Inverse Lansing Effect: Maternal Age and Provisioning Affecting Daughters' Longevity and Male Offspring Production

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Submitted November 23, 2021; Accepted April 28, 2022; Electronically published September 28, 2022

Online enhancements: supplemental PDF.

ABSTRACT: Maternal age effects on offspring life history are known in a variety of organisms, with offspring of older mothers typically having lower life expectancy (the Lansing effect). However, there is no consensus on the generality and mechanisms of this pattern. We tested predictions of the Lansing effect in several *Daphnia magna* clones and observed clone-specific magnitude and direction of the maternal age effect on offspring longevity. We also report ambidirectional, genotype-specific effects of maternal age on the propensity of daughters to produce male offspring. Focusing on two clones with contrasting life histories, we demonstrate that maternal age effects can be explained by lipid provisioning of embryos by mothers of different ages. Individuals from a single-generation maternal age reversal treatment showed intermediate life span and intermediate lipid content at birth. In the clone characterized by the "inverse Lansing effect," neonates produced by older mothers showed higher mitochondrial membrane potential in neural tissues than their counterparts born to younger mothers. We conclude that an inverse Lansing effect is possible and hypothesize that it may be caused by age-specific maternal lipid provisioning creating a calorically restricted environment during embryonic development, which in turn reduces fecundity and increases life span in offspring.

Keywords: Daphnia, Lansing effect, longevity, maternal provisioning, lipids, mitochondrial membrane potential, sex ratio.

Introduction

Maternal age affects health and life history characteristics of offspring in many ways. It has long been recognized that older mothers tend to produce less healthy, shorter-living offspring. The Lansing effect, or maternal effect senescence, was initially thought to be most pronounced in clonal organisms, such as rotifers (Jennings and Lynch 1928; Lansing 1947; Hernández et al. 2020). It has since been demonstrated in a variety of sexually reproducing species, such as *Drosophila* and other insects (Hercus and Hoffman 2000; Kern et al. 2001; Fox et al. 2003; Bloch Qazi et al. 2017; Wylde et al. 2019), birds (Bouwhuis et al. 2015; Schroeder et al. 2015), and humans (Gillespie et al. 2013; Gao et al. 2019). Still, many maternal effect senescence studies use cyclic parthenogenic models, such as rotifers (Bock et al. 2019; Hernández et al. 2020) or *Daphnia* (Plaistow et al. 2015), perhaps because of a special interest in the effects of an uninterrupted chain of mitoses on cross-generational quality of germline, given that mechanisms ensuring rejuvenation of germline are known to be associated with meiosis and fertilization (e.g., Bohnert and Kanyon 2017).

Despite decades of research, there is no consensus on the fundamental mechanisms or even on universal occurrences of maternal effect senescence (Plaistow et al. 2015). Likewise, there is no consensus on why the detrimental effects of maternal age, observed in many species, do not generally accumulate over generations (Monaghan and Metcalfe 2019; Monaghan et al. 2020). In cases where maternal age effects indeed do accumulate over generations (e.g., Wylde et al. 2019), what kind of selection on longevity do they impose (Bonduriansky and Day 2018)? Most importantly, do the data on maternal effects on aging suggest the existence of transgenerational rejuvenation mechanisms, conceivably operating in the germline of younger mothers but gradually failing in the older ones? In nature, lineages frequently pass through a rejuvenating young reproducing generation, which likely causes effects of aging not to accumulate over generations. Laboratory studies of uninterrupted multigenerational accumulation of senescent effects are rare (Wylde et al. 2019).

Several explanations for maternal effect senescence have been proposed (Monaghan et al. 2020), of which only a few apply to asexual organisms: decreased gamete quality (mutational or epigenetic); decreased maternal care, including

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American Naturalist, volume 200, number 5, November 2022. Published by The University of Chicago Press for The American Society of Naturalists. https://doi.org/10.1086/721148

egg provisioning; and compensatory responses in the offspring triggering a growth–life span trade-off with detrimental life expectancy outcomes (Metcalfe and Monaghan 2001, 2003; Lee et al. 2013; Monaghan and Metcalfe 2019). Gamete quality mechanisms could act by accumulation of damage in the nucleus (i.e., germline mutations or abnormal DNA methylation) or in the cytosol. Nonnuclear changes are likely to include mitochondrial DNA (mtDNA) mutations (Monaghan and Metcalfe 2019), accumulation of misfolded proteins and lipid peroxides in membranes, or other organelle damage. These mechanisms are likely to have a unidirectional effect on offspring quality and life span lowering in the offspring of older mothers.

However, there are several potential compensatory mechanisms that may ameliorate these detrimental effects. At least two mechanisms have been suggested that may reverse maternal age effects on offspring longevity. First, maternal provisioning does not necessarily decline with age. Increase in reproductive effort with age is often described as a terminal investment (Clutton-Brock 1984; Velando et al. 2006), with older mothers with low life expectancy redirecting resources from survival to reproduction. Additionally, maternal investments may increase rather than decrease with age when older mothers expect their offspring to compete for seasonal resources (Travis et al. 2021).

Second, even if maternal investments are declining with age, reduced maternal investment might increase the life span of progeny through the dietary restriction imposed on the offspring during embryonic development (Hibshman et al. 2016; Bock et al. 2019). In this scenario, offspring compensatory mechanisms, such as compensatory growth (Metcalfe and Monaghan 2001, 2003; Plaistow et al. 2015), may lead to extended, not shortened, offspring life span. Thus, one may envision situations in which older mothers may produce higher-quality, longer-lived offspring, resulting in an "inverse Lansing effect." It is worth noting that investment-based rather than damage-based mechanisms of the maternal age effects on offspring are expected to be fully reversible via a single generation of propagation through younger mothers (Travis et al. 2021). The goal of this study is to evaluate the effect of age-specific maternal resource investments on offspring longevity in the context of the possibility of an inverse Lansing effect.

There is an additional prediction we consider here, not often reflected in Lansing effect studies—namely, possible changes in offspring sex ratio due to grandmaternal age, operating through age-related decline in the quality of mitochondria daughters inherit from their mothers. There are several lines of evidence that suggest that effects of maternal senescence may operate through changes in mitochondria (Wilding 2015; Monaghan and Metcalfe 2019). It is known that gamete quality can decrease with maternal age because of oxidative stress resulting in mtDNA mutations and membrane damage (Eichenlaub-Ritter et al. 2011; Lord and Aitken 2013; Ross et al. 2013; Pasquariello et al. 2019; Amartuvshin et al. 2020). In mice, the consequences of such damage can be ameliorated by antioxidant intervention (Silva et al. 2015) or electron transport chain–enhancing supplementation of coenzyme Q (Ben-Meir et al. 2015). The lack of protection offered by nuclear repair and recombination mechanisms, the lack of histones, and the proximity of mtDNA to the site of reactive oxygen species production (Ames et al. 1995; Eichenlaub-Ritter et al. 2011; Palozzi et al. 2018) makes mtDNA vulnerable to mutations. At the same time, multicellular organisms possess few options to select against defective or genetically impaired mitochondria (Palozzi et al. 2018; Monaghan and Metcalfe 2019). For organisms with maternally controlled environmental sex determination, such as cyclic parthenogens like rotifers or cladocerans, including *Daphnia*, it could be beneficial for the female to produce daughters earlier in life, ensuring transmission of "young" mitochondria to grandchildren, and sons later in life, when mitochondria quality degenerates. Moreover, if an individual is able to assess the quality of its own mitochondria, it would be beneficial for an organism with impaired mitochondria, other conditions equal, to invest in sons rather than daughters, provided these sons get a chance to mate with females with better mitochondria than their own. In *Daphnia*, sex of the offspring is determined by a suite of environmental cues, such as population density, food intake, temperature, and photoperiod (Stross and Hill 1965; Ferrari and Hebert 1982; Hobaek and Larsson 1990; Kleiven et al. 1992), with a strong within-population genetic variation for the response to these cues (Yampolsky 1992; Deng 1996; Fitzsimmons and Innes 2006; Lampert et al. 2012*a*). Indeed, there is a tendency for an increase in male offspring production with age in *Daphnia* (Hobaek and Larsson 1990; Fitzsimmons and Innes 2006). If a similar effect could be observed in daughters of older versus younger mothers, this could be interpreted as support for the idea that older mothers can transmit damaged mitochondria. Again, it is possible that germline or embryonic caloric restriction conditions may reverse mitochondrial damage. Caloric restriction is known to decelerate (Hepple et al. 2006; Valle et al. 2008) or possibly even reverse (Li et al. 2016; Bi et al. 2018) age-related mitochondrial damage through mechanisms that may include modulating mitochondrial proliferation (Valle et al. 2008), autophagy (Bi et al. 2018), and mTOR (Dai et al. 2014).

