Late-life reproduction in an insect: terminal investment, reproductive restraint or senescence.

Charly Jehan, Camille Sabarly, Thierry Rigaud, Yannick Moret

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1 Late-life reproduction in an insect: terminal investment, reproductive
2 restraint and senescence

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6 Charly Jehan,* Camille Sabarly, Thierry Rigaud, Yannick Moret

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9 UMR CNRS 6282 BioGéoSciences, Équipe Écologie Évolutive, Université Bourgogne-
10 Franche Comté, Dijon, France

11
12 * Corresponding author: charly.jehan@u-bourgogne.fr
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Abstract

1. The Terminal Investment, Reproductive Restraint and Senescence Hypotheses may explain individual late-life patterns of reproduction. The terminal investment hypothesis predicts that individuals increase reproductive allocation late in life as prospects for future survival decrease. The other two hypotheses predict reduced reproduction late in life, but for different reasons. Under the Reproductive Restraint hypothesis, individuals restrain their reproductive effort to sustain future survival and gain more time for reproducing, whereas under the Senescence hypothesis, reproduction is constrained because of somatic deterioration. While these hypotheses imply that reproduction is costly, they should have contrasted implications in terms of survival after late reproduction and somatic maintenance.

2. Testing these hypotheses requires proper consideration of the effects of age-dependent reproductive effort on post-reproduction survival and age-related somatic functions. We experimentally tested these three hypotheses in females of the mealworm beetle, *Tenebrio molitor*, an iteroparous and income breeder insect. We manipulated their age-specific allocation into reproduction and observed the effects of this manipulation on their late life-fecundity, post-reproduction survival and immunocompetence as a measurement of somatic protection.

3. We found that females exhibit age-related declines in fecundity and that this reproductive senescence is accelerated by a cost of early reproduction. The cost of reproduction had not significant effect on female longevity and their ability to survive a bacterial infection, despite that some immune cells were depleted by reproduction. We found that female post-infection survival deteriorated with age, which could be partly explained by a decline in some immune

parameters. Importantly, females did not increase their reproductive effort late in life at the expense of their late-life post-reproduction survival.

4. Late-life reproduction in *T. molitor* females is not consistent with a terminal investment strategy. Rather, our results suggest that females allocate their resources according to a priority scheme favouring longevity at the expense of reproduction, which is in line with the reproductive restraint hypothesis.

5. Such a priority scheme also shows that a relatively short-lived insect can evolve life history strategies hitherto known from long-lived animals only, putting in perspective the role of longevity in the evolution of life history strategies.

Key words: ageing, cost of reproduction, innate immunity, life history, mealworm beetle, senescence, terminal investment.

Introduction

Life history theory assumes that organisms are constrained to optimally trade-off limited energetic and time resources between life history traits, to maximize fitness (Roff, 1992; Stearns, 1992). The most prominent life-history trade-off involves the cost of reproduction (Stearns, 1989). The cost of reproduction is at the core of the disposable soma theory of ageing, which postulates that reproduction is traded-off against somatic protection and maintenance, leading to a progressive decline in future reproduction and survival (Kirkwood, 1977). Thus, under this assumption of trade-offs between current reproduction and subsequent reproductive performance, organisms are expected to optimize their age-specific allocation to reproduction and survival to maximize fitness (Charlesworth & Léon, 1976; Pianka, 1976; Bell, 1980).

So far, theory predicts that organisms may optimise their age-related reproductive investment in two ways. First, organisms are predicted to increase their investment in reproduction as they approach the end of their life (Williams, 1966; Clutton-Brock, 1984; Forslund & Pärt, 1995; Isaac & Johnson, 2005). This hypothesis invokes that current reproduction is costly on future reproduction and/or survival (Alonso-Alvarez et al., 2004; Harshman & Zera, 2007; Reznick, 1985). Young individuals, with high residual reproductive value, i.e. high opportunity for future reproduction (Williams, 1966), are therefore expected to allocate less to current reproduction to ensure future reproductive opportunities, whereas older individuals, with low residual reproductive value, should allocate more to current reproduction. Under this assumption of age-dependent “terminal investment” (Clutton-Brock, 1984), older individuals able to support higher costs of reproduction should be selected, because subsequent reproductive opportunities might be null. In line with this hypothesis, older females of the burying beetle, *Nicrophorus orbicollis*, tend to produce larger brood sizes than young ones during their first reproductive attempt (Creighton, Heflin, & Belk, 2009). However, empirical evidence for this prediction is often inconclusive. Indeed, in some species, reproductive performance can be improved with

age-dependent experience (Curio, 1983; Martin, 1995). In other cases, the differential survival of high and low performing reproducers could be a confounding effect for this pattern (Forslund & Pärt, 1995). In fact, if individuals with low reproductive performance are more likely to die young because of higher ‘frailty’ (see Vaupel, Manton, & Stallard, 1979), individuals with high reproductive performance become over represented at old age because of selective disappearance, thus explaining late increased reproductive output at the cohort or population level (Nussey, Coulson, Festa-Bianchet, & Gaillard, 2008). It is nevertheless possible to correctly analyse age-specific change in a trait from longitudinal data while controlling for between-individual variation in a population using adapted statistical models (van de Pol & Verhulst, 2006; Nussey, Kruuk, Donald, Fowlie, & Clutton-Brock, 2006; Nussey et al., 2011). Furthermore, terminal reproductive investment is expected to be associated to shorter subsequent survival independently of chronological age but this has rarely been tested (Tarwater & Arcese, 2017).

Second, and opposite to the terminal investment hypothesis, the “reproductive restraint” hypothesis predicts that organisms adaptively invest less in reproduction as they age (Curio, 1983; McNamara, Houston, Barta, Scheuerlein, & Fromhage, 2009). As reproductive activity may generate accumulation of damages (see above), thus decreasing survival probability, organisms should benefit by reproducing less intensively late in life to defer death and therefore gain more time for reproducing (McNamara & Houston, 1996; McNamara et al., 2009). Such a reduced reproduction late in life due to reproductive restraint should be distinguished from a simple consequence of senescence. On the one hand, senescing individuals are expected to show reduced reproduction late in life as a side effect of somatic deterioration, associated with an age-dependent decline of somatic protection, including immunity (Loison, Festa-Bianchet, Gaillard, Jorgenson, & Jullien, 1999; Weladji, Mysterud, Holand, & Lenvik, 2002). On the other hand, reduced late-life reproduction due to reproductive restraint should be associated

with maintained somatic protections. Empirical evidence consistent with the reproductive restraint hypothesis is scarce (Cotter, Ward, & Kilner, 2010; Lecomte et al., 2010; Elliott et al., 2014; Smith, Belk, & Creighton, 2014; Morin, Rughetti, Rioux-Paquette, & Festa-Bianchet, 2016; Boonekamp, Bauch, & Verhulst, 2020) and direct experimental evidence is missing. Experimentally disentangling the terminal investment strategy from the reproductive restraint one and senescence requires to assess the effects of age-dependent reproductive effort on post-reproduction survival and age-related somatic functions (e.g., immune protection) (Coulson & Fairweather, 2001; McNamara et al., 2009; Reed et al., 2008). The terminal investment hypothesis assumes that as older organisms reach a state in which prospects for future survival decrease, they should increase their allocation to current reproduction. Therefore, late reproductive allocation should be inversely related to survival and immune protection after the last reproduction. In contrast, under the reproductive restraint hypothesis, late allocation in reproduction is not expected to compromise late post-reproduction survival, and immune protection should be maintained with age. However, as organisms invest in their early reproduction, immune protection and longevity are expected to decline rapidly.

