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Author for correspondence:
M. Nystrand
e-mail: magdalena.nystrand@monash.edu

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Transgenerational interactions involving parental age and immune status affect female reproductive success in *Drosophila melanogaster*

M. Nystrand and D. K. Dowling

School of Biological Sciences, Monash University, Clayton, Victoria 3800, Australia

It is well established that the parental phenotype can influence offspring phenotypic expression, independent of the effects of the offspring's own genotype. Nonetheless, the evolutionary implications of such parental effects remain unclear, partly because previous studies have generally overlooked the potential for interactions between parental sources of non-genetic variance to influence patterns of offspring phenotypic expression. We tested for such interactions, subjecting male and female *Drosophila melanogaster* of two different age classes to an immune activation challenge or a control treatment. Flies were then crossed in all age and immune status combinations, and the reproductive success of their immune- and control-treated daughters measured. We found that daughters produced by two younger parents exhibited reduced reproductive success relative to those of other parental age combinations. Furthermore, immune-challenged daughters exhibited higher reproductive success when produced by immune-challenged relative to control-treated mothers, a pattern consistent with transgenerational immune priming. Finally, a complex interplay between paternal age and parental immune statuses influenced daughter's reproductive success. These findings demonstrate the dynamic nature of age- and immune-mediated parental effects, traceable to both parents, and regulated by interactions between parents and between parents and offspring.

1. Introduction

It is well known that the maternal phenotype and genotype can exert sizeable effects on the offspring phenotype, independent of the offspring's genotype [1], and that such effects may be of considerable evolutionary and ecological significance [2]. These effects can be mediated, for example, through transfer of nutrition via the egg, direct resource provisioning, protection against predators, or via the passive transfer of immune factors to offspring [1,3]. Recently, evidence has also accumulated that suggests sizeable contributions of non-genetic paternal effects to offspring fitness [4–6], such as, for example, the transfer of accessory gland proteins [4,7], epigenetic mechanisms [8] or via the transfer of immune factors [9]. However, compared with the wealth of studies exploring maternal effects, little is still known about the pervasiveness and magnitude of paternal effects, particularly in species that lack overt paternal care [6]. The main reason for this is that the paternal influence in such species was traditionally considered to be limited to the haploid genotype that each sire contributes to the offspring, or in some systems extending to the effects associated with external resource supply such as food provisioning or extra-gamete provisioning such as nuptial gifts [1,2].

Two factors that are core components of organismal life history, and known to be pivotal mediators of parental effects, are individual age and immune capacity. The majority of eukaryotes exhibit declines in physiological performance associated with increasing age [10–12], which renders age-mediated variance in parental condition a likely source of variance contributing to patterns of expression in the offspring phenotype [13,14]. Most notably, it is well

established that old mothers often produce shorter-lived offspring, a phenomenon known as 'the Lansing effect' [13,15,16]. Parental age effects have also been documented for other components of offspring life history, such as indices of reproductive output [10,13,16–18] and development or growth rates [14,19,20]. Moreover, such effects are likely to be exacerbated via interactions with extrinsic sources of environmental stress—such as deviations from the optimal climatic or dietary environment [1,21,22].

Immune capacity is known to be sensitive to age-related senescence [23–27], and immune-related traits are also heavily entwined in non-genetic transgenerational processes [1,3,13]. This is particularly well documented for the adaptive branch of the immune system that is present only in vertebrates [3,28]. In this adaptive branch, studies have demonstrated age-related deterioration caused by alterations of T cells and a decreasing abundance of naive B cells [25], as well as general transgenerational effects in which mothers transfer immune factors to offspring through the placenta, colostrum or breast milk [3,28]. Less is known about the capacity for age-dependent or transgenerational effects in the innate branch of the immune system common to all metazoans [9,25–27,29–31]. Historically, it was assumed that the innate immune system only provided immediate, non-specific immune responses, lacking in immune memory [32] and transgenerational transfer of immune factors [33]. This view is, however, being increasingly challenged by studies across a range of invertebrates [9,33–35], whose findings support the premise of transgenerational transfer of innate immunity [36].