The quality of maternal mitochondria can also have the opposite effect on offspring's longevity. Moderately reduced mitochondrial potential ($\Delta\Psi_m$) is often hypothesized and occasionally demonstrated to have a life-extending effect (Wang and Hekimi 2015; Campos et al. 2021). Therefore, inheriting less efficient mitochondria (e.g., from an aged mother) may result in lowered respiratory

metabolism and translate into longer life span, providing the possibility for an inverse Lansing effect.

What might be the nature of resources differentially provisioned to offspring by young versus older mothers? Previously, Plaistow et al. (2015) detected the Lansing effect in two out of three genotypes tested and observed that reduced life expectancy in daughters of older mothers was correlated with increased early-life reproductive effort. This lead to the conclusion that the nature of the maternal age effect is not necessarily the irreversible senescent state that older mothers transmit to their daughters. Rather, it may be entirely investment based, with better offspring provisioning by older mothers favoring early reproduction over longevity, thus modulating the reproduction-survival trade-off in the offspring. The nature of the resource that is provisioned by mothers to achieve this effect is not yet known, but it is possible to hypothesize that this resource is maternally derived storage lipids. Earlier it had been demonstrated that older (and larger) mothers give birth to larger and better lipid-provisioned offspring (Glazier 1992), leading to earlier maturity and higher early reproduction, which in turn could lead to shortened life span. If this is the case, this is not a particular effect of maternal age per se: the same effect should be observable in better-provisioned offspring independently of maternal age, which may explain intergenotype differences in the magnitude or even directionality of the maternal age effect.

Furthermore, one might also expect that maternal provisioning may affect daughters' propensity to produce male offspring. On the one hand, lower resource availability early in life may signal high population density and/or low food availability, environmental cues that trigger male production (Hobaek and Larsson 1990; Kleiven et al. 1992), so poorly provisioned females should produce more sons. Inversely, lipid metabolism and male production are under the control of the same hormonal regulator, methyl farnesoate, which both increases deposition of triacylglycerides into eggs (Jordão et al. 2016; Fuertes et al. 2018) and upregulates male production (Olmstead and Leblanc 2002; Lampert et al. 2012*b*; Toyota et al. 2015). Thus, mothers with higher levels of methyl farnesoate may both supply their daughters with more lipids and send them a signal to produce males, resulting in better-provisioned daughters producing more sons. It is unclear which of the two opposing signals prevails, but in either case maternal provisioning is expected to affect not just longevity but also the male production propensity of *Daphnia* females.

In this study, we examine the potential of specific *Daphnia* genotypes to buffer the negative effects of increasing maternal age on offspring in the context of lipid provisioning and mitochondrial quality. Specifically, we test the following hypotheses: (1) the Lansing effect in *Daphnia* is genotype specific, and an inverse Lansing effect can occur; (2) the longevity of daughters of younger and older mothers is affected by nutrient (lipid) provisioning by their mothers; (3) daughters of older mothers invest more into their sons than into their daughters; and (4) neonates born to mothers of different ages differ in their mitochondrial properties. We first describe the genotype × maternal age interaction observed in an experiment with five different *Daphnia* clones and then focus on two clones in a smaller-scale experiment with more detailed life history data measured.

Material and Methods

Origin and Maintenance of Clones

Daphnia magna clones used in this study (table S1) were obtained from the Basel University *Daphnia* stock collection in Basel, Switzerland. They are a subset of clones that have been previously characterized for a number of life history traits (Coggins et al. 2021*b*) and were chosen to represent a range of clone-specific life expectancies. Stocks were maintained in the laboratory at 20°C in 200-mL jars with COMBO water (Kilham et al. 1998), 10 adults per jar, and fed a diet of *Scenedesmus acutus* at the concentration of 100,000 cells/mL/day or 2×10^6 cells/*Daphnia*/day. This *Daphnia* and food density was the same in all experiments. Table S1 lists the clones' IDs as recorded by the Basel clone collection. For the sake of brevity, clones will hereafter be referred to by the first two letters of their IDs (FI, GB, IL, FR, and HU), which are indicative of the country of origin (using internet domain two-letter codes).

Life Span Experiments

Because the interactions discussed below span three generations, we will use the following conventions to avoid ambiguity. The age of generation 1 females at the time they give birth to generation 2 females will be referred to as "maternal age" in the analyses of its effects on their daughters (e.g., on the daughters' longevity) and "grandmaternal age" in the analysis of its effects on the grandchildren of generation 1 females (e.g., grandchildren's sex). Generation 2 females will be referred to as "daughters" relative to generation 1 females and as "mothers" relative to generation 3 individuals, which in turn are referred to as "offspring." Males in generation 3 are also referred to as "sons" or "grandsons" relative to generations 2 and 1, respectively.

Details of the life span experiments are summarized in table 1. We first conducted a larger experiment with five clones that were maintained in groups of five in 100-mL jars and in which individual life history parameters were not measured. We then chose two clones for a smaller

Table 1: Summary of experiments, hypotheses, and outcomes

	Experiment 1	Experiment 2	Experiment 3
Design details	Cohorts consisting of daughters of old mothers (78.7 ± 13.70[a] days old) and young mothers (18.6 ± 3.53 days old) maintained in groups of five per 100-mL jar; two consecutive blocks analyzed together	Cohorts consisting of daughters of old mothers (80.1 ± 3.94 days old) and young mothers (14.0 ± 1.89 days old) maintained individually in 20-mL vials; see text for details of maternal age reversal treatment	Neonates born to 75- or 28-day-old mothers[b]
Clones used	Five clones listed in table S1	FI-FSP1-16-2, GB-EL75-69, and HU-K-6[c]	GB-EL75-69
Readouts	Life span, fecundity, offspring sex ratio[d]	Life span, fecundity, offspring sex ratio, lipid deposits in neonate siblings of cohort individuals	$\Delta\Psi_m$ (mitochondrial membrane potential, rhodamine 123 assay)
Hypotheses tested	Maternal age affects longevity of daughters and their offspring sex ratio[d] in a clone-specific manner	Maternal age effects on daughters' life history can be explained by lipid allocation to neonates	Inverse Lansing effect can be explained by reduced Ψ in offspring of older mothers
Main outcome	Maternal age effect on daughters' longevity and male production is genotype specific	Contrasting effects of maternal age on daughters' longevity and male production is consistent with contrasting changes in lipid provisioning to neonates with maternal age	Offspring of older mothers have lower Ψ in excretory tissues but higher Ψ in neural tissue than daughters of young mothers

[a] Maternal age means and standard deviations.
[b] No variation in maternal age.
[c] Only sex ratio data available for the clone HU-K-6.
[d] Offspring sex ratio was an unplanned comparison in experiment 1, block 1.

experiment with individually maintained *Daphnia* and more detailed life history data recorded. The choice of the two clones was influenced by their contrasting life histories: in four separate previous experiments (Coggins at al. 2021*b*; Anderson et al. 2022; Ekwudo et al. 2022) they were shown to differ in early reproduction and life span, with the GB clone characterized by longer life span than the FI clone.

In both experiments, in order to obtain generation 2 daughters of older (hereafter, the O treatment) and younger (hereafter, the Y treatment) generation 1 females simultaneously, two grandmaternal cohorts of each clone were created by collection of neonate females born by 15–20-day-old *Daphnia*, staggered 50–55 days apart (which corresponds to approximately the median life span and which implies that *Daphnia* in the Y treatment went through three 15–20-day-long generations during the lifetime of the O treatment grandmothers; see fig. 1). Additionally, to test the reversibility of any grandmaternal effects though any hypothetical "rejuvenation" effects of being born to younger mothers, a subset of experiment 2, generation 2 females were born to 14.6 ± 2.65 (SD) mothers, who were in turn daughters of 70-day-old mothers (hereafter, the maternal age reversal [OY] treatment; fig. 1, gray). This treatment was limited to only one clone, GB. Experiment 1 was conducted in two blocks with cohort sizes of 353 individuals (in 70 independent jars) and 1,095 individuals (in 220 jars). Experiment 2 consisted of a single cohort of 153 individuals (each in an independent vial).

Sex Ratios, Clutch Size, and Offspring Size

Sex ratio was determined in clutches produced by females between their age at maturity and the age of 40 days. Females that died before that age were excluded from the analysis to avoid bias in sex ratios, as early clutches rarely contain males. Additionally, to check for the sex ratio of offspring produced by older mothers, the sex ratio of offspring was measured for a subset of mothers 55–100 days old in experiment 1 and mothers 40–85 days old in experiment 2.

