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Age-specific fecundity under parasitic threat in an insect: terminal investment *versus* reproductive restraint

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Abstract

1. The terminal investment hypothesis predicts that animals increase their reproductive effort as they age, and their chances for survival and reproduction decline. An infection predicts further diminished residual reproductive value, and could therefore trigger increased breeding effort at the expense of somatic defences and survival. Attempts to test this hypothesis have produced mixed results, which also often relied on potential changes in the breeding effort only. Alternatively, animals may restrain their current reproduction to sustain immunity and so later reproduction.
2. We tested these possibilities in females of the yellow mealworm beetle, *Tenebrio molitor*, an iteroparous insect with reproductive tactics similar to that of long-lived organisms.
3. For this purpose we mimicked pathogenic bacterial infections early or late in life in breeding females by injecting them with a suspension of inactivated *Bacillus cereus*, a known natural pathogen of *T. molitor*, and measured female age-specific fecundity, survival and immunity.
4. Inconsistent with a terminal investment, early or late-life immune challenges of females did not reduce their survival or enhance their reproductive output. Female fecundity declined with age and was reduced by the early but not the late immune challenge. Both early and late-life fecundity correlated positively with life expectancy. Finally, young and old females exhibited similar antibacterial immune responses, suggesting that they both restrained reproduction to sustain immunity.
5. Our results clearly demonstrate that age-specific reproduction of *T. molitor* females under parasitic threat is discrepant with a terminal investment. By contrast, our results rather suggest that females used a reproductive restraint strategy to sustain immunity and therefore subsequent reproductive opportunities. However, as infections were mimicked only, the fitness benefit of this reproductive restraint could not be shown.

26 **Keywords:** Cost of innate immunity, life history shift, reproductive restraint, senescence,
27 terminal investment.

28 **Introduction**

29 Trade-offs are of paramount importance in life history evolution (Roff, 1993; Stearns, 1992).
 30 Because of resource and time scarcity, organisms cannot simultaneously maximize all life
 31 history traits and natural selection should favour optimal combinations of those to maximize
 32 fitness. In iteroparous species, optimal investment between current and future reproduction
 33 likely depends on the relative expected lifespan (Williams, 1966). According to the terminal
 34 investment hypothesis (Clutton-Brock, 1984), individuals increase their current reproductive
 35 effort as their residual reproductive value (i.e., opportunity for future reproduction) decreases.
 36 Such a terminal investment is expressed at the expense of longevity, which would no longer
 37 provide fitness benefit (Clutton-Brock, 1984).

38 Terminal investment may occur as a response to a survival threat such as a parasite or
 39 pathogen infection (Agnew et al., 2000; Duffield et al., 2017; Forbes, 1993; Hochberg et al.,
 40 1992; Minchella, 1985; Perrin et al., 1996). Parasite and pathogen typically reduce host
 41 survival or fecundity (Hamilton & Zuk, 1982) and infection might be a relevant cue of a
 42 rapidly diminishing life span (Grenfell & Dobson, 1995). There is ample empirical evidence
 43 that parasitized host can make adaptive adjustments of their reproductive effort towards a
 44 terminal reproductive investment to reduce the fitness cost of parasitism (Adamo, 1999; Blair
 45 & Webster, 2007; Chadwick & Little, 2005; Giehr et al., 2017; Minchella & Loverde, 1981;
 46 Schwanz, 2008). Similar outcomes were found from immune stimulations with non-living
 47 immune elicitors (Adamo, 1999; Bonneaud et al., 2004; Duffield et al., 2017; Moret &
 48 Schmid-Hempel, 2000). Indeed, the immune system can act as a signalling mechanism
 49 alerting the host about a virulent infection threat (Adamo, 1999; Bonneaud et al., 2004;
 50 Moret, 2003), or about a higher risk of subsequent infection by the same parasite or pathogen
 51 (Lui, 1998). While most of the studies that provided evidence of a terminal investment did so
 52 by revealing increased allocation to current reproduction in response to a parasitic threat

(Duffield et al., 2017), they often overlooked two other key predictions. First, the resulting increased allocation to current reproduction should be negatively correlated to post-reproduction survival (Jehan et al., 2021; Tarwater & Arcese, 2017). Second, the increased reproductive effort should be accompanied by a reduced investment into somatic defences such as immune defence (Duffield et al., 2017; Jehan et al., 2021), because of trade-offs between reproduction and immunity (Norris & Evans, 2000; Sheldon & Verhulst, 1996).