Despite the extensive study of maternal effects *per se* [1,3,13,37], and increasing attention devoted to paternal effects [4,6], the extent to which intrinsic and extrinsic parental sources of variance interact to shape the offspring phenotype remains highly elusive and the subject of limited research [20,38]. However, the presence of such interactions is likely to have evolutionary ramifications [20]. This is because their existence would imply that phenotypic plasticity in the parental generation, and more specifically complex interactions between non-genetically determined parental phenotypes, could be important contributors to offspring phenotypic expression [39]. In fact, the magnitude of such effects might even outweigh the contributions associated with heritable functional allelic variation [39–41].

Here, we explore the degree to which interactions between parental sources of variance interact to shape the offspring phenotype in a laboratory-reared population of the fruitfly *D. melanogaster*. The study population is maintained on a discrete generation cycle in which the entire reproductive fitness of each generation is achieved within the first 5 days of their adult life. Potential implications of this generation cycle are discussed in full in later sections (within the 'Material and methods' and 'Discussion'). We focus on interactions involving the two classic traits that have historically been the focus of parental effects research—age and immune capacity. Specifically, we use an assay of immune activation, designed to assess an individual's ability to activate their immune system in response to a non-replicating pathogen, as our proxy of innate immune capacity. This approach avoids exposure of the individual to a live pathogen, and hence allows us to untie the effects brought about by host upregulation of their immune system on the one hand, and additional confounding effects that would arise if the pathogen was to be transmitted from parents to offspring on the other. Hence, this set-up is salient to the transgenerational context of our study. We experimentally address the extent to which interactions between maternal and paternal age, and maternal, paternal and offspring immune activation, shape early-life reproductive success. By exploring the interaction between parental immune status and offspring immune status, we are also able to explore the potential for parental transfer of innate immunity factors, a controversial premise with emerging experimental support [9,33–36,42,43].

2. Material and methods

(a) Fly stocks

The base stock population was founded by 60 wild-caught non-virgin females that were collected from three different localities within Coffs Harbour, New South Wales, Australia, in February 2010. Each of the wild-caught females contributed 10 sons and 10 daughters to those of the other 59 females, to create a single mass-bred population. This mass-bred population was maintained for 2 years across twelve 10-dram vials, each containing 20 adult pairs, under standardized rearing conditions of 25°C at a 12 L : 12 D cycle, and ad libitum access to live yeast, with a potato–dextrose–agar–yeast substrate. In this laboratory population, each new generation is propagated during a 20 h ovipositioning period that takes place when adult flies are 4 days old. Egg densities are subsequently trimmed to moderate densities of 150 per vial, and then all eclosing offspring are admixed with those of the other 11 vials each generation, prior to their sorting into new vials. Thus, in this population, a female's reproductive success is primarily determined by her ability to produce viable offspring during a 20 h period early in adult life [44]. While this 'discrete generations cycle' is standard for many laboratory-reared populations of *D. melanogaster* [45], it is interesting to consider the possible implications of a culturing procedure that selects so strongly for early-life fitness. According to classic evolutionary theory, selection for early-life fitness should result in the evolution of rapid senescence and shorter longevity [46,47], but curiously, such patterns have not been observed for laboratory populations of fruitflies [45]. In fact, our study population still live on average for around 50 days (45 days post-reproduction), with some individuals living for over 80 days [44]. Thus, while in theory the rearing design might mean that our laboratory population flies will suffer effects of senescence at an earlier adult age than their wild-living counterparts, in practice this is unlikely. We return to this issue in the 'Discussion'.

In February 2012, we created two replicate populations from the population described above. Each of these replicate populations was thereafter reared across three 250 ml bottles, with the life cycle of each replicate population separated temporally by one week, such that experimental focal flies were available to collect on a weekly cycle. To propagate each replicate population, 150–200 flies from each of the three bottles, for each replicate, were admixed every generation at 4–5 days of age, and provided with a 2 h period to oviposit. Egg densities were then controlled at 200–300 eggs per bottle. These populations were maintained like this for 35 generations, and the experiment described below commenced in June 2013.