Nile Red Staining for Lipids

To quantify maternal provisioning of storage lipids to offspring, newborn *Daphnia* (<24 h old) from the same clutches from which generation 2 FI and GB females came from were stained with Nile Red dye for 2 h with

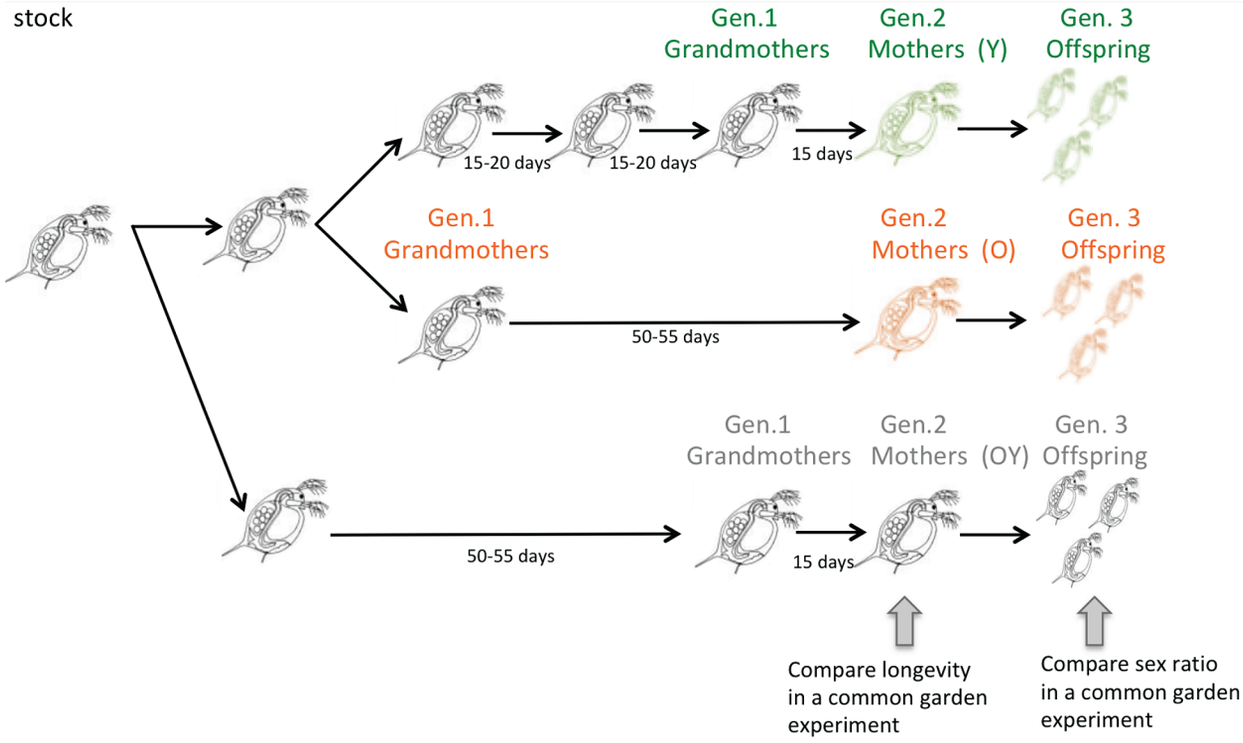

Figure 1: Experimental design: generations 1 (grandmaternal), 2 (maternal), and 3 (offspring). Staggered initiation of progenitor generation allows common garden measurement of longevity among generation 2 individuals (mothers) and sex ratio among generation 3 individuals (offspring) in O (daughters of old mothers), Y (daughters of young mothers), and OY (daughters of maternal age reversal mothers) treatments. The OY treatment was included only for clone GB in experiment 2. Image design based on and *Daphnia* shapes copied from figure 1 of Plaistow et al. (2015) for ease of comparison (© 2015 The University of Chicago).

a final dye concentration of 1 mg/mL, achieved by adding 5 μL of a 200 mg/mL stock solution in acetone to 995 μL of COMBO water. Fluorescence was recorded using an EVOS microscope (4× objective, 0.13 aperture). Fluorescence in the entire body was measured (fig. S1*A*). Because the distribution of storage lipid bubbles is patchy, the histogram of intensities was recorded and the fraction of intensities above an arbitrarily chosen threshold was obtained, with the threshold chosen in such a way that it masked in the lipid bubbles, leaving out the rest of the body. Specifically, the 8-bit image gray value of 200 was chosen as the threshold, with 2.5% of pixels showing intensity above this threshold in three randomly chosen images. The same threshold was applied to all images. This allows analysis of the portion of pixels located inside and the portion of fluorescence intensity emanating from the brightly fluorescent lipid vesicles, excluding background fluorescence emanating from nonstorage lipids in other tissues. The portion of pixels and the portion of fluorescence from the lipid bubbles are therefore proxies for the portion of the body occupied by lipid storage and lipid density in these storage areas, respectively.

Mitochondrial Potential Measurements

Mitochondrial membrane potential ($\Delta\Psi_m$) is commonly used as a measure of mitochondrial quality, with reduced $\Delta\Psi_m$ values indicating loss of membrane integrity and/or reduced function of electron transport chain (Perry et al. 2018) and correlating with apoptosis and aging (Nicholls 2004). Mitochondrial membrane potential is commonly determined by measuring the accumulation of cationic fluorophore rhodamine 123 inside the mitochondria (Emaus et al. 1986; Huang et al. 2007; Perry et al. 2018); the rate of such accumulation is proportional to the magnitude of proton gradient across the inner mitochondrial membrane. Mitochondrial potential was measured by means of rhodamine 123 staining in neonates born to either young or old mothers treated as described above. Newborns <12 h old were placed in groups of five into 1.5-mL tubes containing 0–10 μM

rhodamine 123 in COMBO water for 24 h (Coggins et al. 2021*a*). Fluorescence was measured using a Leica DM3000 microscope with a 10× objective (0.22 aperture) equipped with a Leica DFc450C camera using the 488-nm excitation/broadband (>515-nm) emission filter. The following regions of interest were selected (fig. S1*B*): second antenna and heart (representing muscle tissues), brain and optical lobe (representing neural tissue), second epipodite and nuchal organ (representing excretory/osmoregulatory organs), and nonneural head tissue (where the fluorescence was emitted largely from the head carapace epithelium without any organized tissue beneath). Median fluorescence (background subtracted) was recorded with an exposure of 100 ms with gain 1, except for rhodamine concentration of zero, which was measured with gain 10 (and the resulting measurement divided by this factor), using ImageJ software (Rasband 2018).

Statistical Analysis

All analyses were conducted using JMP statistical software (ver. 10; SAS Institute 2012). Life span data were analyzed by fitting a proportional hazards model (JMP Survival platform), with maternal age and clone as categorical predictors and, where applicable, with lipid abundance at birth and early offspring sex ratio as a continuous covariable. The OY treatment was available only for the GB clone, and the two young-mother treatments (Y and OY) were pooled for the purpose of any two-way full factorial analyses involving maternal age and clone effects. Because the clones were deliberately chosen to represent either the long-life end or the short-life end of the longevity spectrum, in this and further analyses the variable clone was treated as a fixed effect. Juvenile mortality (under the age of 6 days), likely to be caused by accidental damage, was excluded from the proportional hazards model; there were 26 and five such cases in experiments 1 and 2, respectively. In experiment 1, these 26 cases were uniformly distributed across maternal age classes (nominal logistic test $P > .34$) and were slightly enriched in one of the clones (IL; nominal logistic test $P < .05$). Such analysis was not possible in experiment 2 because of the low number of neonate mortality cases. In neither experiment did censoring early mortality affect the main result, that is, a significant clone × maternal age interaction term (see below).

Sex ratio (portion of male offspring or portion of predominantly male clutches) was analyzed using a generalized linear model with a binomial distribution and a logit link function. In addition to the excluded juvenile mortality, in any analysis that included offspring sex ratio or total number of offspring produced, individuals that died prior to 40 days of age of were excluded to eliminate a bias in sex ratio and offspring number estimates. There were 34 such individuals in total, 23 of them in clone GB. The numbers of male and female clutches rather than the total number of males and females produced was used as the response variable. Only 3.9% of clutches contained offspring of both sexes (typically one or two offspring of a different sex); these were listed as male or female clutches by simple majority rule.