As an alternative to terminal reproductive investment, immune-challenged hosts may reduce investment into current reproduction and sustain immune defence to maintain subsequent reproductive opportunities, a strategy in accordance with the reproductive restraint hypothesis (Curio, 1983; McNamara et al., 2009). Such a strategy involves that organisms may develop a long-term immunity to the parasite after recovery, thus deferring death and maintaining later life reproduction. Long-term immunity shall be achieved through the acquired immune system of vertebrates or the immune priming response of invertebrates (Coustau et al., 2016; McDade et al., 2016; Milutinović & Kurtz, 2016).

Here, we tested for these two alternative hypotheses by mimicking bacterial infections in females of the mealworm beetle, *Tenebrio molitor*. *T. molitor* is a cosmopolitan pest of stored grain product that is currently raising considerable interests for its use as an alternative protein source for food and feed (Vigneron et al., 2019). Females begin to reproduce from their fifth day post-eclosion, and can mate many times with several males within weeks of their adult life. *T. molitor* is iteroparous and is an income breeder insect (Zanchi et al., 2019): it reproduces using resources gained as an adult, as opposed to a capital breeder insect, for which reproduction depends on resources cumulated during the juvenile stages (Jönsson, 1997; Stearns, 1992). *T. molitor* feeds at both the larval and the adult stages and females lay eggs in several reproductive bouts along its lifespan (Dick, 1937; Hughes, 2017). These features possibly allow females of this insect to adjust their lifetime reproductive effort

according to variation in their physiological state and environmental conditions. *T. molitor* is a relatively long-lived insect species that exhibits reproductive tactics similar to that of long-lived mammals (Jehan et al., 2021), for which the cost of current reproduction may compromise future reproduction without jeopardizing survival (Hamel et al., 2010). Previous work found that ageing *T. molitor* females tended to restrain reproduction late in life in favour of longevity (Jehan et al., 2021). However, in this study, females were not exposed to cues mimicking a survival threat like would do a pathogenic challenge. It is therefore not known whether *T. molitor* females, exposed to a parasitic threat, are maintaining a reproductive restraint strategy or are switching toward a terminal investment.

In the present study, individual *T. molitor* females were immune challenged with the inactivated bacterium, *Bacillus cereus*, an important natural and virulent pathogen of *T. molitor* (Du & Laing, 2011; Jurat-Fuentes & Jackson, 2012). This challenge was made either early or late in life to examine the effect of a pathogenic threat at different ages on fecundity, post-reproduction survival and immunity. Indeed, since young females (i.e., below c.a. 15 days post-eclosion – Jehan et al., 2020) still have a high residual reproductive value, we expect that an immune challenge occurring early in life should not trigger a reproductive shift toward a terminal investment (Duffield et al., 2017). As the immune response is costly, we even predict that early challenged females should exhibit reduced fecundity (Moreau et al., 2012). By contrast, as the residual reproductive value of old females becomes low, exposure to an immune challenge is expected to trigger a life history shift resulting in an increased reproductive investment in line with the terminal investment hypothesis (Duffield et al., 2017). Such an increased reproductive investment late in life should be accompanied by both a reduced immunity and a reduced survival. If these assumptions are not fulfilled, then *T. molitor* females may alternatively use the reproductive restraint strategy. The reproductive restraint strategy shall be supported if the following results are found. First, late-life

reproductive output should not increase in response to the late-life immune challenge. Second, late-life reproductive output should not correlate negatively with late-life survival (Jehan et al., 2021; Tarwater & Arcese, 2017). Finally, immune investment should be maintained late in life (Duffield et al., 2017).

Material and Methods

INSECT CULTURE AND EXPERIMENTAL DESIGN

Virgin adult beetles of controlled age (10 ± 2 days post-eclosion) were obtained from pupae, randomly sampled from a stock culture and maintained in standard laboratory conditions (see below). Prior to the experiments, all the experimental insects were maintained separately, at $24 \pm 1^\circ\text{C}$, 70% RH in permanent darkness, supplied with *ad libitum* bran flour and water, and supplemented once a week with apple.

This study involved two separate experiments because it was not possible to assess the influence of age-specific immune challenges on reproduction, longevity and immunity on the same individuals. For this reason, a first experiment investigated the influence of age-specific immune challenges on fecundity and longevity, and a second one examined the influence of age-specific immune challenges on induced antibacterial activity.