To commence the experiment, females (4–5-day-old adults) were collected from each of the two bottles per population replicate, and translocated to two replicated ovipositioning chambers (flat media surface for egg laying with a diameter of 80 mm), in which they were allowed to lay eggs for 4 h. These females (the grandparents of the experimental daughters) were then discarded, and 30–50 eggs per ovipositing chamber were collected and transferred to fresh vials containing potato–dextrose–agar–yeast

substrate. Virgin adult males and females, eclosing from these ovipositioning chambers, were collected 9 days later, and stored separately by sex. These flies constituted the parents of the experimental flies to be used in the experiment.

(b) Experimental set-up

(i) Parental age treatment

When parents were collected, they were immediately assigned to an associated maternal or paternal age treatment, each of which consisted of two levels—4 ('younger') or 14 days ('older') post-eclosion, at the time of their entry into the experiment. We created numerous single-sex vials per age class, such that the parents contributing to each replicate of the experiment were sourced from many different vials, thus mitigating any vial-sharing effects from confounding our interpretations. The flies assigned to the 14-day-old age class were transferred to new vials with fresh food thrice weekly.

(ii) Parental immune treatment

All parents were assigned to an immune treatment, consisting of two levels—an immune activation challenge (hereafter 'immune challenged') or a control treatment ('control treated'). Each fly was micro-injected into the abdomen, with a 41.4 nl dose of immune challenge or control, using a nano-injector (Nanoject; Drummond Scientific Company, Broomall, PA). Immune-challenged flies were injected with heat-killed bacteria, which allowed us to specifically focus on effects associated with the upregulation of the host immune system while avoiding the added effects associated with the disease costs of pathogenic infection [33,48]. Specifically, we used a dose of heat-killed *Micrococcus luteus* (provided by Micromone, Monash University, Australia; strain A204, and verified as heat-killed), diluted in phosphate-buffered saline, PBS (Sigma Aldrich tablet P4417, pH 7.4) to a concentration of OD600 = 0.1. *Micrococcus Luteus* is a gram-positive bacterium, which is abundant in the environment, including in soil and on decaying matter, and these are habitats that *Drosophila* are regularly in close contact with [49]. Live *M. Luteus* is relatively non-pathogenic to wild-type *Drosophila*, only inducing mortality rates of approximately 10–20%, 3 days post-injection (compare to mortality rates approximately 50–70% for *Enterococcus faecalis* and *Staphylococcus aureus*) [49]. Gram-positive bacterium mainly activates the *Toll* pathway in *Drosophila*, and primarily stimulates the cellular response through phagocytosis [49]. Control-treated flies received only the PBS. All flies were injected while anaesthetized under light CO_2 exposure for approximately 2 min at each of the respective age classes.

After injections, flies were transferred to fresh vials in single-sex groups of five, in which they were given 72 h to recover, with access to ad libitum live yeast. At the end of the recovery period, four parental flies of each age class and immune treatment and sex (per replicate) were exposed to a corresponding set of flies of the opposite sex for 5 h to enable matings to take place. This was conducted according to a factorial design across sexes, such that all possible combinations of maternal and paternal age (i.e. 'older' and 'younger') and maternal and paternal immune statuses (immune challenged versus control treated) were represented, and replicated per sampling block (×2 replicates per block; five sampling blocks in total, each separated by one week). This crossing scheme generated 16 different combinations of parental age, parental immune treatment and parental sex. Following matings, mothers were transferred collectively to a single vial, containing ad libitum yeast, and allowed to oviposit for 18 h, after which time 25 eggs were collected from each cross combination and placed in a new vial with substrate, to generate the focal offspring.