For the lipid abundance at birth data, both the fraction of pixels above the threshold and the fraction of fluorescence intensity emanating from areas above the threshold were used with nearly identical results (fraction of fluorescence reported here). The differences in lipid abundance between maternal age groups were analyzed using one-way ANOVA with maternal age as the main effect. The joint effect of maternal age and lipid abundance at birth on life history traits was analyzed using heterogeneity of slopes ANCOVA, with maternal age as a categorical main effect and lipid abundance as a continuous covariable.

For the mitochondrial membrane potential analysis, the Michaelis-Menten function was fitted to median fluorescence data separately for offspring of young and old mothers with the horizontal asymptote parameter F_{max} interpreted as total mitochondrial capacity and the Michaelis-Menten constant K_m interpreted as a parameter inversely proportional to membrane potential. The Akaike information criterion was used to determine whether the Michaelis-Menten curve resulted in a better fit than linear regression. When it did not, linear regression was used, with the slope being proportional to membrane potential. The magnitude of difference in the fitted Michaelis-Menten function between maternal age groups was assessed using the relative likelihood of the two fitted models based on the Akaike information criterion.

Results

Because we report the results of several experiments that differed in experimental protocol and tested different hypotheses, the results are summarized in table 1.

Life Span

The effect of maternal age on the life span of generation 2 individuals was clone specific and differed between experiments in which individuals were maintained in groups of five in 100-mL jars (experiment 1) versus individually in 20-mL vials (experiment 2). In experiment 1 (fig. S2), only one out of five clones tested showed a significant reduction in life span (HU clone, $P < .003$) among daughters of older mothers. Unexpectedly, two clones showed the opposite effect: higher life expectancy among daughters of older mothers. In experiment 2, one of the two clones used (GB) showed a significant effect of maternal age on generation 2 life span, again in the opposite direction to the

Lansing effect prediction, with offspring of older mothers surviving longer than offspring of younger mothers. Individuals in the maternal age reversal treatment showed intermediate life span (fig. 2). In both experiments maternal age was therefore not a highly significant main effect, but the clone × maternal age interaction was (table 2).

Offspring Sex Ratio

Maternal age (i.e., the age of generation 2 females) had little effect, if any, on offspring sex ratio (table S2; fig. S4). The grandmaternal age, however, both in the initial unplanned observations and in the subsequent two experiments designed to observe the Lansing effect, showed a clone-specific effect on the grandchildren sex ratio (figs. 3, S3). The effect was not observed in some clones, and when it was observed it had different directions in different clones, resulting in a significant interaction term (tables 3, S2). Daughters of older mothers tended to produce more sons in the least male-producing clone (GB) and fewer sons in the most male-productive clone (FR). Thus, clonal differences in male production were smaller in daughters of older mothers than in daughters of younger mothers (fig. 3).

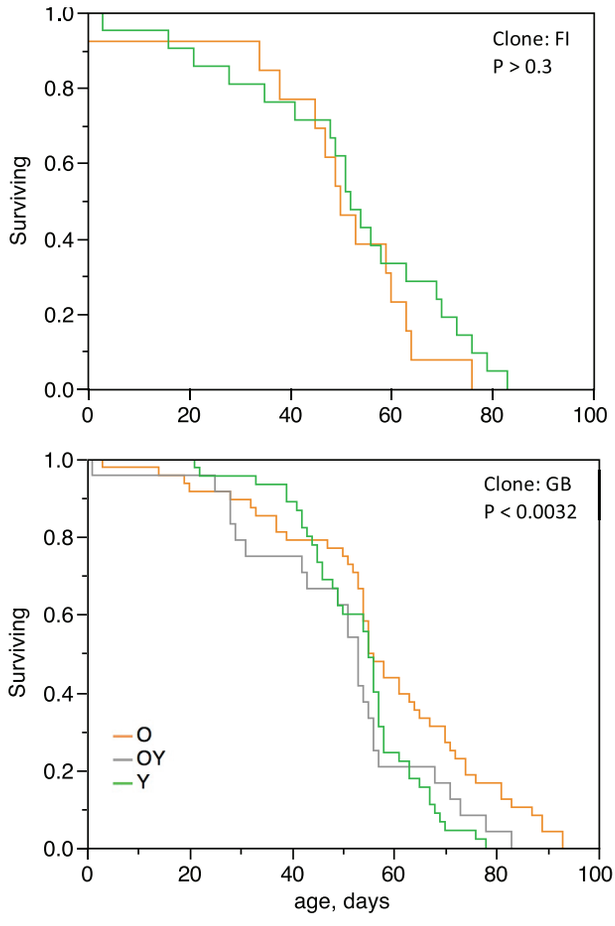

Figure 2: Survival curves of daughters of young (Y; green) and old (O; orange) mothers in two *Daphnia* clones in experiment 2. Gray in clone GB: maternal age reversal treatment (OY). *P* values shown are for the effect of maternal age in a proportional hazards test of daughters' longevity.

Table 2: Proportional hazards test of the effects of clones and generation 1 individuals' age (maternal age [MA]) on the longevity of generation 2 females in two separate experiments (see figs. 2, S2)

Source	df	LR χ^2	*P*
	Experiment 1		
Clone	4	145.74	<.0001
MA	1	3.83	.0503
Clone × MA	4	22.13	<.0001
	Experiment 2		
Clone	1	.57	.13
MA	1	.90	.034
Clone × MA	1	6.34	.012

Note: Y and OY treatments were combined for the GB clone for the purpose of this analysis. LR = likelihood ratio.

Interplay between Male Production and Longevity

We then asked the question of whether the effect of maternal age on the life span of generation 2 individuals was independent from that on the sex ratio among their offspring (fig. 4; table 4). The effect of maternal age on life span was particularly pronounced among generation 2 females that produced no male offspring early in life. Among all generation 2 females, male offspring production early in life positively correlated with life expectancy in daughters of young mothers but remained flat in daughters of older mothers, with the daughters born in the maternal age reversal treatment again showing an intermediate pattern. Of the two clones in experiment 2 analyzed separately, only the GB clone showed a significant effect of early male production on the longevity of generation 2 females (table 4).

Lipid Provisioning

Analysis of the joint effect of maternal age and maternal neonate lipid provisioning is presented in table 5. The same analysis without lipid abundance but utilizing a more complete set of life history data for the two clones used in experiment 2 is shown in table S3. Maternal lipid provisioning of neonates correlated positively with the size of neonates at birth, with older mothers giving birth to larger, more lipid-rich offspring (table 5). This size difference completely disappeared by offspring maturity, with neither maternal age nor lipid provisioning being a good predictor of size at

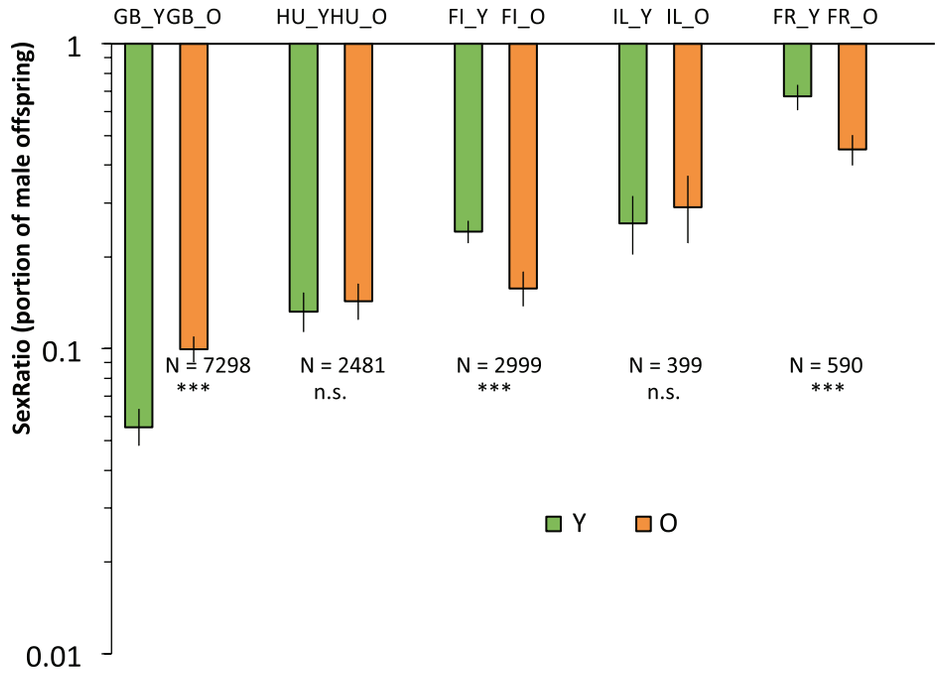

Figure 3: Sex ratio (portion of male offspring produced during the first 35 days of life) in daughters of young mothers (Y; green bars) and old mothers (O; orange bars) in five *Daphnia* clones. Clones are labeled by the first two letters of clones' IDs (table S1) and are ordered from low male producing to high male producing. Shown are the number of offspring sexed and two-tailed *P* values from a Fisher exact test of the effect of grandmaternal age on offspring sex. Data were combined from two experiments. See table 1 for statistical details. See figure S1 for the same data for all maternal ages and for the two experiments separately. $^{***}P < .001$; n.s. = not significant.