EXPERIMENT 1: AGE-SPECIFIC IMMUNE CHALLENGE, FECUNDITY AND LONGEVITY

Here, we assessed the age-specific reproductive performance of *T. molitor* females exposed to early or late-life immune challenges, mimicking reduced expected life span, while tacking into account for variation in individual quality. To this purpose, sixty 10-day-old virgin adult females were individually immune challenged with a single injection of a 5 μL suspension of

the inactivated bacterium, *Bacillus cereus* (10^8 bacteria.mL⁻¹ in Phosphate Saline Buffer: PBS 10 mM, pH 7.4). A second group of 60 females received a 5-μL injection of sterile PBS only as a procedural control (see below for inactivation procedure). Immediately after their respective immune treatment, females were allowed to reproduce for a 20-days period (from day 10 to day 30 post-eclosion) to assess their early-life reproductive performance. To this end, each female was paired with a 10 ± 2 -day old virgin male for 10 days. Then, the male was replaced by a new 10 ± 2 -day old virgin male, with which the female spent 10 additional days. Couples were maintained in a Petri dish containing bleached flour and provided *ad libitum* food and water under standard laboratory conditions (see above). Female reproductive output was estimated by counting the number of eggs produced by the female every 3 or 4 days along the 20-day episode of reproduction. For this purpose, couples were transferred into a new Petri dish and eggs were searched by sieving the flour (600 μm) and counted (Moreau et al., 2012). At the end of this early reproductive episode, each female was kept alone in a new Petri dish with *ad libitum* food and water for 10 days (until day 40 post-eclosion) awaiting their assessment for their late-life reproductive performance.

Prior to the assessment of their late-life reproductive performance, half of the early challenged and control females were challenged with inactivated *B. cereus* whereas the other half was injected with sterile PBS, as described above. Thus, the late-life reproductive performance was assessed on females that have been either immune challenged twice ('early and late' in life), once (either 'early' or 'late' in life) or never ('control'). Late-life reproductive performance of females was assessed from day 40 to day 60 post-eclosion as described above for the early reproductive performance of females. At the end of their late reproductive period, females were isolated into a new Petri dish with *ad libitum* food and water, checked twice a week until death. Prior to each reproductive period and immune

treatment, female body mass was measured to the nearest 1 mg using an OHAUS balance (discovery series, DU114C).

EXPERIMENT 2: AGE-SPECIFIC IMMUNE CHALLENGE ON ANTIBACTERIAL ACTIVITY

T. molitor immunity relies on both constitutive and inducible innate mechanisms with cellular and humoral components (Vigneron et al., 2019). An important aspect of *T. molitor* immunity is immune priming, through which individuals improve their immune response as a function of their immunological experience. This reduces their susceptibility to re-infection (Moret & Siva-Jothy, 2003). In *T. molitor*, the acquired immune protection in response to a bacterial immune challenge is pathogen-dependent and is mainly caused by a persistent antibacterial response, which can last at least 20 days after the immune challenge (Dhinaut et al., 2018). We therefore assessed the effect of age-specific challenge on immunity of *T. molitor* females through the measurement of the antibacterial activity of their haemolymph.

Six groups of females were created, according to their respective age-specific immune treatment and the age at which their haemolymph was sampled to assess antibacterial activity. Four groups of 20 females were treated as described above (immune-challenged early, late, both early and late, and not immune-challenged as control) to estimate antibacterial activity in their haemolymph late in life. Females were allowed to reproduce as previously described, and provided a sample of haemolymph immediately after their late reproductive episode (at 60 days post-eclosion). Two additional groups of 25 females were created to estimate antibacterial activity early in life. Here, the sample of haemolymph was taken at 30 days post-eclosion (i.e., immediately after their early reproductive episode). In one group, females were immune challenged early at 10 days post-eclosion, while in the second group they were not immune challenged. These two additional groups were created to test the effect of the immune

challenge on antibacterial activity according to age when compared to the groups of old females that were immune challenged late only (at 40 days post-eclosion).

BACTERIAL CULTURES FOR IMMUNE CHALLENGES

The bacterium *Bacillus cereus* used to perform the immune challenges was obtained from the Pasteur institute (CIP69.12). The bacteria were grown overnight at 28°C in liquid Broth medium (10 g/L bacto-tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7). Bacteria were then inactivated in 0.5% formaldehyde prepared in phosphate-buffered saline (PBS 10 mM, pH 7.4) for 30 minutes, rinsed three times in PBS, and their concentration adjusted to 10⁸ bacteria per mL using a Neubauer improved cell counting chamber (Dubuffet et al., 2015). The success of the inactivation was tested by plating a sample of the bacterial solution on sterile Broth medium with 1% of bacterial agar and incubated at 28°C for 24 hours. Aliquots were kept at -20 °C until use. Immune challenges were performed by injection through the pleural membrane between the second and the third abdominal tergites using sterile glass capillaries that had been pulled out to a fine point with an electrode puller (Narashige PC-10). Immune challenge with inactivated *B. cereus* using this method has proven its efficacy to efficiently stimulate the immune response of *T. molitor* (Jehan et al., 2021).