Based on these hypotheses, we tested the reproductive allocation strategy of females of the yellow mealworm beetle, *Tenebrio molitor* (Coleoptera: Tenebrionidae). The mealworm beetle is a cosmopolitan stored grain product pest that lives for several months in populations of variable density. In this highly promiscuous insect, adult females are continuously receptive to reproduction from their fifth day post-emergence and can mate many times with several males within their five months of adulthood. *T. molitor* is iteroparous and considered as an income breeder insect (Zanchi, Moret, & Gillingham, 2019), whose adults finance reproduction from resources gained as adults, as opposed to capital breeder insects, for which reproduction depends on resources cumulated as juveniles (Stearns, 1992; Jönsson, 1997). It 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 to adjust their lifetime reproductive effort according to their physiological state and environment (Kasumovic, Brooks, & Andrade, 2009). Usually, females can produce up to 30 eggs per day but egg production often starts to decline after three weeks (Dick, 1937). Such an age-associated decline in female fecundity may reflect either intrinsic reproductive senescence (i.e., chronological decline in reproductive performance that does not necessary result from the cost of a previous reproductive effort) and/or, based on the disposable soma theory of ageing, trade-off between early and late reproduction (Williams, 1966, Kirkwood, 1977, Ricklefs & Wikelski, 2002).

Here, we experimentally manipulated female age-specific allocation into reproduction at the individual level and assessed its consequences on female fecundity, late-life post-reproduction survival and immunocompetence. The immune system of *T. molitor* relies on both constitutive and inducible innate mechanisms with cellular and humoral components (Vigneron, Jehan, Rigaud, & Moret, 2019). Haemocytes are circulating immune cells involved in parasite elimination. *T. molitor* contains four main types of haemocytes that are morphologically and functionally distinct (Chung & Moon, 2004; Urbański, Adamski, & Rosiński, 2018; Vigneron et al., 2019). Granulocytes and plasmatocytes are the two most abundant haemocytes, and are involved in phagocytosis and encapsulation, respectively. Oenocytoids are cells producing enzymes of the melanization cascades, whereas the last category of haemocytes, the prohaemocytes, may function as precursors of haemocytes. The activity of these immune cells is at the core of the inflammatory response, as they may generate cytotoxic compounds (Nappi & Ottaviani, 2000), causing self-damage and lifespan reduction (Sadd & Siva-Jothy, 2006; Daukšte, Kivleniece, Krama, Rantala, & Krams, 2012; Khan, Agashe, & Rolff, 2017; Krams et al., 2013; Pursall & Rolff, 2011). Haemocyte counts were found to decrease after mating

(Fedorka, Zuk, & Mousseau, 2004; McKean & Nunnery, 2001). Haemocyte numbers decline or remain unchanged with age, depending on the insect species studied (Adamo, Jensen, & Younger, 2001; Doums, Moret, Benelli, & Schmid-Hempel, 2002; Horn, Leips, & Starz-Gaiano, 2014; Jehan, Chogne, Rigaud, & Moret, 2020). In addition, microbial challenge also induces the production of antibacterial peptides in the haemolymph (Vigneron et al., 2019), an energetically costly process that may reduce survival (Moret & Schmid-Hempel, 2000). However, previous work found that variation in immune parameters, as a function of age and reproductive effort, does not always predict organism resistance or tolerance to an immune challenge by natural pathogens (Adamo et al., 2001; Khan et al., 2017). Therefore, a direct measure of immune protection, through a pathogenic exposure, is required in addition to the assessment of immune parameters for a comprehensive understanding of their variation. Hence, confronting putative up- or down-regulation of immune components with organism resistance as a function of age and reproductive allocation will help the interpretation of variation of immune parameters in terms of somatic protection or dysregulation (Khan, Prakash, & Agashe, 2016; Licastro et al., 2005).

In this study, individual *T. molitor* females were allowed to reproduce either early and/or late in life to examine the effect of ageing and reproduction on fecundity, late post-reproduction survival and immunity. Immunity was assessed through the measurement of haemocyte counts, antibacterial activity and resistance to a bacterial infection. Under the hypothesis that early reproduction is costly, we expect that females reproducing early would exhibit reduced late fecundity, immunity and longevity. If female late-life reproductive strategy corresponds to a terminal investment, they are then expected to favour late reproductive investment at the expense of a reduced post-reproduction immunity and survival. By contrast, if females adopt a

reproductive restraint strategy, late reproduction should not be associated with reduced late post-reproduction immunity and survival.

Material and methods

INSECT CULTURE AND EXPERIMENTAL DESIGN

Virgin adult beetles of controlled age (10 ± 2 days post-emergence) were obtained from ca. 700 pupae randomly sampled from a stock culture maintained in standard laboratory conditions at Dijon, France. Prior to the experiments, all the experimental insects were maintained separately, at $24 \pm 1^\circ\text{C}$, 70% RH in permanent darkness (as this insect is highly photophobic), supplied *ad libitum* with bran flour and water, and supplemented once a week with apple.

The following experiments had two objectives. First, we assessed the influence of age-specific reproduction and the intensity of the reproductive effort on patterns of actuarial, reproductive and immune senescence. For this purpose females were allowed to reproduce either early (from 10 to 20 days old), late (from 40 to 60 days old) or both early and late in life and then assessed for their longevity, fecundity, resistance to a bacterial infection and variation in their immune parameters. The early-life reproduction period corresponds to the age range during which all females are sexually mature and produce a large number of eggs (up to 30 eggs) per day (Dick, 1937; Drnevich, Papke, Rauser, & Rutowski, 2001). The late-life reproductive period corresponds to the age range during which survival is still high (Jehan et al., 2020), preventing selective disappearance, and for which egg production is strongly declining. Indeed, from 40 to 60 days old, egg production is usually about one egg a day and negligible after 80 days old (Dick, 1937; Jehan et al., 2020). Second, we assessed the immediate cost of one single reproductive event performed by young and old females, and immune defences with the aim of explaining the patterns of senescence previously observed.

To address these two questions, we conducted three successive experiments (Fig. S1) because it was not possible to measure the three main parameters - namely longevity/reproduction, resistance to bacterial infection and measure immune parameters – in a single series.

EXPERIMENT 1: AGE-SPECIFIC REPRODUCTIVE EFFORT, FECUNDITY AND LONGEVITY

The influence of early and/or late reproduction on longevity and the cost of early reproduction on late reproduction were estimated from 4 groups of 30 females of *T. molitor* experimentally varying in their age-specific reproductive effort. In a first group, females were allowed to reproduce during 20 days early in life only: between day 10 and day 30 post-emergence. Females of the second group were allowed to reproduce during 20 days late in life only: between day 40 and day 60 post-emergence. Females in the third group were allowed to reproduce both for 20 days early and 20 days late in life, whereas a last group of control females were never allowed to reproduce.

Each reproducing female was paired with a 10 ± 2 days old virgin male for 10 days. This male was then replaced by a new 10 ± 2 days 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. Female reproductive output was estimated by counting the number of eggs laid by the female every 3 or 4 days along the 20 days episode of reproduction. Couples were transferred into a new Petri dish and eggs were counted by sieving the flour (mesh size $600\mu\text{m}$) (Moreau, Martinaud, Troussard, Zanchi, & Moret, 2012). Body mass of the females was measured to the nearest 1 mg using an OHAUS balance (discovery series, DU114C) at the date of the first episode of reproduction. When females were not allowed to reproduce during the above specified reproductive episodes, they were treated as those allowed to reproduce, except they were paired with 10 ± 2 days old virgin

females instead of males to control for a potential effect of density of individuals in the Petri dish. From the end of each reproductive episode, females were maintained alone in a new Petri dish with *ad libitum* food and water, and checked twice a week until death.