the time the first clutch of eggs is produced. The only factor affecting this parameter was clones (table 5). It should be noted that this difference was significant only when lipid provisioning was accounted for (tables 5, S3). Despite there being no difference in adult size either between daughters of old and young mothers or between individuals originating in high- versus low-lipid-provisioned clutches, the amount of lipids at birth was a good predictor of the total number of offspring produced in the first 40 days of life (table 5). Finally, late-age fecundity showed no effect of lipid abundance at birth but demonstrated a slight effect of maternal age, with daughters of older mothers producing more offspring per surviving individual, indicating that they did not just live longer but remained reproductively more active late in life. It should be noted that only two individuals among the daughters of younger mothers produced any offspring within this age class, so this result may be highly biased.

In one of the two clones tested (GB; fig. 5), maternal age affected lipid provisioning to offspring. The offspring of younger mothers received approximately three times more lipids than the offspring of older mothers, with the maternal age reversal offspring showing intermediate values (fig. 5). The other clone, FI, showed the opposite tendency, which did not reach statistical significance. The maternal age effect of generation 2 individuals' offspring sex ratio in both clones completely disappeared once lipid provisioning was accounted for (table 6), indicating that this effect could be entirely accounted for by lipid abundance at birth. In turn, lipid abundance at birth as a continuous main effect was not a predictor of either longevity or male production when analyzed across both clones. The only terms that remain significant in the model with lipid abundance at birth as a covariable are the interaction terms, indicating that lipid provisioning and its effects on offspring were clone

Table 3: Differences among clones, differences between grandmaternal age classes (GMA), and interaction effects in the sex ratio of offspring (see figs. 3, S3)

Source	df	LR χ^2	*P*
Clone	4	934.7	<.0001
GMA	1	2.66	.10
GMA × clone	4	111.3	<.0001

Note: Shown is a generalized linear model with nominal logistic fit assuming a binomial distribution and logit link function (JMP; SAS Institute 2012). LR = likelihood ratio.

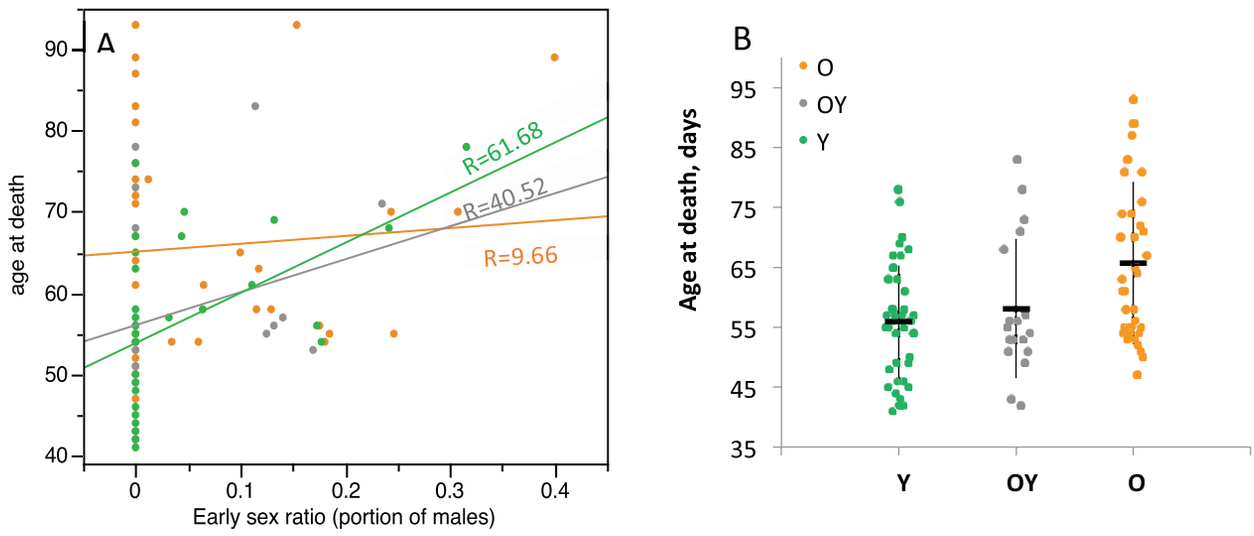

Figure 4: *A*, Correlation between male production and longevity in daughters of young and old mothers in the GB clone. O (orange) = daughters of older mothers; Y (green) = daughters of young mothers; OY (gray) = reversal treatment. Early mortality (age at death <40 days) is excluded. Regression for all data combined: $R = 37.6$, $P < .009$ (line not shown); regression coefficients for grandmaternal age classes are shown separately next to the regression lines. See table 3 for the effects of offspring sex ratio on maternal survival in proportional hazards tests. *B*, Subset of data for females producing no males (sex ratio = 0 in *A*). Individual data points and means and standard deviations are shown. ANOVA difference between groups: $df_{den} = 2$, $df_{num} = 38$, $F = 8.43$, $P < .001$). Tukey test at $\alpha = .05$: O is different from Y and OY. Tukey test at $\alpha = .01$: Y is different from O, OY is not significantly different from either.

specific, at least in the two clones for which lipid abundance data are available. Specifically, in clone GB older mothers supplied fewer lipids to their daughters than younger mothers, while in clone FI it was the other way around (fig. 5). As a result, in each clone generation 2 individuals coming from better-provisioned clutches had a shorter life span regardless of maternal age (fig. 5*B*), although only in GB was this difference significant. This observation was concordant with higher early-life offspring production in generation 2 females from better-provisioned clutches (table 5), indicating a possible trade-off.

Similarly, inclusion of lipid abundance at birth as a covariable eliminated the observed sex ratio effect, leaving only the three-way clone × maternal age × lipids interaction (table 6), consistent with the idea that genotype-specific maternal provisioning explains the propensity of daughters to produce male clutches and not maternal age per se.

Mitochondrial Potential in Offspring

Mitochondrial membrane potential was measured in a separate experiment 3 (table 1), with no life history data available. The only analysis possible was simply the comparison between daughters of young versus daughters of old mothers, obtained and measured in a common garden experiment. There was no interpretable difference in rhodamine 123 fluorescence between newborn daughters of older and younger mothers in either striated muscle (antenna) or heart muscle (fig. 6*A*, 6*B*) as well as in nonneural head tissue (fig. 6*G*). There was no sign of saturation over rhodamine 123 concentration in the heart muscle and no difference between maternal age groups; in the striated muscle in the antenna there was significant saturation in daughters of younger mothers but not in daughters of older mothers, indicating either higher total mitochondrial capacity in daughters of older mothers or higher membrane potential in daughters of younger mothers. In both

Table 4: Proportional hazards test of the effects of maternal age (MA) and early offspring sex ratio (SR; age <40 days) on longevity in experiment 2, generation 2 females

		Proportional hazards test	
Source	df	LR χ^2	*P*
		Clone = FI	
MA	1	.583	.45
SR	1	.041	.84
MA × SR	1	.562	.45
		Clone = GB	
MA	1	11.347	.0008
SR	1	5.107	.0238
MA × SR	1	1.381	.24

Note: Individuals that died prior to 40 days of age were censored to exclude bias in sex ratio estimates. Compare with figure 4. Y and OY treatments were combined for the GB clone. LR = likelihood ratio.