HAEMOLYMPH COLLECTION AND ANTIBACTERIAL ACTIVITY

Haemolymph collection was done as described by (Dhinaut et al., 2018). Briefly, beetles were chilled on ice for 10 minutes before a 3-μL sample of haemolymph was collected from a wound made in the beetle's neck. This sample was flushed into an N-phenylthiourea-coated microcentrifuge tube (P7629; Sigma-Aldrich, St Louis, MO, USA), flash-frozen in liquid nitrogen and then stored at -80 °C for later examination of its antibacterial activity.

Antimicrobial activity of the haemolymph was measured using the inhibition zone assay described by (Moret, 2006). Briefly, haemolymph samples were thawed on ice, and 2 μ L of the sample solution was used to measure antibacterial activity on zone inhibition plates seeded with *Arthrobacter globiformis* from the Pasteur Institute (CIP105365). An overnight culture of the bacterium was added to Broth medium containing 1 % agar to achieve a final concentration of 10⁵ cells.mL⁻¹. Six millilitres of this seeded medium were subsequently poured per Petri dish. After solidification, sample wells were made using a Pasteur pipette fitted with a ball pump. Two microliters of sample were deposited in each well. Plates were then incubated overnight at 28°C and the diameter of each zone of inhibition was measured.

STATISTICS

Female longevity was analysed as a function of their immune treatment (occurring early or late in life) using a mixed effects Cox model (Therneau, 2020) including early-life immune treatment (yes/no), late-life immune treatment (yes/no) and their interaction as independent categorical covariates. This allowed us to test, in a full factorial design, whether the impact of the late immune challenge could be dependent on the early immune challenge, which would be revealed as a significant interaction term since the effect of early and late immune challenge on survival might not be a priori additive. We also included female body mass measured prior to each reproductive period (at 10 and 40 days old) as an independent continuous covariate, which was tested as a main effect only. To account for potential variation in frailty among female individuals, their identity was included as a random effect. This Cox regression analysis was performed using R version 3.6.3 (R Core Team, 2020) and COXME package version 2.2-16. Model reduction was limited to non-significant interaction terms, whereas non-significant main effects were kept in the final model. Proportional

assumption was checked using `cox.zph()` function in the R Survival package version 3.1-12 (Therneau, 2020).

We investigated potential change in female body mass according to age at which they initiate each reproductive period (10 day old *versus* 40 day old, respectively) using a mixed linear model that included female age, the early immune challenge (yes/no) and their interaction term as independent categorical variables. Female identity was included as a random effect. Note that the late immune challenge was not included as an explanatory variable in the model as this treatment was applied after body mass measurements.

Age-specific female fecundity was analysed using a mixed linear model as a function of age at which females initiated each reproductive period (10 day old *versus* 40 day old), whether the females were exposed to an early immune challenge (yes/no), a late immune challenge (yes/no) and their interaction as fixed categorical factors. Female body mass measured prior to each reproductive period was included as an independent continuous covariate tested as a main effect only. Based on the survival data, we calculated life expectancy (in days) of each female at the beginning of each reproductive period (here corresponding to the total adult lifespan minus 10 for early-life expectancy and minus 40 for late-life expectancy) as an indication of the general state of each individual. We included it in the model as an independent continuous covariate tested as a main effect and as two-way and three-way interaction terms with the early and late immune challenges. A negative relationship between fecundity and life expectancy would be indicative of a terminal investment (Jehan et al., 2021; Tarwater & Arcese, 2017).

Antibacterial activity of the haemolymph was analysed as a function of immune challenge (occurring early or late in life), age and initial body mass using Generalized Linear Models (GLM) with a Tweedie distribution and a Log link function. Fecundity, body mass and antibacterial activity data were analysed using IBM® SPSS® statistics 19 for Macintosh.

Model simplification was achieved by removing higher order non-significant interactions based on Akaike's information criterion.

Results

LONGEVITY REMAINED UNALTERED BY AGE-SPECIFIC IMMUNE CHALLENGE

Whether the immune challenge occurred early or late in life, it had no effect on female longevity either as main effects (Fig. S1, Table S1, mixed effects Cox regression analysis: early challenge $z = 0.61$, $df = 1$, $P = 0.540$, late challenge $z = 0.83$, $df = 1$, $P = 0.410$) or as an interaction (effect before being removed from the last model $z = 0.52$, $df = 1$, $P = 0.610$), while accounting for inter-individual variation by including female identity as random effects. Female body mass measured prior to each reproductive period did not explain any variation of longevity (mass $z = -1.01$, $df = 1$, $P = 0.310$).

BODY MASS DECREASED WITH AGE

Female body mass significantly decreased with age (estimate of 10 day old *versus* 40 day old $\pm$ s.e. 5.66 ± 3.61 , $F_{1,187} = 6.50$ $P = 0.012$, Fig. 1). However, the early immune challenge did not affect female body mass either as a main effect ($F_{1,187} = 0.12$ $P = 0.734$) or in interaction with age ($F_{1,187} = 0.07$ $P = 0.793$).