EXPERIMENT 2: INFLUENCE OF AGE AND REPRODUCTION ON RESISTANCE TO A BACTERIAL INFECTION

Potential changes in somatic maintenance in response to a female's lifetime reproductive effort were examined in terms of resistance to a bacterial infection. For this purpose, groups of 30 females that did not reproduce, that reproduced early in life only, that reproduced late in life only and that reproduced both early and late in life, as described above (Fig. S1), were exposed at the age of 60 days old (immediately after the late sequence of reproduction) to a systemic bacterial infection. The infection was done through wounding with a fine sterilized needle (0.03 mm, Fine Science Tools® n°26000-25) dipped into a pellet of live *Bacillus cereus* (Dhinaut, Chogne, & Moret, 2018). *B. cereus* is a natural pathogen of *T. molitor* (Du Rand & Laing, 2011; Jurat-Fuentes & Jackson, 2012) known to stimulate its immune response and to induce significant mortality upon infection (Dhinaut, Balourdet, Teixeira, Chogne, & Moret, 2017). In addition to these groups of females that were infected late in life, two additional groups of 30 females were exposed to the infection at 30 days old (immediately after the early sequence of reproduction). These additional groups were kept virgin or allowed to reproduce early in life only, to assess the age-specific cost of reproduction on resistance to an infection when compared to the groups of old (60 days old) females that were kept virgin and those that reproduced late in life only (Fig. S1).

After exposure to the infection, survival was checked every hour during the first 12 hours post-infection and then once a day for 14 days (Dhinaut et al., 2017, 2018).

EXPERIMENT 3: INFLUENCE OF AGE AND REPRODUCTION ON THE IMMUNE RESPONSE

Potential variation in resistance to the infection by *B. cereus* was further examined by testing the effect of female age and reproductive effort on various immune effectors, before and after an immune challenge. For this purpose, we took a 5-μL sample of haemolymph in females of 60 days old, coming from groups that varied in their reproduction (virgin, early reproduction only, late reproduction only and both early and late reproduction – Fig. S1). This sample taken at the end of their reproductive treatment, was used to test for the concentration of the main haemocytes of *T. molitor* and its antibacterial activity while the females were unchallenged (corresponding to basal levels of these immune parameters). Immediately after this first sample of haemolymph, the females were immune challenged through a single injection of a 5-μL suspension of inactivated *B. cereus* at a concentration of 10^8 cells per mL of phosphate buffer saline solution (PBS 10 mM, pH7.4). A second 5-μL sample of haemolymph from each female was collected 3 days after this challenge, corresponding to the peak of the immune response (Haine, Moret, Siva-Jothy, & Rolff, 2008; Haine, Pollitt, Moret, Siva-Jothy, & Rolff, 2008) for the concentration of the main haemocytes and the antibacterial activity of their haemolymph while they were immune challenged.

In addition to these groups of females that were assessed late in life, two additional groups of 15 to 25 females (kept virgin or allowed to reproduce in early life only - Fig. S1) were assessed at 30 days old (immediately after the early sequence of reproduction). These additional groups were used to assess the age-specific effect of reproduction on immune parameters, when compared to the groups of old (60 days old) females that were kept virgin and those that reproduced late in life only. In all groups, animals were sacrificed after the second haemolymph sample.

BACTERIAL CULTURES FOR IMMUNE CHALLENGES AND INFECTIONS

The bacterium *Bacillus cereus* used in this study to perform immune challenges and infections was obtained from the Pasteur institute (CIP69.12). The bacterium was grown overnight at 28°C in liquid Broth medium (10 g bacto-tryptone, 5 g yeast extract, 10 g NaCl in 1000 mL of distilled water, pH 7). Bacteria used to perform immune challenges were inactivated in 0.5% formaldehyde prepared in PBS for 30 minutes, rinsed three times in PBS, and their concentration adjusted to 10⁸ bacteria per mL using a Neubauer improved cell counting chamber. 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). For infection, overnight bacterial cultures (20 mL) were centrifuged at 3500 g at 4°C for 30 min. The supernatant was discarded and the bacteria pellet was used for infection.

HAEMOLYMPH COLLECTION AND IMMUNE PARAMETERS

Haemolymph collection was done as described by Dhinaut et al. (2018). Briefly, beetles were chilled on ice for 10 min before the sampling of 5 µL of haemolymph from a wound made in the beetle's neck. Samples were flushed in a microcentrifuge tube containing 25 µL of anticoagulant buffer (Lee et al., 1999). A 10-µL subsample was immediately used for haemocyte count using a Neubauer improved haemocytometer under a phase-contrast microscope (magnification x 400). The count of the main haemocyte types found in *T. molitor* haemolymph was further done using the description made by Urbański et al. (2018). We especially measured the concentration of granulocytes, plasmatocytes and prohaemocytes. The observation of oenocytoïds was anecdotic in our study. Another 5-µL were transferred in

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 µL 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 10^5 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 microlitres 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

All the statistical approaches are summarized in Table 1.

Female fecundity from Experiment 1 was analysed using Generalized Linear Models (GLMs) fitted with a Poisson distribution and a Log link function. We first tested whether female reproductive performance in a single reproductive attempt differs with age. For this purpose, the number of eggs laid by females that reproduced only once in life: either early or late in life; was analysed as a function of age (30 days old versus 60 days old), as a fixed categorical factor, and female initial body mass, measured at 10 days old, as an independent continuous covariate tested as main effect only (Table 1). Then, we tested whether early reproduction, the general state of females or the imminence of their death predict late reproductive effort (Tarwater & Arcese, 2017; Table 1). For this purpose, late female fecundity was analysed as a function of early reproduction (yes/no) as a categorical fixed factor, the duration in days from the late

reproductive episode to female death, and female initial body mass as continuous independent covariates. Finally, among female that reproduced twice (early and late in life) the number of eggs laid late in life was analysed as a function of the number of eggs laid early in life to test whether early reproduction affects late reproductive output at the individual level (Table 1). Data on fecundity were analysed using IBM® SPSS® statistics 19 for Macintosh.

We analysed the differences in female longevity from Experiment 1 as a function of their reproductive treatment using a mixed effects Cox model (Therneau, 2012) including early-life reproduction (yes/no), late-life reproduction (yes/no) and their interaction as independent categorical covariates. This allowed us to test, in a full factorial design, whether the impact of late reproduction could be dependent on late reproduction, which would be revealed as a significant interaction term, since the effects of early reproduction and late reproduction on late survival might not be a priori additive. We also included female initial body mass 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 random effect (Table 1).

Mixed effects Cox regressions were also used to examine resistance to a bacterial infection from Experiment 2 as a function of age and reproductive treatment. We first analysed the survival of females exposed to a bacterial infection late in life (at 60 days old) according to early-life reproduction (yes/no), late-life reproduction (yes/no) and their interaction as independent categorical covariates in a full factorial design. Female initial body mass was included as an independent continuous covariate, which was tested a main effect only (Table 1). Then, we tested the effects of age and a single reproduction attempt on female post-infection survival. For this purpose, the Cox regression analysis included female age at which they were exposed to the bacterial infection (30 days old or 60 days old), reproduction (yes/no) occurring just before being exposed to the bacterial infection and their interaction as categorical

independent covariates in a full factorial design. Note that old females that reproduced both early and late in life were not used in this analysis. Female initial body mass was included as an independent continuous covariates, which was tested as a main effect only (Table 1). In this experiment, survival was assayed for 14 days. Therefore, beetles that survived beyond this period were censored. These analyses also took into account potential inter-individual variation by including female identity as random effect (Table 1). All Cox regression analyses were performed using R version 3.6.3 (R Core Team, 2020) and the ‘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.

In Experiment 3, individual variation in haemocyte counts and antibacterial activity, measured before and after an immune challenge, were analysed using analyses of variance for repeated measures. Data on haemocyte counts were standardized to z scores using the rank inverse-normal transformation procedure from Templeton (2011), whereas data on antibacterial activity were log-transformed to satisfy the conditions of use of parametric tests. We first tested, in old females (60 days old), for changes in immune defences resulting from an immune challenge as a function of early-life reproduction (yes/no), late-life reproduction (yes/no) and their interaction as independent categorical fixed factors (Table 1). Then, we tested the effects of female age and a single reproduction attempt on changes in immune defences before and after an immune challenge. For this purpose, the analysis included female age (30 days old or 60 days old), reproduction (yes/no) occurring just before being assayed and their interaction as categorical independent covariates (Table 1). For all the parametric tests, non-significant interactions were removed for all models. Data on immune parameters were analysed using IBM® SPSS® statistics 19 for Macintosh.