Table 5: ANCOVA of the joint effects of clones, maternal age (MA), and lipid abundance at birth (L) on life history parameters of generation 2 females in two clones with contrasting life histories (GB and FI)

Source	df	SS	*F* ratio	Prob > *F*	Sign of change
			Response: body length at birth		
MA	1	.0311	12.90	**.0025**	O > Y
L	1	.0213	8.84	**.0185**	$R = 1.884$ (.634)
MA × L	1	.0015	.62	.43	
Clone	1	.0048	1.98	.16	
MA × clone	1	.003	1.25	.27	
L × clone	1	.0005	.22	.64	
MA × L × clone	1	.001	.43	.52	
Error	96	.0024			
			Response: body length at maturity		
MA	1	.00002	.0009	.98	
L	1	.02713	1.04	.31	
MA × L	1	.02725	1.04	.31	
Clone	1	.50881	19.43	**<.0001**	FI > GB
MA × clone	1	.00293	.11	.74	
L × clone	1	.02518	.96	.33	
MA × L × clone	1	.01726	.66	.42	
Error	76	.02619			
			Response: age at first clutch birth		
MA	1	22.166	12.09	**.0032**	O < Y
L	1	.95	.52	.47	
MA × L	1	.609	.33	.57	
Clone	1	1.509	.82	.37	
MA × clone	1	2.812	1.53	.22	
L × clone	1	12.483	6.81	*.05*	
MA × L × clone	1	4.721	2.57	.11	
Error	93	1.834			
			Response: total offspring <40 days		
MA	1	117.613	.42	.52	
L	1	3,002.522	10.77	**.006**	$R = 707.6$ (215.6)
MA × L	1	1,802.12	6.46	*.063*	
Clone	1	101.567	.36	.55	
MA × clone	1	644.754	2.31	.13	
L × clone	1	1,533.318	5.50	*.084*	
MA × L × clone	1	273.884	.98	.32	
Error	96	278.859			
			Response: total offspring >40 days		
MA	1	219.255	4.42	.12	O > Y
L	1	14.924	.30	.58	
MA × L	1	24.735	.50	.48	
Clone	1	51.578	1.04	.31	
MA × clone	1	112.522	2.27	.14	
L × clone	1	156.681	3.16	.08	
MA × L × clone	1	147.433	2.97	.09	
Error	96	49.6			

Note: Sequential Bonferroni multiple test correction was applied to *P* values <.05 (for each main effect or interaction separately). R = regression coefficient (standard error). Lipid abundance was measured as the sum of intensities above an arbitrary threshold in neonate siblings from the same clutch. The maternal age variable includes only two levels (young and old). Bonferroni-adjusted *P* values <.01 are in boldface, and those <.1 are in italics. The rightmost column shows the directionality of the main effects. For categorical maternal age and clone effects, the sign of the difference shown (e.g., "O > Y" in the body length at birth section indicates that daughters of older mothers were larger at birth than those of younger mothers). For continuous effects, regression coefficients (and standard errors) are shown, with a positive regression coefficient indicating a positive correlation.

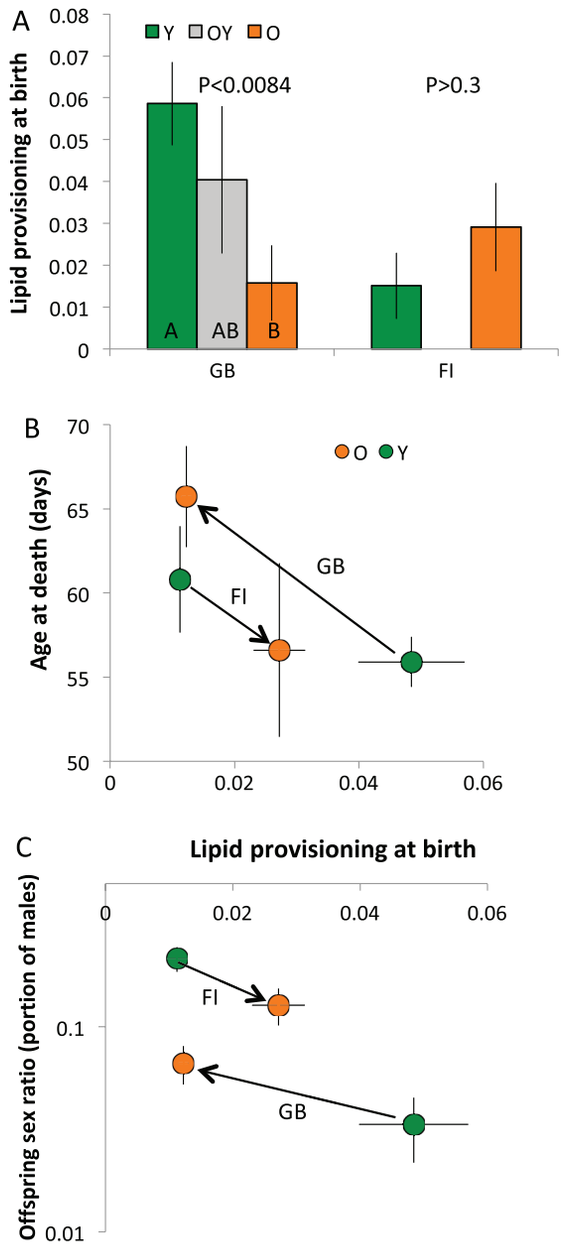

Figure 5: Lipid provisioning at birth in two clones with contrasting life histories (*A*) and its effects on offspring longevity (*B*) and male production (*C*). Lipid provisioning was measured in clutchmate sisters of generation 2 females as the portion of Nile Red stain image intensity above an arbitrary threshold chosen to capture fluorescence within lipid vesicles. Offspring sex ratio was measured as the portion of (predominantly) male clutches. Arrows show the direction of change with maternal age in each clone. Vertical and horizontal bars show standard errors based on variance among generation 2 individuals. Y and OY treatments in the GB clone were pooled in *B* and *C*. In *A*, *P* values are from a one-way ANOVA within each clone; letters indicate significant differences in the Tukey post hoc test (levels not significantly different at $P < .05$ share a letter). See table 5 for statistical analyses of data in *B* and *C*.

neural tissues analyzed, however, daughters of older mothers showed earlier saturation (higher K_m), indicative of higher $\Delta\Psi_m$ (fig. 6*B*, 6*C*). For excretory tissues (epipodite and nuchal organ), newborn daughters of younger mothers showed higher fluorescence across the range of rhodamine 123 concentrations, possibly indicating higher membrane potential, but the high variability of estimates did not allow an unequivocal comparison of K_m and F_{max} estimates.

Discussion

We have shown that in *Daphnia*, the Lansing effect (i.e., the effect of maternal age on daughters' life span and other life history characteristics) can act in both directions, in agreement with a recent similar finding in *Caenorhabditis elegans* (Travers et al. 2021). The inverse Lansing effect is genotype and condition specific, with only one of the genotypes showing it in each of the experiments. Furthermore, maternal age affects the propensity of daughters to produce males in a genotype-specific manner. Early male production correlated with greater life span, with both likely being correlates of maternal provisioning. Thus, in *Daphnia* there is no fully penetrant detrimental effect of maternal age on daughters' life history. Rather, age-related changes in maternal lipid provisioning appear to shape the magnitude and even direction of the effect.

There is a substantial caveat to this conclusion: we have lipid-provisioning and detailed life history data only for

Table 6: Analysis of the joint effects of generation 1 females' age and lipid provisioning to daughters on daughters' longevity (top) and male production (bottom)

Source	df	LR χ^2	*P*
	Proportional hazards model: longevity		
Clone	1	2.91	.09
MA	1	5.88	.015
Clone × MA	1	1.31	.25
L	1	1.04	.31
Clone × L	1	6.22	.013
MA × L	1	1.25	.26
Clone × MA × L	1	.66	.42
	Generalized linear model: portion of male clutches		
Clone	1	20.51	<.0001
MA	1	1.78	.18
Clone × MA	1	1.79	.18
L	1	8E−5	.99
Clone × L	1	.028	.87
MA × L	1	2.03	.15
Clone × MA × L	1	5.64	.0175

Note: L = lipids; LR = likelihood ratio; MA = maternal age.