REPRODUCTION DECLINED WITH AGE AND AS A RESULT OF AN IMMUNE CHALLENGE EARLY IN LIFE

Overall, female fecundity significantly depended on female age and female life expectancy as main effects (Table 1). On the one hand, female fecundity declined with age (Fig. 2) and, on the other hand, females that exhibited the longest life expectancy were also those that produced the largest number of eggs (Fig. 3). The early immune challenge significantly

affected female fecundity, but as two-way interaction terms with age and with life expectancy (Table 1). The interaction term between the early immune challenge and age shows that the early immune challenge induced a decline in female fecundity at early age only (Fig. 2, Fig. 3A, B). The significant interaction term between early challenge and life expectancy (Table 1) shows that females with the longest life expectancy were those that exhibited the largest decline in fecundity after an immune challenge early in life (Fig. 3A). The late immune challenge had no significant effect on late-life female fecundity, whether they were previously exposed to an early challenge or not (Table 1, Fig. 2). Female body mass significantly explained variation in female fecundity, as the heaviest females laid more eggs than the lightest ones (Table 1).

ANTIBACTERIAL IMMUNE RESPONSE

The challenge with inactivated *B. cereus* significantly induced an antibacterial immune response in young (assessed at 30 days post-eclosion) and old (assessed at 60 days post-eclosion) females (Table 2a, Fig. 4). The analysis revealed a significant interaction between female age and the immune challenge (Table 2a), which mainly results from the fact that old control females exhibited a slightly higher antibacterial activity than young control ones (Fig. 4). This is also the reason why antibacterial activity was marginally increased with age (Table 2a, Fig. 4), since the level of antibacterial activity in young and old immune-challenged females was similar (Fig. 4).

Among old females (assessed at 60 days post-eclosion), antibacterial activity was marginally increased by both the early and the late immune challenge (Table 2b, Fig. 4). The analysis revealed a significant interaction between the early and the late immune challenges (Table 2b). Indeed, old females that were exposed to an early immune challenge (occurring 50

days earlier) still exhibited high level of antibacterial activity in their haemolymph (Fig. 4), possibly as a result of an immune priming response (Dhinaut et al., 2018).

Discussion

Challenging the immune system of breeding *T. molitor* females early or late in life did not induce measurable effects on their longevity in our experimental conditions. Reduced longevity was expected if the immune response imposed a substantial survival cost or if the challenged females shifted toward a terminal reproductive investment. The immune function is known to be costly for organisms (Moret & Schmid-Hempel, 2000) and could cause self-damage (named immunopathology) (Sadd & Siva-Jothy, 2006), which is expected to reduce longevity (Pursall & Rolff, 2011; Urabe et al., 1994). Previous studies found that the immune response causes self-damages in *T. molitor* (Khan et al., 2017; Sadd & Siva-Jothy, 2006) and that females exposed to an early life immune challenge suffered from a reduced longevity (Khan et al., 2017). Our results, contrasting with those of these previous studies, might have at least two main causes in addition to many other small differences. First, Khan et al. (2017) did not allow females to reproduce, whereas our experiment allowed all the females to reproduce early and late in life. It was previously found that reproduction significantly stimulates immune activity in *T. molitor* females (Jehan et al., 2021), which may have buffered the effect of the immune challenge in our experiment. Second, Khan et al. (2017) kept their insects at a suboptimal temperature condition (30°C) compared to the optimum range of temperature of 25-28°C usually used to grow *T. molitor* (Punzo, 1975; Ribeiro et al., 2018) and that we have used in the present study. Actually, in the study by Khan et al. (2017), control (non-immune challenged) females of *T. molitor* showed a 90th percentile survival time of 83.1 days, whereas it was 112 days in our study, a difference suggesting that in their study, females were maintained under stressful conditions, allowing the expression of the cost of an

early immune activation on longevity. In general, detrimental effects of the immune response on survival are revealed in stressful conditions (Moret & Schmid-Hempel, 2000). Rearing conditions in our study were probably too favourable to highlight such a survival cost of the immune response.

Nevertheless, despite the relatively favourable conditions of our study, the immune challenge had an age-specific effect on female reproduction. Only the early-life immune challenge had a measurable effect on female reproduction by reducing their early-life fecundity, a result consistent with the predicted trade-off between reproduction and immunity (Sheldon & Verhulst, 1996). Accordingly, early challenged females exhibited stronger level of antibacterial activity in their haemolymph than control females. However, despite having induced an antibacterial immune response of similar magnitude to that of the early challenge, the late-life challenge had no further impact on the following female's clutch size. This suggests that old females paid a lower cost to the immune response than young ones.