Results

AGE-SPECIFIC REPRODUCTIVE EFFORT AND LATE-LIFE HISTORY TRAITS

Longevity

Early and late-life reproduction had no effect on female longevity either as main effects (Table S1, Mixed effects Cox Regression analysis: early reproduction $z = 0.03$ $df = 1$ $P = 0.980$, late reproduction $z = 1.22$, $df = 1$, $P = 0.220$) or as an interaction (effect before being removed from the last model: $z = -0.24$ $df = 1$ $P = 0.810$), while accounting for inter-individual variation by including female identity as random effects. Only the initial body mass of females explained variation in longevity, with the heaviest females surviving slightly longer than the lightest ($z = -2.34$, $df = 1$, $P = 0.019$, Odd ratio = 0.98). Furthermore, as age-specific survival within the 70 first days post-emergence, which covers the two reproductive periods during which females were allowed to reproduce, was very high (ranging from 100% to 96 % - Fig. S2). Therefore, any later difference in life histories could not be attributed to selective disappearance of frail individuals during this period of female lifespan.

Fecundity

When considering females that reproduced only once in life, those that reproduced late in life produced almost nearly half the number of eggs than those that reproduced early (Table S2, GLM $\chi^2_{1,56} = 52.06$ $P < 0.001$, Fig. 1). Heavier females laid more eggs than lighter ones (initial body mass $\chi^2_{1,56} = 3.93$ $P = 0.048$).

Considering only the reproduction late in life, the number of eggs laid by females was lower when they had first reproduced early in life than when they had not (Table S3, GLM $\chi^2_{1,53} = 9.87$ $P = 0.002$, Fig. 2). Female initial body mass did not explain late fecundity (initial body mass $\chi^2_{1,53} = 0.53$ $P = 0.465$). However, late post-reproduction survival (in days) was

positively correlated with female late fecundity ($\chi^2_{1, 53} = 6.00$ $P = 0.014$), demonstrating that females that had the shortest post-reproductive survival were also those exhibiting the lowest fecundity late in life (Fig. 2).

Finally, among the females that reproduced twice, there was a positive correlation between the number of eggs laid early and late in life (Table S4, GLM $\chi^2_{1, 27} = 9.15$ $P = 0.002$, Fig. 3). Female initial body mass did not explain variation in this relationship ($\chi^2_{1, 27} = 0.05$ $P = 0.820$).

Post-infection survival

Female resistance to a bacterial infection occurring late in life (at 60 days old) was not affected by reproduction either early in life (Table S5, Mixed Effects Cox regression analysis, $z = -1.26$ $df = 1$ $P = 0.210$), late in life ($z = 2.08$ $df = 1$ $P = 0.089$) or their interaction (effect before being removed from the last model: $z = -0.41$ $df = 1$ $P = 0.680$), while accounting for inter individual variation by including female identity as random effects. Heavier females exhibited greater resistance to infection than light ones ($z = -2.28$, $df = 1$, $P = 0.023$, Odd ratio = 0.98).

Immune parameters

Female total haemocyte count was similar whenever they had reproduced early or late in life (Table 2, Fig. 4a). The immune challenge with the dead bacterium *B. cereus* resulted in a general increase in the total haemocyte count, irrespective of their age-specific reproductive effort (Table 2, Fig. 4a).

Some of the haemocyte types exhibited contrasted levels of variation according to the age-specific reproductive effort of the females and the immune challenge. Granulocytes represented about 60 to 70 % of the total haemocyte count, near to the range previously

described in this insect species (Urbański et al., 2018). While early reproduction did not change the granulocyte count, late reproduction resulted in a decline of this type of haemocyte (Table 2, Fig. 4b). However, granulocyte count was insensitive to the immune challenge either as a main effect or as an interaction with early or late reproduction (Table 2, Fig. 4b). Plasmatocyte count, which varied between 29 and 69 % of the total haemocyte count, was affected by the interaction between early and late reproduction (Table 2). While plasmatocyte count tended to increase when females had reproduced only once (early reproduction only or late reproduction only), it was at the lowest value in females that had reproduced both early and late in life (Fig. 4c). The immune challenge increased the plasmatocyte count irrespective of early or late reproduction (Table 2, Fig. 4c).

The prohaemocyte count (about 0.5% of the total haemocyte count) was unaffected by early reproduction whereas it was reduced by late reproduction (Table 2, Fig. 4d). The immune challenge induced a decline in the count of this haemocyte type independently of early or late reproduction (Table 2, Fig. 4d).

Reproduction, either early or late, had no effect on the haemolymph antibacterial activity, which was stimulated by the immune challenge only (Table 2, Fig. 4e).

EFFECT OF AGE AND REPRODUCTION ON IMMUNOCOMPETENCE

Post-infection survival

Old *T. molitor* females had less resistance to infection by *B. cereus* than young females (Table S6, Mixed Effects Cox Regression analysis: $z = 8.12$, $df = 1$, $P = 0.002$, Odd ratio = 1.03, Fig. 5). Whether the females reproduced or not before being exposed to the infection had no effect on their resistance ($z = 2.73$, $df = 1$, $P = 0.073$). The interaction term between age and reproduction did not explain any variation of resistance to the infection (not retained by the model selection procedure) as well as initial female body mass ($z = -1.14$ $df = 1$ $P = 0.240$).

455

456 *Immune parameters*

457 Female total haemocyte count was not affected by age (Table 3, Fig. 6a). As above, the immune
458 challenge with *B. cereus* raised the total haemocyte count, but this increase was much stronger
459 in females that reproduced than in females that did not reproduce, as revealed by the Challenge
460 × Reproduction interaction, independently of age (Table 3, Fig. 6a).

461 The various types of haemocytes that constitute the total haemocyte count showed contrasted
462 levels of variation according to age, reproduction and the immune challenge. Whereas the
463 granulocyte count (here about 64% of the total haemocyte count), was unaffected by age,
464 reproduction or the immune challenge (Table 3), plasmatocyte (about 37 % of the total
465 haemocyte count) and prohaemocyte (about 1.2 % of the total haemocyte count) counts showed
466 variation according to age and reproduction (Table 3).

467 Plasmatocyte count was higher in old females than in young ones and was further increased
468 by reproduction (Table 3, Fig. 6b). The plasmatocyte count increased upon the immune
469 challenge, but this increase was much stronger in females that reproduced than in females that
470 did not reproduce, irrespective of their age (Table 3, Fig. 6b). Prohaemocyte count declined
471 with female age and as an effect of reproduction in an additive manner (Table 2, Fig. 6c).

472 Upon the immune challenge, prohaemocyte count decreased, and this decrease was much
473 stronger in young females than in old ones (Table 3), mostly because the basal level of
474 prohaemocytes decreased with female age (Fig. 6c).

475 Antibacterial activity in female haemolymph was not affected by reproduction or age (Table
476 3). It only increased as an effect of the immune challenge with the dead bacterium *B. cereus*.
477 (Table 3, Fig. 6d).

478

Discussion

This study aimed at experimentally examining the effect of ageing and reproduction on fecundity, longevity and immunocompetence of *T. molitor* females. More specifically, we tested whether late life reproduction corresponds to a terminal investment or a reproductive restraint pattern or senescence.