Whenever possible, 16 virgin daughters from each parental cross combination (×2 replicates per block) were collected upon eclosure into adulthood, 9 days following the parental ovipositing period, and transferred to fresh vials. At 48 h post-eclosion, the daughters were themselves assigned to an 'offspring immune treatment', which consisted of the same two levels as per the parental equivalent described above—immune challenged or control treated (i.e. injection with 41.4 nl of *M. Luteus* in PBS, or PBS alone). The offspring immune treatment was assigned such that half of the daughters of each parental cross combination received the immune challenge, and half the control. Focal daughters were then transferred to individual vials with access to ad libitum live yeast. After a 72 h recovery period, each daughter was exposed to a same-aged virgin male collected from the general Coffs Harbour population for 4 h, to enable mating. Each daughter was then transferred to an individual ovipositioning vial, without live yeast, in which she could lay eggs. Daughters were transferred to fresh vials every 24 h for 4 days, and then discarded. A pilot experiment exploring the correlation between reproductive output across 10 days showed a strong positive correlation between reproductive success summed over days 1–4, and summed over days 1–10 ($R^2 = 0.82$, $z = 1.49$, $n = 300$, $p \leq 0.001$). All offspring (i.e. grand-offspring to the flies subjected to the parental treatments described above) eclosing from these four ovipositionng vials (spanning a 96 h laying period) were counted 11 days post-ovipositioning. This trait thus represents early-life reproductive success of daughters and is also consistent with the culturing protocol of the laboratory population in which each generation is propagated by 4-day-old females [44]. A total of 581 daughters were assayed. For logistic reasons, we focused our sampling on daughters only.

(c) Statistical analysis

Analyses were run in R v. 2.15.3 [50]. The data (i.e. number of eclosed offspring) were overdispersed and zero-inflated, as evident from residual graphs and comparisons of information criteria between models. In addition, simulations of the 95% confidence intervals around the number of zero values expected from a full Poisson model with correction for overdispersion indicated zero-inflation of the data. Hence, we adopted a zero-inflated, negative binomial model to the data (NB1 fit: variance is calculated as $\phi\mu$), thus allowing for the zero-count outcomes to be a mixture of structural and sampling zeros using the package glmmADMB (http://glmmadmb.r-forge.r-project.org/glmmADMB.html) [51]. The response variable was daughter reproductive success; fixed factors were maternal and paternal age, maternal and paternal immune treatment, and daughter immune treatment; and random factors were sampling block and parental vial nested in sampling block. We tested for all interactions, up to second order, and progressively simplified a full model by removing non-significant parameters one at a time by comparing log-likelihood values between nested models. We note that a simpler model, limited to inferences based on first-order interactions, provides qualitatively similar results (electronic supplementary material, table S1).

3. Results

Although daughters produced by older mothers generally exhibited higher reproductive success than those produced by younger mothers, this effect was in part contingent on the age of the sire (table 1 and figure 1). Specifically, daughters produced by pairings of younger mothers and younger fathers had lower reproductive success than those produced by any other combination of parental ages. By contrast, daughters produced by older mothers had generally higher reproductive success if they had been sired by a younger rather than an older father (figure 1).

Table 1. Parental effects on daughter reproductive output. Model reduction was based on comparing log-likelihood values between nested models. Significant effects are denoted by italic type.

effects	d.f.	log-likelihood	deviance	$Pr\ (>\chi^2)$
maternal age	1	−3237.4	4.7	0.0302
paternal age	1	−3232.4	0.9	0.3428
maternal treatment	1	−3232.9	0.94	0.3323
paternal treatment	1	−3232.0	0.26	0.6101
offspring treatment	1	−3235.1	4.36	0.0368
maternal age × paternal age	*1*	*−3226.2*	*6.6*	*0.0102*
paternal age × maternal treatment	1	−3221.5	0.28	0.5967
paternal age × paternal treatment	1	−3221.7	0.4	0.5271
maternal treatment × paternal treatment	1	−3222.9	2.42	0.1198
maternal treatment × offspring treatment	*1*	*−3231.8*	*11.28*	*0.0008*
paternal age × maternal treatment × paternal treatment	*1*	*−3218.1*	*4.86*	*0.0275*