It should be noted that fecundity of old females was about half that of young ones among controls. This is consistent with previous observations, showing that despite being continuously sexually receptive during adulthood, *T. molitor* female fecundity declines after about two weeks but does not necessary stop (Dick, 1937; Drnevich et al., 2001; Jehan et al., 2020). This might be due to senescence. The age-specific decline of female body mass, which positively predicted female fecundity, may support this view. However, ageing females are also expected to exhibit reduced somatic protection, such as a reduced immune activity. This was clearly not the case here, although our measure of immune activity was restricted to the ability to produce an antibacterial immune response. Antimicrobial activity found in early challenged females is still high late in life. This suggests that old females are still protected by the antibacterial activity induced 50 days earlier, which is, at least, twice as long as previously observed in this insect species (Dhinaut et al., 2018; Haine et al., 2008) and is in line with

other observations in dragonflies (Bulet et al., 1992). Such a relatively high level of immune activity maintained late in life is not consistent with the terminal investment hypothesis, but is rather in support of the reproductive restraint hypothesis. Indeed, it seems that reduced fecundity late in life allows females to afford the cost of a late-life immune response, which presumably helps to prolong lifespan and, consequently, future reproductive opportunities.

Another important prediction of the terminal investment hypothesis is that individuals should increase their reproductive investment at the expense of their subsequent lifespan. A negative relationship between reproduction and life expectancy from the moment at which the terminal investment is initiated is therefore expected (Tarwater & Arcese, 2017). We found no evidence of such a relationship either early or late in life in *T. molitor* females. At the opposite, the relationship between female fecundity and life expectancy was always positive, illustrating that the females with the lowest life expectancy were also those that had the lowest fecundity. This result is similar to the one previously found in another experimental context (Jehan et al., 2021). The slope of this positive relationship was decreased in response to the early immune challenge, suggesting that females with the longest life expectancy were also those that exhibited the largest reduction in fecundity caused by the early challenge. These females, presumably also those of the best relative quality, were likely those that invested the most into immunity in response to the early challenge. This suggests that they might have diverted resources from current reproduction to sustain somatic defence. We may hypothesise that these females may gain fitness by improving their survival to the pathogenic threat to reproduce later, as predicted by the reproductive restraint hypothesis. However, since our immune treatment only mimicked a survival threat without being real, our experimental design cannot reveal the true associated fitness benefit of diverting resources from current reproduction to immunity. In any case, this result, and the fact that female close to death did

not produce the highest clutch-size, strongly suggests that *T. molitor* females did not perform a terminal investment.

As noted earlier, our study comforts the results of a previous study which found no evidence of a terminal investment with age in *T. molitor* females (Jehan et al., 2021). However, this previous study did not involve cues, such as an immune challenge, that would represent a terminal investment trigger (Duffield et al., 2017). Here, we have used *B. cereus*, recognized as an important natural pathogen of *T. molitor* (Du & Laing, 2011; Jurat-Fuentes & Jackson, 2012), to challenge the females' immune system. Such an immune challenge was expected to mimic a systemic infection by the bacterium, representing a significant cue predicting imminent death. Accordingly, experimental infections with this bacterium can kill 90% of *T. molitor* females in less than three days (Dhinaut et al., 2018; Jehan et al., 2021). Here, the use of *B. cereus* immune challenge did not trigger terminal investment either in young or old females. However, we cannot dismiss that the way the immune challenge was performed might have been too artificial to accurately mimic a deadly infection by *B. cereus*. *B. cereus* infects *T. molitor* mostly orally and more accidentally by wounding through the cuticle. The host immune response can greatly vary according to the route of infection (Behrens et al., 2014), as host adaptation can be contingent on the infection route taken by pathogens (Martins et al., 2013). Future studies may need to perform oral immune challenges to mimic the trigger that would induce terminal investment. Nevertheless, *Bacilli* bacteria that include numerous entomopathogens such as *B. cereus*, appear to have been of paramount importance in the evolution of *T. molitor* immunity, especially through the evolution of immune priming (Dubuffet et al., 2015). This is shown by the fact that they induce highly persistent antibacterial immune responses compared to other bacterial pathogens (Dhinaut et al., 2018). The very long-lasting antibacterial response resulting from the *B. cereus* challenge

observed in this study suggests that the immune system of *T. molitor* females integrated the infectious signal appropriately.