T. molitor females are sexually mature from the fifth day post-emergence. While they are continuously receptive during their adulthood, egg production may decline from the third week onward of adult life (Dick, 1937). Such an age-associated decline in female fecundity may reflect either intrinsic reproductive senescence and/or trade-off between early and late reproduction (Ricklefs & Wikelski, 2002). In our study, the assessment of female age-specific fecundity demonstrated clear evidence for reproductive senescence in *T. molitor* females, as the number of eggs laid for the first time by older females (from 40 to 60 days old) is nearly half that laid by younger females (from 10 to 30 days old), all else being equal (Fig. 1). This decline in egg production is similar to results identified in other insect species, such as the fruit fly, *Drosophila melanogaster* (Churchill, Dytham, & Thom, 2019), and the cockroach, *Nauphoeta cinerea* (Moore & Sharma, 2005). In addition, older female fecundity is further reduced when they had reproduced early in life, demonstrating the occurrence of a trade-off between early and late reproduction, which accelerated reproductive senescence. However, our results failed to provide evidence for such a trade-off at the individual level, as females with the highest fecundity early in life were also those with the highest fecundity late in life. This relationship cannot be attributed to selective disappearance of frail individuals since, within this period of time, age-specific mortality is very low. Interestingly, initial body mass, which was found to be a good indicator of body condition and potential female fecundity (Zanchi et al., 2019), did not explain variation in the female trade-off between early and late reproduction. Body mass in *T. molitor* is both genetically determined (Morales-Ramos,

504 Kelsrup, Rojas, & Emery, 2019) and explained by proximal food acquisition (e.g., Kim, Kim,
505 Yoon, Lee, & Kim, 2017). Our result therefore suggests that genetically determined body size
506 is not correlated with the trade-off, or that conditions of resource acquisition in our
507 experiment were rather optimal. Indeed, females were fed *ad libitum* and kept individually,
508 preventing competition for food. Because the experiment was too short to select for body size,
509 we believe that optimal feeding conditions likely prevented the observation of individual
510 trade-off in resource allocation between early and late reproduction, as theorized by van
511 Noordwijk and de Jong (1986). This is especially relevant in an income breeder insect like *T.*
512 *molitor* whose reproduction is fuelled by resources gained as adults, as opposed to capital
513 breeder insects, for which reproduction depends on the limited resources cumulated as
514 juveniles (Jönsson, 1997). Therefore, under unlimited access to food during adulthood,
515 individual trade-off between early and late reproduction might remain undetectable.
516 Nevertheless, it is noteworthy that late fecundity was positively associated with post-late
517 reproduction survival time, which is opposite to the prediction from the terminal investment
518 hypothesis. One could argue that, as we have imposed the late reproductive event for all the
519 females at the same age, most females may have not reached the critical physiological state
520 that would induce enhanced fecundity at the expense of late survival. However, numerous
521 females had died soon after their late reproduction (within the first 30 days post-late
522 reproduction, Fig. 2), indicating they have reached this critical state. These females are clearly
523 not those that exhibited the highest fecundity, suggesting that the relationship we have found
524 is rather strong. All together, these results demonstrate that *T. molitor* females are
525 experiencing reproductive senescence and that early reproduction is costly on future
526 reproduction, which could accelerate the rate of reproductive senescence. In addition, the
527 observed pattern of lifetime reproductive allocation does not support the occurrence of a
528 terminal investment.

529

530 As the cost of early reproduction accelerated reproductive senescence, it was expected that
531 longevity could have been affected too (Flatt, 2011; Harshman & Zera, 2007; Lemaître et al.,
532 2015). However, our results do not support this view as females that reproduced early, late or
533 both did not exhibit reduced longevity compared to females kept as virgins. These results
534 suggest the occurrence of an allocation priority scheme (Rigby & Moret, 2000), where
535 allocation of resources to reproduction is sacrificed before the allocation to survival. It
536 therefore means that the fitness of *T. molitor* females is, to some extent, primarily achieved
537 through longevity, which would potentially support the reproductive restraint hypothesis.
538 It is usually predicts that costs of reproduction are reflected in future reproduction rather than
539 survival in long-lived species, whereas the opposite is expected for short-lived breeders

540 (Hamel et al., 2010). Our results suggest that *T. molitor* females present comparable life
541 history tactics to that of long-lived large mammals, despite its longevity of few months.
542 Iteroparity of this income breeder insects characterised by a low adult mortality rate and
543 living in a stable environment (Vigneron et al., 2019) may potentially explain the selection of
544 such a priority scheme (Bouwhuis, Choquet, Sheldon, & Verhulst, 2012; Hamel et al., 2010;
545 Lemaître & Gaillard, 2017). Further study is required on this matter. Moreover, in line with
546 this priority scheme hypothesis, we also found that female reproduction never jeopardized
547 their survival to a bacterial infection, either early, late or both. Nonetheless, older females
548 were less tolerant to the infection than younger ones. Hence, somatic protection is therefore
549 intrinsically senescing in *T. molitor* females, but the cost of reproduction appeared
550 insufficient to accelerate its pace, at least in our study. As explained above, it is possible that
551 conditions were too favourable for females to exhibit any significant reproduction cost on
552 resistance to infection. However, our results are supported by a previous study using the
553 ecologically and phylogenetically related species, *Tribolium castaneum*, which found a

similar decline in post-infection survival with age and only a small negative effect of reproduction on female survival after infection (Khan et al., 2016).

Measured immune parameters do not fully explain the above pattern of resistance to a bacterial infection, although changes in some of them according to age, reproduction and the immune challenge are significant. Our results show that female post-infection survivorship is unlikely dependent on antibacterial activity, as its level was not found to be affected by age or reproduction prior to infection. Only the immune challenge increased its activity in the haemolymph, and previous studies have demonstrated that it only provides a survival benefit to a bacterial infection when it has been previously induced (Dhinaut et al., 2018; Moret & Siva-Jothy, 2003).

Haemocyte counts, and especially variation of the different haemocyte types, may partly explain variation in female post-infection survivorship. Nonetheless, an important insight from our data is that measuring total-haemocyte count, without taking into account the variation of the different types of haemocytes, appears insufficient to characterize female immunocompetence according to age and reproductive effort. Plasmatocytes are considered as the second most important haemocytes, after granulocytes, mainly involved in phagocytosis, encapsulation and wound healing (Theopold, Schmidt, Söderhäll, & Dushay, 2004; Vigneron et al., 2019). Their recruitment was induced by the immune challenge and reproduction, putatively explaining variation in total-haemocyte count as a function of these factors. Such a reproduction-dependent increase of plasmatocytes after an immune challenge suggests that females are likely wounded during mating and/or oviposition, which may have primed their immune system, resulting in an increased concentration of plasmatocytes. Indeed, priming was previously found associated with enhanced circulation of haemocytes (Dhinaut et al., 2018; Zanchi, Troussard, Martinaud, Moreau, & Moret, 2011). It is not known

579 whether increased plasmatocyte count results from the synthesis of new cells (presumably
580 from prohaemocytes) and/or the release of sessile haemocytes into circulation, which each
581 process may involve different costs. Interestingly, plasmatocyte count also increased with
582 female age, as can be explained by a higher probability of having been wounded with age.
583 Old females that had experienced early and late reproduction had less circulating
584 plasmatocytes than females that had reproduced only once, either early or late. This suggests
585 that the two successive reproductive episodes were costly for the females, which were not
586 able to produce or release plasmatocytes anymore. Reproduction also had an apparent cost on
587 granulocyte and prohaemocyte counts, which were reduced in old females after a late
588 reproductive effort. Nevertheless, these apparent costs of reproduction on circulating
589 granulocytes and prohaemocytes did not jeopardize female post-infection survivorship.
590 In contrast to plasmatocyte count, prohaemocyte count was found to decline with female age,
591 after reproduction and after an immune challenge. The decline of prohaemocytes after the
592 immune challenge is smaller in old females than in young ones, as old females had a lower
593 number of circulating prohaemocytes. Together these results provide two important insights.
594 On the one hand, prohaemocytes contribute to the production of plasmatocytes as females
595 grow in age, after reproduction and after the immune challenge. Considering the different
596 proportion of each of these cell types in the haemolymph (plasmatocytes 37% and
597 prohaemocytes 1.2 % of the total haemocyte count), prohaemocytes are unlikely to be
598 contributing to the production of plasmatocytes at a one to one ratio, although the dynamics
599 of production of the prohaemocytes and their differentiation into other new immune cells is
600 not known. On the other hand, there is a clear demonstration that prohaemocyte count, which
601 represents the ability of the insects to produce new immune cells, is senescing. Therefore, in
602 the long term, age may limit the ability of the females to protect themselves from pathogens
603 late in life, which is consistent with our finding that old females had less resistance to

bacterial infection compare to young ones. Overall, the interpretation of immune measures in the context of immunosenescence is complex and may require the assessment of numerous parameters to better understand such a phenomenon. Note that our study allowed assessing a limited number of immune parameters and it is likely that other unmeasured immune components, such as the activity of the prophenoloxidase system or lysozyme activity could be helpful to better explain the pattern of longevity and post-infection survivorship of *T. molitor* females as a function of age and reproductive effort.