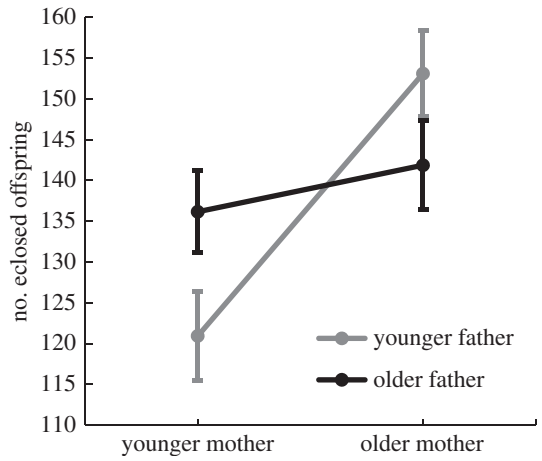

Figure 1. Daughter reproductive success (mean ± s.e.) across each combination of maternal and paternal age.

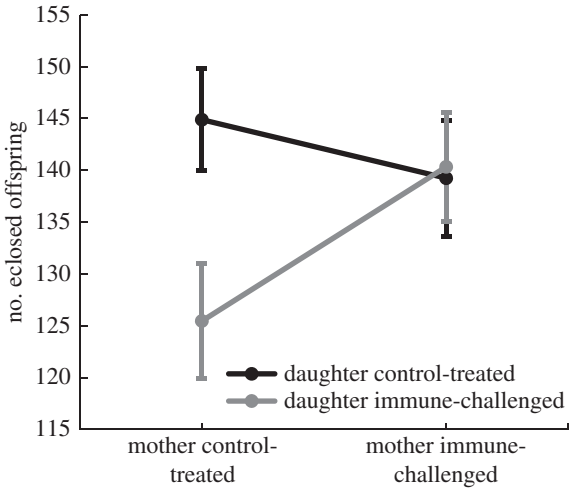

Figure 2. Daughter reproductive success (mean ± s.e.) across combinations of maternal and offspring immune treatment.

An interaction between maternal and daughter immune treatments affected daughter reproductive success. A striking pattern emerged among daughters whose mothers had received the control treatment. Among these, daughters that received the immune-challenge treatment exhibited markedly lower reproductive success than daughters who received the control (table 1 and figure 2). On the other hand, daughters produced by mothers who had received the immune-challenge treatment enjoyed relatively high reproductive success, regardless of whether they themselves received the immune challenge or the control (figure 2). This is consistent with what would be predicted under a scenario of transgenerational transfer of innate immune factors from immune-challenged mothers to daughters.

Daughter reproductive success was also shaped by a complex interaction between maternal and paternal immune treatments and paternal age (table 1 and figure 3*a*,*b*). Here, daughters sired by older control-treated fathers exhibited higher reproductive success than those sired by older immune-challenged fathers, with this pattern being insensitive to the immune status of the mothers (figure 3*a*). However, patterns involving younger fathers were contingent on the maternal immune treatment. In particular, daughters exhibited highest reproductive success when produced by the combination of immune-challenged mothers and younger but control-treated fathers (figure 3*b*), and reduced reproductive success when produced by control-treated mothers and younger control-treated males (figure 3*b*).

4. Discussion

We identified dynamic parental effects on transgenerational reproductive success, encompassing age- and immuno-mediated interactions between mothers and fathers, as well as maternal–offspring immuno-mediated interactions. In particular, two clear patterns emerged. First, the combination of two parents of the younger age class produced daughters that exhibited lower reproductive success relative to daughters produced by other maternal–paternal age combinations. Reproductive success was always higher for daughters that had at least one older parent. Second, daughter reproductive success was highest when both daughters and their mothers had received the control immune treatment, and were thus naive to an immune activation challenge. However, daughters that received the immune challenge had higher reproductive success when produced by mothers that had also received the immune challenge, relative to mothers that had received the control, and

