suggest that multigenerational maternal age affects may be a part of Drosophilid life histories. Few studies involving parental condition measure changes over three generations - extending beyond direct maternal effects (but see Mondotte et al., 2020 for an effect of immune priming in Drosophila), although there exists robust evidence of multigenerational effects of maternal condition including obesity (Brookhart and Duncan, 2016), heavy metal exposure (Yang et al., 2020), age (Bloch Qazi et al., 2017; Layton et al., 2019), and nutrition (Osborne and Dearden, 2017). Maternal age effects extending across generations can contribute to phenotypic variation within populations. This is important for insect populations in at least two ways. First, in laboratory studies, transgenerational effects introduce phenotypic variation thereby interfering with detection of small effects in experimental outcomes. Second, in natural populations, this variation may impact how populations respond to: a) other environmental stressors (Barrere-Cain and Allard, 2020), and b) management techniques to augment or limit population sizes.

Genotype effects

Our results show that while maternal senescence has a profound negative effect on early embryonic viability, genetic variation underlies a significant portion of the variation in embryonic response. When comparing the two strains of females used, both early and late embryonic stages had elevated rates of atypical development. However, while Canton-S flies had large changes in levels of atypical development across embryonic stages, Oregon-R flies had more uniform level of atypical development across the embryonic stages. This suggests that strain-specific differences in egg provisioning may underlay these effects. These results are consistent with previous work describing the same lines' differing sensitivities to the effects of maternal senescence (Bloch Qazi et al., 2017) as well as early maternal fertility, embryo hatchability, and female sperm storage (McGraw et al., 2009). Increased embryonic sensitivity to maternal age in Canton-S could be part of a trade-off with higher early documented fertility in Canton-S relative to Oregon-R (McGraw et al., 2009). Additionally, although modest, transcriptional responses to mating differed between these two strains (McGraw et al., 2009). While the observed strain differences may be influenced by different gene-by-environment (GxE) interactions, they are unlikely to be due to direct environmental effects on maternal populations as they had been maintained under the same laboratory conditions for at least 200 generations. Additionally, experiments on the two lines were conducted at the same time, raised on the same batches of medium, and under common incubator conditions. Even among closely-related populations, responses to maternal condition can differ.

Processes underlying maternal age effects

Maternal provisioning

The large, direct negative impact of maternal (F_2) senescence on embryonic development at several embryonic stages reflects the critical and dominant role of maternal provisioning on embryogenesis. This result is consistent with the negative impact maternal age had on the earliest stage, pre-cleavage, as well as both early and late gastrulation, when cell differentiation and morphogenesis requires sufficient "raw materials" to construct a larva. Provisioning occurs through the molecules and organelles that are incorporated into the developing oocyte and can be both quantitative and qualitative in effect. Previous studies show how disrupted provisioning affects egg quality at the levels of morphology, composition, organelle status, and molecular structure. Older females produce eggs differing in size from, and more variable in size than, young females (Parsons, 1962; Azevedo et al., 1997; Bloch Qazi et al., 2017). This could be due to a reduction in a number of molecules including water, proteins, carbohydrates, fats, and nucleic acids. For example, oocytes of aged females have smaller yolk sacks than those of young females (Zhao et al., 2007). Oocytes of older females also exhibit decreased molecular homeostasis in the form of increased carbonylation of proteins (Fredriksson et al., 2012). However, there is no evidence that oocytes from older mothers have increased rates of aneuploidy (Greenblatt et al., 2019). At the level or the organelle, mitochondrial quality may decline (Kann et al., 1998), although the number of mitochondria increases with maternal age (Mengel-From et al., 2019). Additionally, changes in female responses to male seminal fluid proteins may affect oocyte development or provisioning. 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). Any and all of these mechanisms could be at play in the present study. The robust role for maternal provisioning on embryonic development is reinforced by finding that young mothers largely, although not completely, compensate for the negative effects of older females in previous generations.

Genetic effects: epigenetics and mutation accumulation

Our detection of a grandmaternal (F_1) age effect on embryogenesis provides modest support for the existence of maternal age effects acting through epigenetic mechanisms. This effect was detected in one strain, Canton-S. While not statistically significant, the possible impact of grandmaternal age on embryogenesis in Oregon-R warrants further investigation. Epigenetics have well-established roles in senescence and disease (reviewed in Skinner, 2011; Yu et al., 2019) and can have multigenerational effects on embryogenesis. For example, the

maternally-inherited repressor H3K27me3, stabilizes early development by repressing early activation of developmental genes in Drosophila embryos (Zenk et al., 2017). Perturbation in H3K27me3 function as a result of increasing maternal age could destabilize early embryogenesis. The absence of a detectable persistent or accumulating negative effect of increasing maternal age across three generations fails to support the existence of accumulated mutational load as a result of advancing maternal age. Together, the potential epigenetic influence(s) complementing the larger effects of maternal oocyte provisioning support the existence of multiple mechanisms underlying observed negative maternal age effects.

Potential male contributions to maternal age effects

This study focused on maternal reproductive senescence, but males also make important contributions to early developmental events (Bonduriansky and Head, 2007) - sometimes in response to female condition. Paternal age effects exist and can interact with maternal effects to influence offspring viability (Ducatez et al., 2012; Tan et al., 2013; Noguera, 2021), fertility (Mossman et al., 2019), and behavior (Noguera, 2021). For example, the centriole inherited from the sperm is necessary for initial cleavage events and disrupted coordination of karyogamy is consistent with potential perturbations at the pre-cleavage (oocyte) stage. Disruptions in centriole dynamics and/or ejaculate allocation in response to maternal age (Lüpold et al., 2011; Sirot et al., 2011; Wigby et al., 2016) could potentially affect maternal oocyte provisioning and fertilization dynamics at the earliest stages of embryonic development.

Conclusion

Advancing maternal age has multiple negative impacts on embryonic development. In laboratory populations, maternal condition negatively affects offspring fitness and is, therefore, an important multigenerational source of phenotypic plasticity affecting experimental populations. A combination of factors including both direct resources and possibly less influential epigenetic influences appear to mediate these negative maternal age effects. Disruption of developmental homeostasis, such as that observed here, may also stochastically sensitize embryos to effects of other disruptors. These impacts could be magnified in less controlled natural populations. Finally, the level of sensitivity to maternal age effects is influenced by genetic background. Small, isolated populations that include older reproductively active females may be particularly vulnerable to maternal condition effects. Elucidating mechanisms underlying multigenerational maternal effect senescence contributes to a foundational understanding of the mechanisms of maternal effects. These outcomes also have value for informing management (both conservation and control) of natural populations of mixed ages and genotypes as well as rearing practices for laboratory animals.

CRediT authorship contribution statement

Halie Ostberg: Conceptualization, Data curation, Investigation, Methodology, Resources. **Laura Boehm Vock:** Formal analysis, Methodology, Software, Visualization, Writing – original draft, Writing – review & editing. **Margaret C. Bloch-Qazi:** Conceptualization, Data curation, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data are included in Supplemental Materials Table S3.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cris.2023.100068.

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