To summarize, we confirmed that *T. molitor* females exhibit age-related decline in fecundity and that this reproductive senescence could be accelerated by a significant cost of early reproduction. Females did not increase their reproductive effort with age at the expense of their late post-reproduction survival. Although we found that females become less able to survive a bacterial infection with age, reproductive effort (even late in life) did not negatively impact resistance. While some cellular immune parameters, such as prohaemocytes, declined with age and reproductive effort, others, like plasmatocytes and antibacterial activity, remained at high levels or still exhibited a strong response potential to an immune challenge late in life, irrespective of reproduction. Therefore, while we found some evidence of immune senescence, some components of immunity remained relatively high late in life. Together, these results suggest that *T. molitor* female late life reproductive effort is not achieved at the expense of post-reproduction survival and somatic functions, which is not consistent with the terminal investment hypothesis (Williams, 1966; Clutton-Brock, 1984; Forslund & Pärt, 1995; Isaac & Johnson, 2005). Conversely, these results, in addition to the observation of a life history priority scheme (Rigby & Moret, 2000), in which longevity is favoured at the expense of reproduction, suggest that *T. molitor* reproduction late in life is more consistent with the reproductive restraint hypothesis (McNamara et al., 2009). Therefore, our study

provides, to our knowledge, the first direct experimental support for this hypothesis, for which only scarce empirical evidence from natural populations (Lecomte et al., 2010; Elliott et al., 2014; Morin et al., 2016; Boonekamp et al., 2020) or indirect experimental support in insects (Cotter et al., 2010; Smith et al., 2014) were previously available.

Life history priority schemes, in which life history traits might be favoured at the expense of others, may have important implications for the evolution of reproductive allocation strategies. Life-history theory predicts that costs of reproduction in long-lived species with low mortality should impact future reproduction rather than survival, while the opposite is expected for short-lived species (Hamel et al., 2010). Here, we found evidence that *T. molitor* females exhibit comparable life history tactics than that of long-lived animals, despite being relatively short-lived. Here, we propose that a priority scheme favouring longevity at the expense of reproduction could be frequent in income breeder insects such as *T. molitor*, in comparison to capital breeder insects. Indeed, longevity may provide the opportunity for income breeder insects to optimise their reproductive effort overtime according to their resource access. This is supported by the fact that *T. molitor* adult females kept in rich food conditions can maintain higher fecundity over time than females kept with low food, irrespective of the nutritive environment they were exposed to as larvae (Zanchi et al., 2019). Further studies are needed to formally test this priority scheme hypothesis between longevity and reproduction in capital and income breeder insects, as it would highlight different life history strategies with regards to senescence. More over, the fact a relatively short-lived insect could evolve life history tactics similar to that of long-lived large mammals change our consideration about the relative importance of longevity (at least its absolute value) in the evolution of life history strategies.

653

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659 **Authors'contributions**

660 CJ, TR and YM conceived the ideas and designed methodology; CJ and CS collected the

661 data; CJ and YM analysed the data; CJ, TR and YM led the writing of the manuscript. All

662 authors contributed critically to the drafts and gave final approval for publication.

663

664 **Data accessibility**

665 Data will be available from the Dryad Digital Repository.

666

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Table 1. Summary of statistical tests and designs used to address the questions asked in each experiment of the study. Expected outcomes are also listed for all tests, including expected results under terminal investment (TI), reproductive restraint (RR) or senescence (S).

Experiments	Questions	Predictions	Statistical tests	Models used
Experiment 1	Does age affect female egg output?	Old females may produce fewer eggs than young ones under RR and S. Old females may produce as many or more eggs than young ones under TI. .	GLM fitted with a Poisson distribution and a log link function	Eggs ~ age at reproduction (young/old) + female initial body mass (in mg)
Experiment 1	Does female late fecundity is predicted by early reproduction and post-late reproduction survival?	Female late fecundity should be reduced by early reproduction under TI, RR and S. It should be negatively correlated to survival after late reproduction under TI, not correlated or positively correlated under RR.	GLM fitted with a Poisson distribution and a log link function	Eggs laid late ~ early reproduction (yes/no) + post-late reproduction survival time (in days) + female initial body mass (in mg)
Experiment 1	Do females trade-off early and late reproductive output?	A negative relationship between early and late fecundity is expected, unless lab conditions are good.	GLM fitted with a Poisson distribution and a log link function	Eggs laid late ~eggs laid early + female initial body mass (in mg)
Experiment 1	Does female longevity depend on early and late reproduction?	Shorter longevity should be associated to early and late reproduction under TI, but not under RR.	Mixed Effects Cox Regression	Age ~ early reproduction (yes/no) + late reproduction (yes/no) + early x late reproduction + female initial body mass (in mg) + female ID (as random effect)
Experiment 2	Is survival to a bacterial infection occurring late in life dependent on reproduction?	Reproduction may reduce late-life infection survival under TI but not under RR.	Mixed Effects Cox Regression	Time in days ~ early reproduction (yes/no) + late reproduction (yes/no) + early x late reproduction + female initial body mass (in mg) + female ID (as random effect)
Experiment 2	Do age and a single reproductive attempt influence survival to infection?	Old females may resist less the infection than young ones. Reproduction is expected to further reduce the survival of old females under TI but not under RR.	Mixed Effects Cox Regression	Time in days ~ age + reproduction (yes/no) + female initial body mass (in mg) + female ID (as random effect)
Experiment 3	Does immune responsiveness late in life is dependent on early and late reproduction?	Overall, reproduction may negatively affect immunity. Late reproduction may further decline immunity under TI but not under RR.	Analyses of variance for repeated measures.	Within and between subjects change in the immune parameter ~ early reproduction (yes/no) + late reproduction (yes/no) + early x late reproduction
Experiment 3	Do age and a single reproductive attempt influence immune responsiveness?	Old females may show lower immune responsiveness than young ones. Reproduction is expected to further reduce the immune	Analyses of variance for repeated measures.	Within and between subjects change in the immune parameter ~ age (young/old) + reproduction (yes/no) + age x reproduction

		responsiveness of old females under TI but not under RR.		
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Table 2. Summary of the analyses for repeated measures testing the effects of a bacterial immune challenge in 60 days old *Tenebrio molitor* females as function of their age-specific reproductive effort (early and late reproduction) in a full factorial design. N.R. refers to the effects not retained by the stepwise procedure. Values where $P \leq 0.05$ are given in bold.

	Total Haemocytes			Granulocytes			Plasmatocytes			Prohaemocytes			Antibacterial activity		
<i>Between subjects</i>															
	<i>d.f.</i>	<i>F</i>	<i>P</i>	<i>d.f.</i>	<i>F</i>	<i>P</i>	<i>d.f.</i>	<i>F</i>	<i>P</i>	<i>d.f.</i>	<i>F</i>	<i>P</i>	<i>d.f.</i>	<i>F</i>	<i>P</i>
Early reproduction	1	0.80	0.373	1	2.68	0.105	1	1.02	0.316	1	0.21	0.649	1	0.08	0.776
Late reproduction	1	2.49	0.118	1	4.78	0.032	1	0.00	0.997	1	7.40	0.008	1	0.06	0.816
Early*Late reproduction	N.R.			N.R.			1	5.79	0.018	N.R.			N.R.		
Error	84			84			83			85			88		
<i>Within subjects</i>															
	<i>d.f.</i>	<i>F</i>	<i>P</i>	<i>d.f.</i>	<i>F</i>	<i>P</i>	<i>d.f.</i>	<i>F</i>	<i>P</i>	<i>d.f.</i>	<i>F</i>	<i>P</i>	<i>d.f.</i>	<i>F</i>	<i>P</i>
Challenge	1	7.71	0.007	1	0.01	0.915	1	23.51	<0.001	1	20.75	<0.001	1	28.57	<0.001
Challenge*Early reproduction	1	0.41	0.525	1	0.42	0.520	1	0.03	0.868	1	0.11	0.743	1	0.01	0.929

Challenge*Late reproduction	1	1.74	0.191	1	1.18	0.281	1	1.06	0.306	1	0.95	0.332	1	0.80	0.372
Challenge*Early*Late reproduction	N.R.			N.R.			1	2.09	0.152	N.R.			N.R.		
Error	84			84			83			85			88		

Table 3. Summary of the analyses for repeated measures testing the effects of a bacterial immune challenge in 30 days old and 60 days old *Tenebrio molitor* females immediately after a 20 days reproductive period. Values where $P \leq 0.05$ are given in bold.