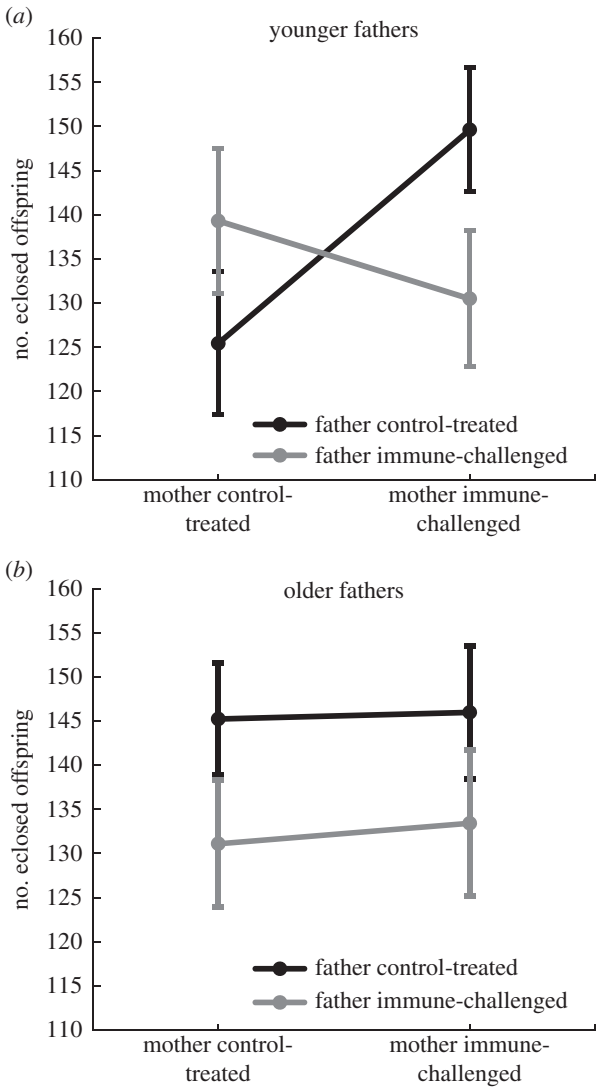

Figure 3. Daughter reproductive success (mean ± s.e.) across combinations of paternal and maternal immune treatments and paternal age with respect to (*a*) younger fathers and (*b*) older fathers.

this is consistent with some level of transfer of immune factors from mothers to daughters—a phenomenon that has not been previously demonstrated in *Drosophila* [31]. Finally, a complex interaction involving paternal age, paternal immune status and maternal immune status affected the expression of transgenerational reproductive success.

Senescence of immune function with increasing age is well documented [23,24,26]. However, information is scarce as to the contribution of immunity-by-age interactions to phenotypic expression, and the extent to which their effects may be transmitted across generations, hence contributing to the non-genetic variance underpinning offspring phenotypic expression. This is surprising considering the tangible parallel research attention devoted to the study of transgenerational age effects on the one hand [6,13,15,17,52], and transgenerational immune effects on the other hand, demonstrating immune responses resulting from both live pathogens and non-replicating immune elicitors [3,9,28,33]. We contend that the existence of parental age–immunity interactions could have important evolutionary and ecological implications. For instance, the inherent variation in age structure found in natural populations could drive widespread genotype-by-age interactions over immune capacity, and this could feed into our understanding and tests of current theories of pathogen-mediated adaptation, such as parasite–host coevolution [53,54] and parasite-mediated sexual selection [55,56]. For example, it is possible that transgenerational plasticity to the pathogenic environment might contribute more to immunocapacity and life-history expression in subsequent generations than does pathogen-mediated selection on allelic variation. Disentangling contributions of genetic and non-genetic parental influences on offspring phenotypic expression in response to pathogen-mediated selection remains a challenge for the future. Furthermore, attention should be invested in further disentangling the transgenerational effects of immune activation *per se*, as studied here, and transgenerational effects associated with the overall organismal immune response (i.e. including the disease responses associated with a live pathogenic infection). The use of live pathogens, however, requires careful experimental manipulation, such that effects of immune transfer can be disentangled from effects of pathogen transfer from parent to offspring.