In conclusion, our work does not support the triggering of a terminal investment in *T. molitor* females in response to a challenge with a natural bacterial pathogen of this insect. Indeed, we showed that, while investing in their immune function, immune-challenged females early in life restrained their reproduction and maintained their longevity. Moreover, when immune challenged late in life, females maintained their immunity and longevity, which may provide future reproductive opportunities instead of performing terminal investment. On the basis of this work and a previous one (Jehan et al., 2021), the reproductive strategy of *T. molitor* females appears to rely on reproductive restraint rather than on terminal investment. This strategy also seems to be the opposite of the one used by males, which were found to produce a terminal reproductive effort in comparable circumstances (Kivleniece et al., 2010; Krams et al., 2011; Sadd et al., 2006). However, it has been proposed that males may have higher fitness payoffs from terminal investment than females (Bateman, 1948; Kivleniece et al., 2010; Sadd et al., 2006), a statement that looks true in the light of the present study.

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Authors' contributions

CJ, TR and YM conceived the ideas and designed methodology; CJ and CS collected the data; CJ and YM analysed the data; CJ, TR and YM led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

421

422 **Data accessibility**

423 Data will be available from the Dryad Digital Repository.

424

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Table 1. Mixed linear model investigating variance in female egg production as a function of their age, their experience of an immune challenge occurring early and/or late in life, body mass measured before each reproductive episode and residual life expectancy calculated at each reproductive episode. Female identity was included as a random factor. Values $P \leq 0.05$ are given in bold.

Source	Estimate $\pm$ s.e.	d.f. num.	d.f. den.	F	P
Age ^a	-49.93 $\pm$ 6.06	1	183	79.28	< 0.001
Early challenge ^b	-8.45 $\pm$ 10.02	1	183	0.07	0.796
Late challenge ^b	-2.69 $\pm$ 4.12	1	183	0.43	0.515
Life expectancy	0.77 $\pm$ 0.12	1	183	57.35	< 0.001
Body mass	0.26 $\pm$ 0.13	1	183	4.26	0.040
Age *Early challenge	21.60 $\pm$ 8.64	1	183	6.25	0.013
Early challenge*Life expectancy	0.38 $\pm$ 0.15	1	183	6.16	0.014

^aEstimate of old female relative to young females as the reference (estimate = 0).

^bEstimate of challenged female relative to control females as the reference (estimate = 0).

Table 2. Generalized Linear Model (GLM, Tweedie distribution, Log link function) analysing antibacterial activity of (a) *Tenebrio molitor* females assessed early (30 day old) and late (60 day old) in life after a single immune challenge occurring at 10 day old and 40 day old, respectively, as a function of their age and their age-specific immune treatment (N = 113), and of (b) females assessed late in life (60 days old) as a function of their immune treatment early and late in life (N = 77). Body mass did not explain any variation in antibacterial activity and was therefore removed from the models. Values $P \leq 0.05$ are given in bold.

Source of variation	β	s.e.	Wald χ^2	d.f.	P
(a) Young and old females					
Age ^a	1.87	0.81	3.64	1	0.056
Challenge ^b	3.54	0.71	32.67	1	< 0.001
Age* Challenge	-2.07	0.88	5.58	1	0.018
(b) Old females					
Early Challenge ^b	1.43	0.43	7.14	1	0.008
Late Challenge ^b	1.51	0.43	8.65	1	0.003
Early Challenge* Late Challenge	-1.37	0.56	6.02	1	0.014

^aEstimate of old female relative to young females as the reference (estimate = 0).

^bEstimate of challenged female relative to control females as the reference (estimate = 0).

Figure 1. Mean body mass ($\pm$ s.e.) of females at the age at which they started their early (at 10 days post-eclosion) and their late (at 40 days post-eclosion) reproductive episodes. Numbers at the bottom of the bars are sample size.

Figure 2. Mean number of eggs ($\pm$ s.e.) produced by females early in life (between day 10 and day 30 post-eclosion) and late in life (between day 40 and day 60 post-eclosion) as function of their life time immune treatment: control (white bars), immune challenged early (grey bars), immune challenged late (hatched white bar) and immune challenged both early and late in life (hatched grey bar). Numbers at the bottom of the bars are sample size.

Figure 3. Relationship between fecundity (number of eggs) and age-specific life expectancy in days of (A) young and (B) old females that have been exposed to an early immune challenge. In (A), the relationships between fecundity and life expectancy were $y = 0.85 \times \text{days of life expectancy} + 38.32$, $r^2 = 0.34$, $P < 0.0001$ and $y = 0.40 \times \text{days of life expectancy} + 34.04$, $r^2 = 0.15$, $P = 0.0017$ among controlled and early-challenged females, respectively. In (B), the relationships between fecundity and life expectancy were $y = 0.47 \times \text{days of life expectancy} + 5.21$, $r^2 = 0.21$, $P = 0.0033$ and $y = 0.50 \times \text{days of life expectancy} - 0.62$, $r^2 = 0.20$, $P < 0.0084$ among controlled and early-challenged females, respectively.