	Total Haemocytes			Granulocytes			Plasmatocytes			Prohaemocytes			Antibacterial activity		
<i>Between subjects</i>															
	<i>d.f.</i>	<i>F</i>	<i>P</i>	<i>d.f.</i>	<i>F</i>	<i>P</i>	<i>d.f.</i>	<i>F</i>	<i>P</i>	<i>d.f.</i>	<i>F</i>	<i>P</i>	<i>d.f.</i>	<i>F</i>	<i>P</i>
Age	1	1.19	0.279	1	0.02	0.888	1	4.49	0.037	1	26.47	<0.001	1	1.90	0.172
Reproduction	1	1.06	0.307	1	0.59	0.444	1	6.65	0.012	1	7.80	0.007	1	0.08	0.774
Error	77			76			76			77			76		
<i>Within subjects</i>															
	<i>d.f.</i>	<i>F</i>	<i>P</i>	<i>d.f.</i>	<i>F</i>	<i>P</i>	<i>d.f.</i>	<i>F</i>	<i>P</i>	<i>d.f.</i>	<i>F</i>	<i>P</i>	<i>d.f.</i>	<i>F</i>	<i>P</i>
Challenge	1	4.62	0.035	1	0.34	0.560	1	27.53	<0.001	1	18.85	<0.001	1	27.53	<0.001
Challenge*Age	1	0.23	0.632	1	0.02	0.891	1	1.76	0.826	1	4.44	0.038	1	0.01	0.916
Challenge*Reproduction	1	8.36	0.005	1	2.63	0.109	1	11.07	0.001	1	2.59	0.112	1	0.72	0.398
Error	77			76			76			77			77		

Figure Legends

Fig. 1. Raw mean number of eggs ($\pm$ s.e.) laid early and late in life by *Tenebrio molitor* females allowed to reproduce either early in life ('Early': from 10 to 30 days old) or late in life ('Late': from 40 to 60 days old) only.

Fig. 2. Relationship between female late fecundity (number of eggs) and female post-late reproduction survival (in days) as a function of whether they were allowed to reproduce early in life (filled circles and continuous line, $y = 0.23 \times \text{post-late reproduction survival days} + 35.82$, $r = 0.27$) or not (opened circles and dashed line, $y = 0.40 \times \text{post-late reproduction survival days} + 48.32$, $r = 0.42$). Shades correspond to the 95% confidence intervals for the relationships.

Fig. 3. Relationship between the number of eggs laid early in life (from 10 to 30 days old) and the number of eggs laid late in life (from 40 to 60 days old) by *Tenebrio molitor* females allowed to reproduce both early and late in life ($y = 0.34 \times \text{number of eggs laid early in life} + 1.88$, $r = 0.52$). Shade corresponds to the 95% confidence intervals of the relationship.

Fig. 4. Total haemocyte count (a), granulocyte count (b), plasmatocyte count (c), prohaemocyte count (d) and antibacterial activity (e) in old females (60 days old) that were either virgin, allowed to reproduce 20 days early in life, reproduce 20 days late in life or to reproduce both 20 days early and 20 days late in life. White bars show basal levels of the immune parameters when the females are unchallenged and grey bars show levels of these immune parameters 3 days after being immune challenged. Sample size: 23 Virgin, 27 to 28 Early, 18 to 20 Late and 20 to 22 Early and Late.

Fig. 5. Survival to an infection with the bacterium *Bacillus cereus* of young (30 days old) and old (60 days old) *Tenebrio molitor* females, independently of their reproduction treatment.

Lines above and below the main curves correspond to the 95 % confidence intervals.

Fig. 6. Total haemocyte count (a), plasmatocyte count (b), prohaemocyte count (c) and antibacterial activity (d) in young (30 days old) and old (60 days old) females that were either allowed ('Reproduced') or not ('Virgin') to reproduce 20 days prior to be assessed for their immune defences. White bars show basal levels of the immune parameters when the females are unchallenged and grey bars show levels of these immune parameters 3 days after being immune challenged. Sample size: 23 young/virgin, 14 to 16 young/reproduced, 23 old/virgin and 18 to 19 old/reproduced females.

Supporting Information

Fig. S1. Common experimental design to the three experiments studying individual female fecundity, longevity (experiment 1), post-infection survival (experiment 2) and changes in immune parameters (experiment 3) as a function of their age-specific reproduction treatment. Females were either allowed to reproduce early in life (from day 10 to day 30) or /and late in life (from day 40 to day 60), as illustrated by copulating beetles, and then used for the respective measurements of the experiments they were allocated to. Note that for experiment 1, fecundity was assessed during the periods the females were allowed to reproduce and that longevity was recorded from the day the female had emerged. Some females were exposed to the early reproductive treatment only and then allocated to the experiment 2 or 3 for the assessment of their survival to a bacterial infection or changes in their immune parameters.

Fig. S2. Adult age-specific survival rates ($\pm$ standard error) of *Tenebrio molitor* females. Data are from Experiment 1 (N = 120).

Table S1. Mixed effects Cox regression analysis for *T. molitor* female longevity according to early reproduction ('early reproduction': yes/no), late reproduction ('Late reproduction': yes/no) and their interaction as independent categorical covariates in a full factorial design. Female initial 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 revealed not significant, whereas main effects were always kept in the final model. Data for this analysis are from Experiment 1 and involve females that did not reproduced at all, those that reproduced early in life only, those that reproduce late in life only and those that reproduced both early and late in life (N = 120).

Table S2. Generalised linear model (GLM) for *T. molitor* female fecundity (number of eggs laid during a 20 days period) when reproducing for the first time according to age ('Age') that is either early in life (from 10 to 30 days old) or late in life (from 40 to 60 days old) and female initial body mass ('Mass': measured at 10 days old). Data for this analysis are from Experiment 1 and involve females that reproduced early in life only and those that reproduced late in life only (N = 59).

Table S3. Generalised linear model (GLM) for *T. molitor* female late life fecundity (number of eggs laid during a 20 days period – from age of 40 to 60 days old) according to whether they had or not reproduced early in life ('Early reproduction': from 10 to 30 days old), late post-reproduction survival ('Late survival' in days) and initial body mass ('Mass', measured at 10

days old). Data for this analysis are from Experiment 1 and involve females that reproduced late in life only and those that reproduced early and late in life (N = 57).

Table S4. Generalised linear model (GLM) testing for the correlation between the number of eggs laid by *T. molitor* females late and early in life accounting for female initial body mass. Data for this analysis are from Experiment 1 and involve females that reproduced both early and late in life (N = 30).

Table S5. Mixed effects Cox regression analysis for *T. molitor* female survival to bacterial infection late in life according to early-life reproduction (yes/no), late-life reproduction (yes/no) and their interaction as independent categorical covariates in a full factorial design. Female initial body mass (measured at 10 days old) was included as an independent continuous covariate, which was tested a main effect only. To account for female individual heterogeneity, female identity was included as a random effect in the model. As survival was assayed for 14 days post-infection, beetles that survived beyond this period were censored. We limited model reduction to the interaction terms when revealed not significant, whereas main effects were always kept in the final model. Data for this analysis are from Experiment 2 and involve females that were exposed to a bacterial infection at 60 days old and that were either never allowed to reproduce, reproduce once early in life, reproduce once late in life and reproduce both early and late in life (N= 101).