Our study also demonstrated clear interacting effects of parental age that were independent of immune status, by way of a maternal age-by-paternal-age interaction on transgenerational reproductive success. Traditionally, parental age effects have mainly been explored from the perspective of the mother [1,2]. Recently, however, and contrary to traditional belief, research has emerged to show that general paternal effects can affect a number of life-history traits in the offspring [4,6], and that these effects may also be sensitive to age-related senescence in fathers [6]. For example, studies have associated older fathers with the frequency of birth defects in humans [57], with reduced reproduction and longevity in laboratory mice (*Mus musculus*) [58], and with reduced fertility, offspring weight and increased offspring mortality in brown Norway rats (*Rattus norvegicus*) [59]. Paternal age effects have also been recorded in insects; old fathers have been noted to produce longer-lived offspring in *D. melanogaster* [52], and older fathers exhibited lower reproductive output when exposed to metabolic stress in the butterfly *Pieris brassicae* [20].

The parental age effects we document in our study are striking, considering that the two age classes used were not extreme in their disparity (4 and 14 days at the time of application of the immune treatment). This suggests that relatively modest differences in parental age can influence the offspring phenotype. It is unclear whether flies had begun to senesce physiologically by two weeks of adult age in our study. Mean lifespans of females kept in sex-specific groups are around 50 days in this population of *D. melanogaster*, and mortality rates do not begin to increase exponentially until around day 35 of life [44]. On the one hand, this suggests that flies generally might not enter a strongly senescent phase of their life, characterized by marked compromises in physiological function, until long after we measured the older age class (flies were approx. 17 days when laying eggs that produced the focal daughters in the older age class). If so, then our results are not necessarily attributable to effects tied to reductions in parental physiological function *per se*. On the other hand, the laboratory population that we used is under strong selection for early-life performance, because only females at 4 days of age (and males between the ages of 1 and 4 days old) have the opportunity to transmit their genes to the subsequent generation [44]. In theory, this selection regime should result in evolution of accelerated senescence, commencing earlier in life [47]. In practice, this

theoretical prediction has not been observed for flies maintained on this life cycle [45]. Note, however, that other studies in *Drosophila* have found that age-related effects on trait expression are largely trait-dependent; many traits do not exhibit a pronounced deterioration in function during the first two weeks of life, including metabolic rate, protein synthesis, geotaxis and fertility [60,61]. By contrast, other traits do display reductions at this age—sometimes notable within the first few days of adult life—such as olfactory sensitivity, locomotive activity and sensitivity to oxidative stress [60]. However, the transgenerational effects attributable to dedicated parental effects were generally positive in their effects, at least when traced to maternal age, and these patterns at face value would be the opposite in direction than expected if older flies were physiologically senescent. While any discussion of underlying mechanism is at this stage speculative, such patterns might be driven by general age-dependent increases in condition among the parents.

We also found a strong interaction between maternal immune status and offspring immune status, independent of parental age. This result is particularly interesting because, despite increasing evidence supporting transfer of innate immunity in both vertebrates (fish and birds [62–65]) and invertebrates [9,33,34], there is still an ongoing debate regarding the validity, generality and interpretation of parental immune transfer in invertebrates [42,43,66,67]. Nonetheless, even without knowing the underlying immunological mechanism [42,43], the results generated in our study are consistent with the hypothesis that transgenerational transmission of innate immune factors does occur in *D. melanogaster*. This contention is based on the finding that daughters were better able to withstand an immune activation challenge (by maintaining high reproductive success) when produced by mothers that had also been immune-challenged prior to reproduction. This result is striking because it is generally opposite in sign to that which would be expected if it was mediated by a general condition-dependent maternal effect. In such a scenario, we would have expected daughters to consistently perform better when produced by highly conditioned mothers that never faced an immune challenge. Furthermore, the observed patterns are not reconcilable with what would be expected under terminal investment, a scenario whereby the immune-challenged daughters would generally have outperformed the control daughters. Potential mechanisms that could drive this result include age- or immune-mediated transgenerational epigenetic regulation, or selection on standing allelic variation in the parental generation [4,8], the latter of which we discuss below.