Figure 4. Mean antibacterial activity ($\log_{10}$ (mm) $\pm$ s.e.) in the haemolymph of young (30 days post-eclosion) and old (60 days post-eclosion) females as function of their life time immune treatment: control (white bars), immune challenged early (at 10 post-eclosion, grey bars), immune challenged late (at 40 days post-eclosion, hatched white bar) and immune challenged both early and late in life (at 10 and 40 days post-eclosion, hatched grey bar). Numbers at the bottom of the bars are sample size.

Supplementary information

Table S1. Mixed effects Cox regression analysis for *T. molitor* female longevity according to early challenge (‘early reproduction’: yes/no), late challenge (‘Late reproduction’: yes/no) and their interaction as independent categorical covariates in a full factorial design. Female body mass (‘Mass’) was also included as an independent continuous covariate, which was tested as a main effect only. To account for female individual heterogeneity, female identity was included as a random effect in the model. We limited model reduction to the interaction terms when found not significant, whereas main effects were always kept in the final model (N = 120).

Figure S1. Survival of *Tenebrio molitor* females as a function of their life-time immune treatment: C0 control, C1 early-life, C2 late-life and C12 early and late immune challenges with the inactivated bacterium *Bacillus cereus*.

Figure 1. Mean body mass ($\pm$ s.e.) of females at the age at which they started their early (at 10 days post-eclosion) and their late (at 40 days post-eclosion) reproductive episodes. Numbers at the bottom of the bars are sample size.

70x70mm (300 x 300 DPI)

Figure 2. Mean number of eggs ($\pm$ s.e.) produced by females early in life (between day 10 and day 30 post-eclosion) and late in life (between day 40 and day 60 post-eclosion) as function of their life time immune treatment: control (white bars), immune challenged early (grey bars), immune challenged late (hatched white bar) and immune challenged both early and late in life (hatched grey bar). Numbers at the bottom of the bars are sample size.

64x65mm (300 x 300 DPI)

Figure 3. Relationship between fecundity (number of eggs) and age-specific life expectancy in days of (A) young and (B) old females that have been exposed to an early immune challenge. In (A), the relationships between fecundity and life expectancy were $y = 0.85 \times \text{days of life expectancy} + 38.32$, $r^2 = 0.34$, $P < 0.0001$ and $y = 0.40 \times \text{days of life expectancy} + 34.04$, $r^2 = 0.15$, $P = 0.0017$ among controlled and early-challenged females, respectively. In (B), the relationships between fecundity and life expectancy were $y = 0.47 \times \text{days of life expectancy} + 5.21$, $r^2 = 0.21$, $P = 0.0033$ and $y = 0.50 \times \text{days of life expectancy} - 0.62$, $r^2 = 0.20$, $P < 0.0084$ among controlled and early-challenged females, respectively.

109x68mm (600 x 600 DPI)

Figure 4. Mean antibacterial activity ($\log_{10}$ (mm) $\pm$ s.e.) in the haemolymph of young (30 days post-eclosion) and old (60 days post-eclosion) females as function of their life time immune treatment: control (white bars), immune challenged early (at 10 post-eclosion, grey bars), immune challenged late (at 40 days post-eclosion, hatched white bar) and immune challenged both early and late in life (at 10 and 40 days post-eclosion, hatched grey bar). Numbers at the bottom of the bars are sample size.

65x66mm (300 x 300 DPI)

Supplementary Information for:

Age-specific fecundity under parasitic threat in an insect: terminal
investment versus reproductive restraint

Table S1. Mixed effects Cox regression analysis for *T. molitor* female longevity according to early challenge (‘early reproduction’: yes/no), late challenge (‘Late reproduction’: yes/no) and their interaction as independent categorical covariates in a full factorial design. Female body mass (‘Mass’) was also included as an independent continuous covariate, which was tested as a main effect only. To account for female individual heterogeneity, female identity was included as a random effect in the model. We limited model reduction to the interaction terms when found not significant, whereas main effects were always kept in the final model (N = 120).

Variable in the best model	β	$\pm$ s.e.	z	d.f.	P	Odd ratio
Mass	- 0.011	0.010	-1.01	1	0.310	0.99
Early challenge	0.218	0.356	0.61	1	0.540	1.24
Late challenge	0.295	0.357	0.83	1	0.410	1.34

Random effects (Female ID): SD 1.36, Variance 1.85

Proportional hazard assumption was checked as a non-significant relationship between residuals and time using the `cox.zph()` function in the R survival package (global test $\chi^2_{65,9} = 3.70$ P = 1, the test is also non significant for each of the covariates).

Fig. S1. Survival of *Tenebrio molitor* females as a function of their life-time immune treatment: C0 control, C1 early-life, C2 late-life and C12 early and late immune challenges with the inactivated bacterium *Bacillus cereus*.