Table S6. Mixed effects Cox regression analysis for *T. molitor* female survival to bacterial infection according to age of exposure to the infection ('Age': 60 days old/30 days old), whether they were allowed to reproduce ('Reproduction': yes/no) just before being exposed to the bacterial infection, and their interaction as categorical independent covariates in a full factorial

design. Female initial body mass (measured at 10 days old) was included as an independent continuous covariate, which was tested a main effect only. To account for female individual heterogeneity, female identity was included as a random effect in the model. As survival was assayed for 14 days post-infection, beetles that survived beyond this period were censored. We limited model reduction to the interaction terms when revealed not significant, whereas main effects were always kept in the final model. Data for this analysis are from Experiment 2 and involve 30 days old infected females that were either or not allowed to reproduce early in life, and 60 days old infected females that were either or not allowed to reproduce late in life (N= 95).

Supporting information

Late life reproduction in an insect: terminal investment versus reproductive restraint

Charly Jehan*, Camille Sabarly, Thierry Rigaud, Yannick Moret

UMR CNRS 6282 BioGéoSciences, Équipe Écologie Évolutive, Université Bourgogne-Franche Comté, Dijon, France

* Corresponding author: charly.jehan@u-bourgogne.fr

Fig. S1. Common experimental design to the three experiments studying individual female fecundity, longevity (experiment 1), post-infection survival (experiment 2) and changes in immune parameters (experiment 3) as a function of their age-specific reproduction treatment. Females were either allowed to reproduce early in life (from day 10 to day 30) or /and late in life (from day 40 to day 60), as illustrated by copulating beetles, and then used for the respective measurements of the experiments they were allocated to. Note that for experiment 1, fecundity was assessed during the periods the females were allowed to reproduce and that longevity was recorded for the day the female had emerged. In addition some females were exposed to the early reproductive treatment only and then allocated to the experiment 2 or 3 for the assessment of their survival to a bacterial infection and changes in their immune parameters, respectively.

Table S1. Mixed effects Cox regression analysis for *T. molitor* female longevity according to early reproduction ('early reproduction': yes/no), late reproduction ('Late reproduction': yes/no) and their interaction as independent categorical covariates in a full factorial design. Female initial 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 revealed not significant, whereas main effects were always kept in the final model. Data for this analysis are from Experiment 1 and involve females that did not reproduced at all, those that reproduced early in life only, those that reproduce late in life only and those that reproduced both early and late in life (N = 120).

Variable in the best model	β	$\pm$ s.e.	z	d.f.	P	Odd ratio
Mass	- 0.015	0.006	-2.34	1	0.019	0.98
Early reproduction	0.006	0.208	0.03	1	0.980	1.01
Late reproduction	0.253	0.208	1.22	1	0.220	1.30

Random effects (Female ID): SD 0.39, Variance 0.15

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 $\chi^2_{20.4} = 1.41$ P=1, the test is also non significant for each of the covariates).

Fig. S2. Adult age-specific survival rates ($\pm$ standard error) of *Tenebrio molitor* females. Data are from experiment 1 (N = 120).

Table S2. Generalised linear model (GLM) for *T. molitor* female fecundity (number of eggs laid during a 20 days period) when reproducing for the first time according to age ('Age') that is either early in life (from 10 to 30 days old) or late in life (from 40 to 60 days old) and female initial body mass ('Mass': measured at 10 days old). Data for this analysis are from Experiment 1 and involve females that reproduced early in life only and those that reproduced late in life only (N = 59).

Effect	β	$\pm$ s.e.	χ^2	d.f.	P	Exp(β)
(Intercept)	3.45	0.34	103.65	1, 56	< 0.001	31.53
Age ^a	0.58	0.08	52.06	1, 56	< 0.001	1.82
Mass	0.005	0.003	3.93	1, 56	0.048	1.01

^a relative to the old females.

Table S3. Generalised linear model (GLM) for *T. molitor* female late life fecundity (number of eggs laid during a 20 days period – from age of 40 to 60 days old) according to whether they had or not reproduced early in life ('Early reproduction': from 10 to 30 days old), late post reproduction survival ('Late survival' in days) and initial body mass ('Mass', measured at 10 days old). Data for this analysis are from Experiment 1 and involve females that reproduced late in life only and those that reproduced early and late in life (N = 57).

Effect	β	$\pm$ s.e.	χ^2	d.f.	P	Exp(β)
(Intercept)	3.32	0.38	78.30	1, 53	< 0.001	27.66
Early reproduction ^a	0.33	0.11	9.87	1, 53	0.002	1.40
Late survival	0.005	0.002	6.00	1, 53	0.014	1.01
Mass	0.002	0.003	0.53	1, 53	0.465	1.00

^a relative to the females that were not allowed to reproduce early in life.

Table S4. Generalised linear model (GLM) testing for the correlation between the number of eggs laid by *T. molitor* females late and early in life accounting for female initial body mass. Data for this analysis are from Experiment 1 and involve females that reproduced both early and late in life (N = 30).

Effect	β	$\pm$ s.e.	χ^2	d.f.	P	Exp(β)
(Intercept)	2.77	0.72	14.75	1, 27	< 0.001	15.91
Early egg number	0.009	0.003	9.15	1, 27	0.002	1.01
Mass	-0.001	0.006	0.05	1, 27	0.820	0.99

Table S5. Mixed effects Cox regression analysis for *T. molitor* female survival to bacterial infection late in life according to early-life reproduction (yes/no), late-life reproduction (yes/no) and their interaction as independent categorical covariates in a full factorial design. Female initial body mass (measured at 10 days old) was included as an independent continuous covariate, which was tested a main effect only. To account for female individual heterogeneity, female identity was included as a random effect in the model. As survival was assayed for 14 days post infection, beetles that survived beyond this period were censored. We limited model reduction to the interaction terms when revealed not significant, whereas main effects were always kept in the final model. Data for this analysis are from Experiment 2 and involve females that were exposed to a bacterial infection at 60 days old and that were either never allowed to reproduce, reproduce once early in life, reproduce once late in life and reproduce both early and late in life (N= 101).

Variable in the best model	β	$\pm$ s.e.	z	d.f.	P	Exp(β)
Mass	- 0.017	0.007	-2.28	1	0.023	0.98
Early reproduction	-0.261	0.208	-1.26	1	0.210	0.77
Late reproduction	0.348	0.205	2.08	1	0.089	1.42

Random effects (Female ID): SD 0.0199, Variance 0.0004

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 $\chi^2_{7.04} = 2.58$ P = 0.92, the test is also non significant for each of the covariates).

Table S6. Mixed effects Cox regression analysis for *T. molitor* female survival to bacterial infection according to age of exposure to the infection ('Age': 60 days old/30 days old), whether they were allowed to reproduce ('Reproduction': yes/no) just before being exposed to the bacterial infection, and their interaction as categorical independent covariates in a full factorial design. Female initial body mass (measured at 10 days old) was included as an independent continuous covariate, which was tested a main effect only. To account for female individual heterogeneity, female identity was included as a random effect in the model. As survival was assayed for 14 days post infection, beetles that survived beyond this period were censored. We limited model reduction to the interaction terms when revealed not significant, whereas main effects were always kept in the final model. Data for this analysis are from Experiment 2 and involve 30 days old infected females that were either or not allowed to reproduce early in life, and 60 days old infected females that were either or not allowed to reproduce late in life (N= 95).

Variable in the best model	β	$\pm$ s.e.	z	d.f.	P	Exp(β)
Age	0.024	0.008	8.12	1	0.002	1.03
Reproduction	0.384	0.214	2.73	1	0.073	1.47
Mass	-0.005	0.005	-1.14	1	0.240	0.99

Random effects (Female ID): SD 0.0199, Variance 0.0004

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 $\chi^2_{7.03} = 5.39$ P =0.62, the test is also non significant for each of the covariates).