Additionally, we found more complex parental interactions on the reproductive success of daughters, which involved paternal age, and the paternal and maternal immune status. Intriguingly, the consequences of immune activation differed across fathers of the different age classes. Older fathers appeared to suffer a higher cost of immune challenge relative to older fathers that received the control treatment, given their daughters had lower reproductive success, and this was independent of the immune status of the mothers to which the fathers were mated. This pattern would suggest that older fathers were less resilient to the costs associated with activation of the immune system than their younger counterparts, in turn indicating a probably condition-dependent mechanism underpinning this pattern. Yet these patterns were more complex in younger fathers and sensitive to the immune status of the interacting mothers. Younger fathers produced daughters with higher reproductive success when they themselves received the control treatment, but when their mates had received the immune challenge. At this stage, it is difficult to ascribe a likely mechanistic driver of these complex patterns. Experiments designed to distinguish between various alternative possible drivers, such as sex-specific parental condition dependence or maternally mediated and context-dependent terminal investment, would represent worthy avenues for further investigation.

While the transgenerational effects identified in this study are putatively non-genetic in origin, we acknowledge that the results may have been driven by selection on allelic variation in the population at one of several life stages. Note, however, that we assayed parents of each age class well before the population-level exponential increase in mortality rates (which occur around day 35), and thus differential mortality of adults across the age classes would have been negligible, at least in the absence of the immune treatment. This assumption finds anecdotal support in the results of a similar study on *D. melanogaster,* which failed to find any substantial injury-induced differences in short-term mortality (3- versus 10-day-old flies) [68]. Yet, we do not have data on total differential adult mortality or larval viability for individuals in the older age classes that received the immune treatment relative to the other age-class-by-immune-treatment levels. We acknowledge that it is plausible that selection resulted in a biased sample of 'better-performing' genotypes contributing to the pool of assayed offspring in the older age classes subjected to immune treatment, relative to other experimental units. Nonetheless, our inability to rule out genetic explanations as mediators of the reported patterns does not diminish the evolutionary relevance of our findings (i.e. complex interactions between parental traits are important contributors to offspring phenotypic expression). It simply serves the point that future research is required to disentangle the proximate mechanisms underlying the transgenerational effects mediated by maternal and paternal age-by-immunity interactions.

It is clear that maternal and paternal effects are prevalent across taxa, and likely to be of evolutionary importance [1,4]. In the light of this, it is striking just how few studies have examined the potential for parental sources of variance to interact across the sexes to shape expression of the offspring phenotype [5,20,37,38]. Of those that have, the results suggest that paternal-by-maternal interactions are both common and non-trivial in their effect. For example, offspring immune responses in the cricket *Teleogryllus oceanicus* were shown to be affected by interactions between maternal and paternal immune status [38], and offspring development rates in *P. brassicae* by interactions between maternal age and paternal age [20]. Our results reinforce these findings, but provide a novel extension by showing that core life-history traits that are closely tied to survival can interact at multiple levels—not just between respective parents, but between parents and traits in the offspring—to affect the expression of a key life-history trait in the offspring, in reproductive success. An important avenue of future research will be to explore whether these parental interactions involving age and immunity, and evidence of immune priming effects, extend to sons, or whether the patterns are sex-specific in their magnitude, or even in their direction.

Finally, we point out that we adopted a heat-killed bacterium, rather than a replicating pathogen, as the immune elicitor in this study. We can thus directly surmise that the observed immune-mediated effects are directly attributable to

effects of host activation of the immune system *per se*, rather than any indirect costs associated with ongoing pathogenic infection or transmission of the pathogen across generations. Hence, the very perception of an infection, and specifically the host's response to this perceived challenge, was sufficient to induce age-sensitive transgenerational effects on offspring reproductive success, at least in females.

Data accessibility. Data are available at Dryad: doi.org/10.5061/dryad.7cf02/1.

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