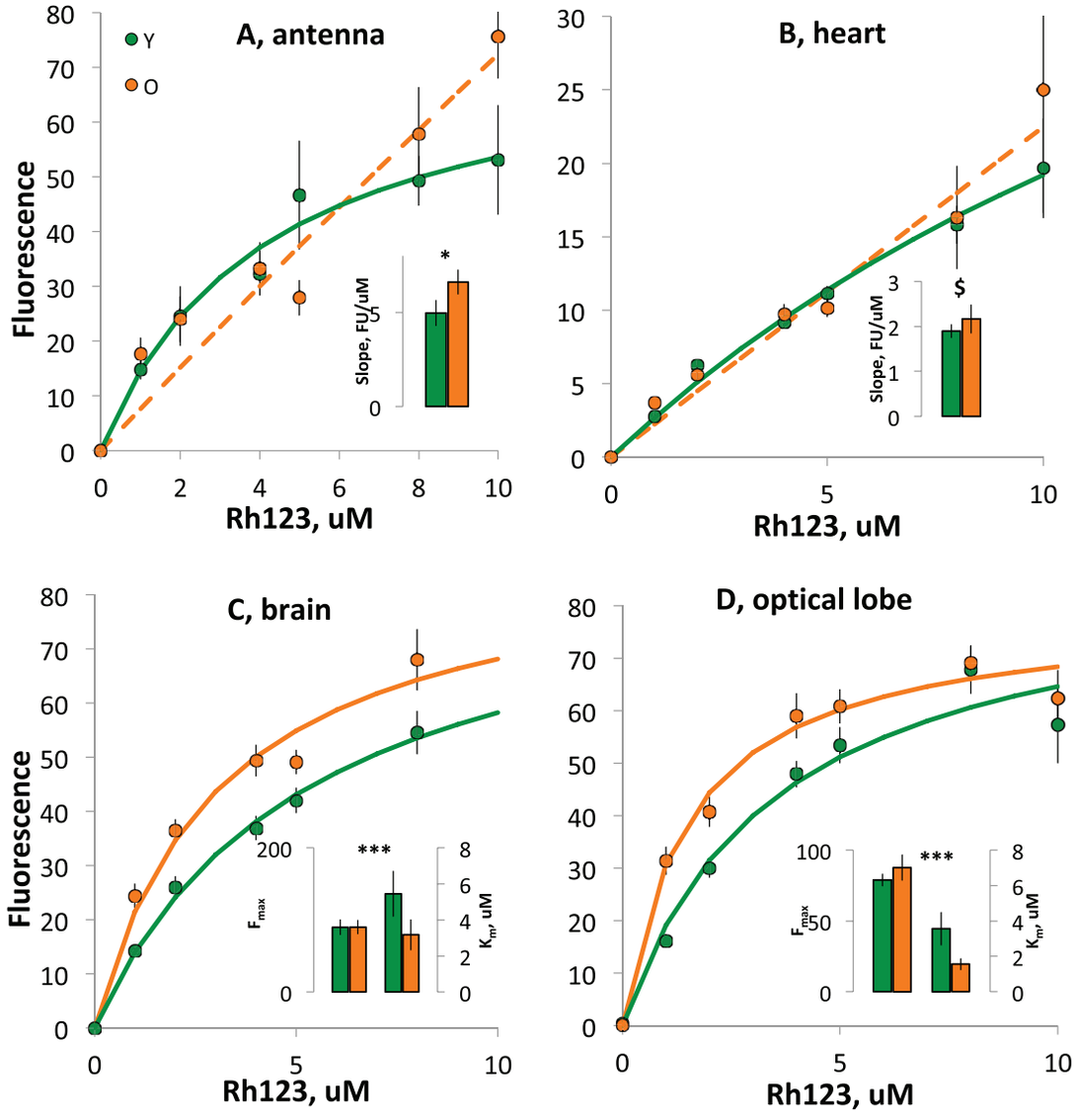

Figure 6: Rhodamine 123 (Rh123) fluorescence (arbitrary units) in neonates born to young (Y; green) and old (O; orange) mothers after 24 h of exposure. Shown are fitted Michaelis-Menten curves (dashed where there is no improvement over linear fit). *A*, *B*, muscular tissues; *C*, *D*, neural tissues; *E*, *F*, excretory/osmoregulatory organs; *G*, nonneural head tissue. Insets show fitted estimates of Michaelis-Menten or linear fit parameters, as appropriate (with approximate standard errors): F_{max} = maximal fluorescence (arbitrary units; interpretable as total mitochondrial capacity); K_m = concentration at half F_{max} (μM; interpretable as the inverse of mitochondrial potential); slope = slope of linear regression (fluorescence units μM^{-1}; interpretable as a correlate of mitochondrial potential). Akaike information criterion–based relative likelihoods (relL) of the two models being different are shown as follows: ***relL < .0001, **relL < .001, *relL < .05, $^{\$}$relL > .01. Note the different fluorescence scale.

clones leaning toward the inverse Lansing effect, not for clones showing the classic relationship. This limits the applicability of these results to Plaistow et al.'s (2015) conclusion about maternal provisioning being causative to the Lansing effect, particularly given that this conclusion has been obtained in a different species of *Daphnia*, one with a much shorter life span. This deficiency could be satisfied by further studies with genotypes showing both the classic and the inverse Lansing effect. There is an additional caveat to our main result. Since there are no studies of the effects of Nile Red staining on life expectancy, lipid provisioning was measured in siblings from the same clutch, not in the females used in the life table experiment. Although the within-clutch variance in lipid abundance is small relative to the among-clutch variance, this still may be a source of uncertainty. Yet despite these caveats,

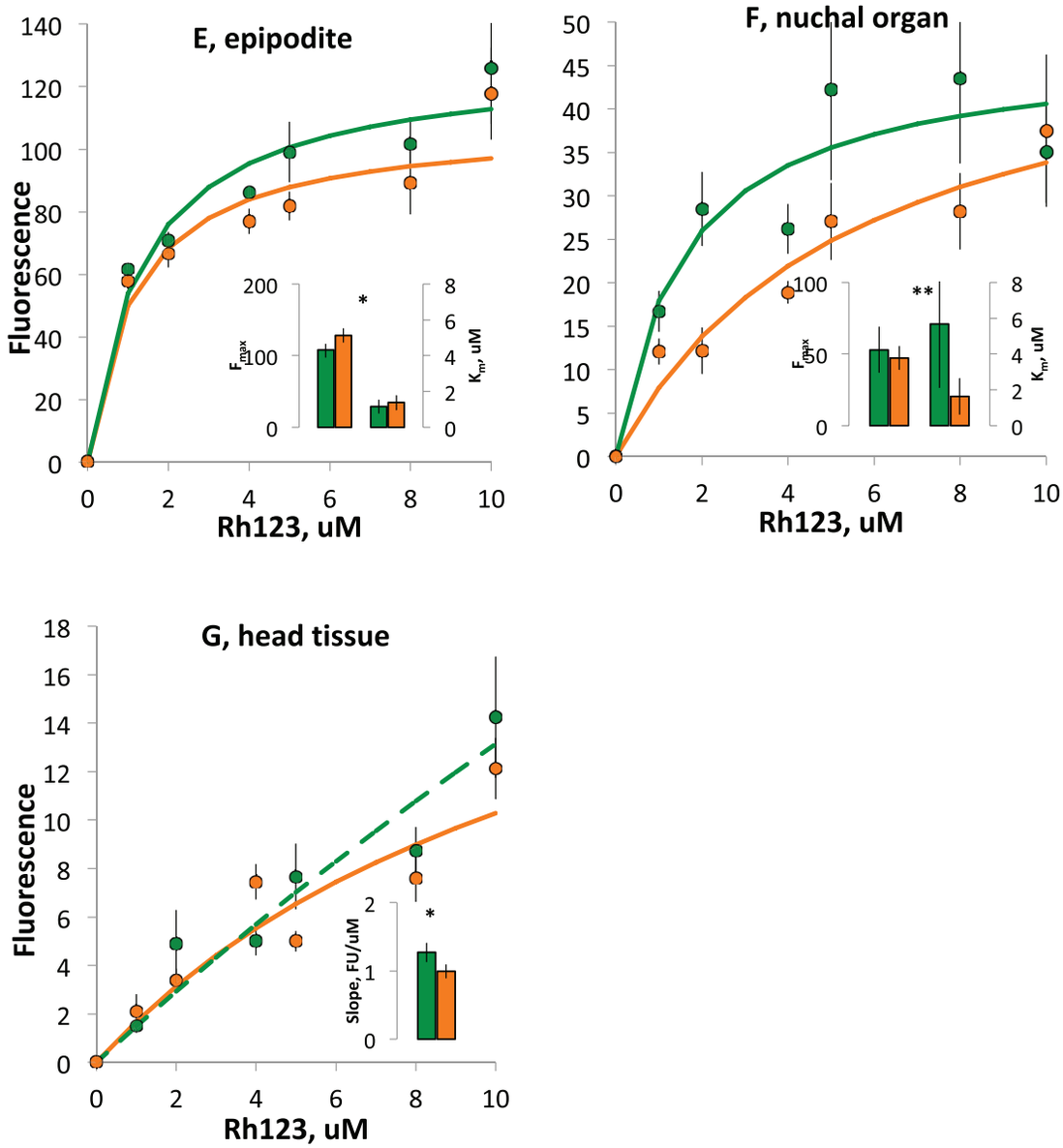

Figure 6: (*Continued*)

the observed inverse Lansing effect can be fully explained by the clonal differences in maternal lipid provisioning to neonates: in the GB clone in which older mothers provide substantially fewer lipids to neonates, the offspring live longer and produce more sons.

It should be noted that this reduced provisioning is almost certainly independent from maternal body size. While larger females are known to produce larger, better lipid-provisioning daughters (Glazier 1992), in this study we see the opposite effect, at least for the two clones with lipid-provisioning data. The GB clone has consistently smaller body size at maturity than the FI clone (in this experiment, 2.59 ± 0.17 [SD] mm vs. 2.98 ± 0.15 [SD] mm; see also Coggins et al. 2021*b*; Anderson et al. 2022). Yet females from the GB clone supply their offspring with higher amounts of lipids than those from the FI clone at young age and decrease this provisioning as they get larger. While we did find that older, larger females give birth to larger daughters, this size difference disappears by the time the daughters reach maturity. This body size compensation in the daughters of younger mothers probably occurred through longer development time to maturity.

There is an additional consideration related to well-characterized life history differences between the two clones for which we have lipid-provisioning data, namely, differences in the habitat of origin. Of course, no generalization is possible based on just two clones, but one might hypothesize that age-specific investments into offspring is the product of previous seasonality-dependent selection. The GB clone originated from a permanent water body, Regents Park pond in London, United Kingdom, in which while there are certainly seasonally variable conditions, *Daphnia* nevertheless occur throughout the year (D. Ebert, personal communication). In contrast, the FI clone originated from a highly intermittent rock pool that experiences frequent drying out in summer and regular freezing in winter (D. Ebert, personal communication) and in which *Daphnia* populations are likely to be eliminated frequently, reestablishing themselves from resting eggs (Pajunen and Pajunen 2003; Altermatt and Ebert 2010). It is conceivable that the ancestral population of the FI clone has never been under selection for fecundity and offspring quality at an advanced age, as the short life span of a habitat precludes *Daphnia* from ever reaching it. In contrast, the GB clone is likely to have been selected for persisting through winters, when survival is possible but reproduction is limited. It would therefore be the oldest overwintering females that resume reproduction in spring during high availability of algae, when the trade-off between offspring number and offspring provisioning is likely to be resolved in favor of higher numbers and lower per-offspring provisioning. It is worth noting that this seasonality-based scenario is somewhat resemblant of that recently described in *C. elegans*, where offspring competition for seasonal resources appears to be the reason for a longer, not shorter, life span of offspring of older mothers (Travers et al. 2021).

Higher life expectancy in less provisioned neonates can be readily interpreted as a manifestation of caloric restriction—probably the most reliable environmental intervention extending life span across almost all animals studied (Fontana et al. 2010; Kapahi et al. 2017). There is a growing body of evidence that caloric restriction may extend longevity not only in organisms experiencing it continuously and throughout the life span but also in organisms experiencing it early in life or even in the parental germline (Brakefield et al. 2005; Barnes and Ozanne 2011; Davis et al. 2016). Lipids are the main storage nutrients in many crustaceans, including *Daphnia* (Goulden and Place 1993; Smirnov 2017). Maternal provisioning of oocytes supplies them with a significant amount of mostly triacylglycerides, which have a profound effect on offspring life history (LaMontagne and McCauley 2001; Garbutt and Little 2014; Sperfeld and Wacker 2015). It is therefore to be expected that reduced lipid provisioning by older mothers can mimic caloric restriction during embryonic development.

What could the relationship be between lipid provisioning and the production of male offspring by generation 2 females (fig. 5*C*)? Increase in male production by poorly provisioned daughters of older mothers in the GB clone appears to be a manifestation of lower caloric intake during these individuals' early life, something that is known to increase male production (Hobaek and Larsson 1990; Klieven et al. 1992). We can suggest a very specific mechanism of such a link. Accumulation of lipid droplets (Jordão et al. 2016) and allocation of glycerolipids into eggs (Fuertes et al. 2018) in *Daphnia* are both regulated by methyl farnesoate, a member of the juvenile hormone family found in many crustacean species. Methyl farnesoate also regulates male production (Olmstead and Leblanc 2002; Lampert et al. 2012*b*; Toyota et al. 2015). Specifically, methyl farnesoate treatment increases deposition of triacylglycerides in somatic tissues and reduces their deposition into the eggs (Jordão et al. 2016; Fuertes et al. 2018). Thus, it is expected that females that deposit fewer lipids into their eggs, such as old-age GB females, also send a high male production hormonal signal to their offspring. It is noteworthy that the observed effect of advanced maternal age—increase of male production in low-male-producing clones and vice versa—exactly mirrors the effect of methyl farnesoate treatment with a long photoperiod reported by Lampert et al. (2012*b*). It should be noted, however, that a previous study (LeBlanc et al. 2013) did not find a second-generation effect of hormonal male production induction, at least when a more potent juvenile hormone mimic, pyriproxyfen, is used. This must mean that the transgenerational effects of maternal age on daughters' male production must engage some additional epigenetic mechanism with longer-lasting effects than simple exposure to juvenile hormones.

Reduced methyl farnesoate synthesis is also consistent with reduced mitochondrial function. Methyl farnesoate as well as other sesquiterpenoid juvenile hormones is synthesized via the so-called JH branch of the universal mevalonate pathway (Noriega 2014). In insects, a critical early step of this pathway is the transport of citrate from the mitochondria to the cytoplasm: inhibiting this step inhibits juvenile hormone biosynthesis (Sutherland and Feyereisen 1996; Nouzova et al. 2015). One may hypothesize that increased mitochondrial membrane permeability may result in increased citrate diffusion into the cytoplasm, resulting in upregulation of the mevalonate pathway. It may be a general mechanism of hormonal response to increased mitochondrial membrane permeability due to toxicants, hypoxia, or age-related damage. We have recently shown that mitochondrial membrane potential in mitochondria-rich epipodite (gill) tissue is reduced with age (Anderson et al. 2022), consistent with the reduced membrane potential in the same tissue in daughters of older mothers reported here (fig. 6*E*). It is tempting to suggest that older mothers do transmit "leaky" mitochondria

to their daughters, at least in some genotypes, which might provide a possible explanation for the inverse Lansing effect, as leaky mitochondria generate less reactive oxygen species. However, if this were true, one would expect the same reduced $\Delta\Psi_m$ in all daughter tissues. This is not what we observe: in excretory and osmoregulatory tissues $\Delta\Psi_m$ was lower, but in neural tissue it was higher in the neonates born to older mothers (fig. 6*C*, 6*D*). This difference requires an explanation. Either germline mitochondria are not in any way compromised by advanced maternal age and the observed differences develop through some other tissue-specific maternal age effects or, possibly, mitochondria present in different parts of an oocyte and therefore allocated to different embryonic tissues are characterized by a different degree of age-related damage or repair.

Conclusions

The Lansing effect in *Daphnia* can be genotype specific, with an inverse effect possible (i.e., longer life in daughters of older mothers). This inverse effect may be related to the lower lipid provisioning of neonates by older mothers, resulting in caloric restriction during embryonic development. Such a mechanism is consistent with the observed regression to the species mean in male offspring produced by daughters of older mothers.

Acknowledgments

We thank Camille Homa and Catherine Pearson for laboratory assistance and D. Kumar and G. Arceo-Gomez (East Tennessee State University) and the Nikon Imaging Center (Harvard Medical School) for access to laboratory equipment. This work was supported by the Paul G. Allen Frontiers Group, the McKenzie Family Charitable Trust, and the Impetus Foundation. The authors declare no competing interests.

Statement of Authorship

Conceptualization: L.Y.Y., M.W.K., L.P., Y.C., R.A.J.-C.; funding acquisition: M.W.K., L.P.; methods development/experimental design: L.Y.Y., C.E.A.; data collection: C.E.A., L.Y.Y.; data analysis: L.Y.Y.; data validation: L.Y.Y.; data visualization: L.Y.Y., C.E.A.; provision of resources (including specimens, reagents, and equipment): M.W.K., L.P., Y.C., R.A.J.-C.; model analysis: L.Y.Y.; supervision: L.Y.Y., L.P.; writing—original draft: L.Y.Y.; writing—review and editing: L.Y.Y., M.W.K., C.E.A.

Data and Code Availability

Data sets and fluorescent microscopy images are available in the Dryad Digital Repository (https://doi.org/10.5061/dryad.q83bk3jj6; Yampolsky 2022).

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Associate Editor: Jeff Leips
Editor: Daniel I. Bolnick

"Footprints of Dinornis (Moa)." From "On the Occurrence of Footprints of Dinornis at Poverty bay, New Zealand" by C. D. Voy (*The American Naturalist*, 1880, 14:682–684).