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► **To cite this version:**

Charly Jehan, Camille Sabarly, Thierry Rigaud, Yannick Moret. Late-life reproduction in an insect: terminal investment, reproductive restraint or senescence.. *Journal of Animal Ecology*, 2021, 90 (1), pp.282-297. 10.1111/1365-2656.13367 . hal-03148633

HAL Id: hal-03148633

<https://hal.science/hal-03148633>

Submitted on 27 May 2021

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

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16

17 **Abstract**

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

27
28 **2.** Testing these hypotheses requires proper consideration of the effects of age-dependent
29 reproductive effort on post-reproduction survival and age-related somatic functions. We
30 experimentally tested these three hypotheses in females of the mealworm beetle, *Tenebrio*
31 *molitor*, an iteroparous and income breeder insect. We manipulated their age-specific
32 allocation into reproduction and observed the effects of this manipulation on their late life-
33 fecundity, post-reproduction survival and immunocompetence as a measurement of somatic
34 protection.

35
36 **3.** We found that females exhibit age-related declines in fecundity and that this reproductive
37 senescence is accelerated by a cost of early reproduction. The cost of reproduction had not
38 significant effect on female longevity and their ability to survive a bacterial infection, despite
39 that some immune cells were depleted by reproduction. We found that female post-infection
40 survival deteriorated with age, which could be partly explained by a decline in some immune

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

43

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

48

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

52

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

55

56

57 **Introduction**

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

67 So far, theory predicts that organisms may optimise their age-related reproductive investment
68 in two ways. First, organisms are predicted to increase their investment in reproduction as they
69 approach the end of their life (Williams, 1966; Clutton-Brock, 1984; Forslund & Pärt, 1995;
70 Isaac & Johnson, 2005). This hypothesis invokes that current reproduction is costly on future
71 reproduction and/or survival (Alonso-Alvarez et al., 2004; Harshman & Zera, 2007; Reznick,
72 1985). Young individuals, with high residual reproductive value, i.e. high opportunity for future
73 reproduction (Williams, 1966), are therefore expected to allocate less to current reproduction
74 to ensure future reproductive opportunities, whereas older individuals, with low residual
75 reproductive value, should allocate more to current reproduction. Under this assumption of age-
76 dependent “terminal investment” (Clutton-Brock, 1984), older individuals able to support
77 higher costs of reproduction should be selected, because subsequent reproductive opportunities
78 might be null. In line with this hypothesis, older females of the burying beetle, *Nicrophorus*
79 *orbicollis*, tend to produce larger brood sizes than young ones during their first reproductive
80 attempt (Creighton, Heflin, & Belk, 2009). However, empirical evidence for this prediction is
81 often inconclusive. Indeed, in some species, reproductive performance can be improved with

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

95 Second, and opposite to the terminal investment hypothesis, the “reproductive restraint”
96 hypothesis predicts that organisms adaptively invest less in reproduction as they age (Curio,
97 1983; McNamara, Houston, Barta, Scheuerlein, & Fromhage, 2009). As reproductive activity
98 may generate accumulation of damages (see above), thus decreasing survival probability,
99 organisms should benefit by reproducing less intensively late in life to defer death and therefore
100 gain more time for reproducing (McNamara & Houston, 1996; McNamara et al., 2009). Such
101 a reduced reproduction late in life due to reproductive restraint should be distinguished from a
102 simple consequence of senescence. On the one hand, senescing individuals are expected to
103 show reduced reproduction late in life as a side effect of somatic deterioration, associated with
104 an age-dependent decline of somatic protection, including immunity (Loison, Festa-Bianchet,
105 Gaillard, Jorgenson, & Jullien, 1999; Weladji, Mysterud, Holand, & Lenvik, 2002). On the
106 other hand, reduced late-life reproduction due to reproductive restraint should be associated

107 with maintained somatic protections. Empirical evidence consistent with the reproductive
108 restraint hypothesis is scarce (Cotter, Ward, & Kilner, 2010; Lecomte et al., 2010; Elliott et al.,
109 2014; Smith, Belk, & Creighton, 2014; Morin, Rughetti, Rioux-Paquette, & Festa-Bianchet,
110 2016; Boonekamp, Bauch, & Verhulst, 2020) and direct experimental evidence is missing.
111 Experimentally disentangling the terminal investment strategy from the reproductive restraint
112 one and senescence requires to assess the effects of age-dependent reproductive effort on post-
113 reproduction survival and age-related somatic functions (e.g., immune protection) (Coulson &
114 Fairweather, 2001; McNamara et al., 2009; Reed et al., 2008). The terminal investment
115 hypothesis assumes that as older organisms reach a state in which prospects for future survival
116 decrease, they should increase their allocation to current reproduction. Therefore, late
117 reproductive allocation should be inversely related to survival and immune protection after the
118 last reproduction. In contrast, under the reproductive restraint hypothesis, late allocation in
119 reproduction is not expected to compromise late post-reproduction survival, and immune
120 protection should be maintained with age. However, as organisms invest in their early
121 reproduction, immune protection and longevity are expected to decline rapidly.

122

123 Based on these hypotheses, we tested the reproductive allocation strategy of females of the
124 yellow mealworm beetle, *Tenebrio molitor* (Coleoptera: Tenebrionidae). The mealworm beetle
125 is a cosmopolitan stored grain product pest that lives for several months in populations of
126 variable density. In this highly promiscuous insect, adult females are continuously receptive to
127 reproduction from their fifth day post-emergence and can mate many times with several males
128 within their five months of adulthood. *T. molitor* is iteroparous and considered as an income
129 breeder insect (Zanchi, Moret, & Gillingham, 2019), whose adults finance reproduction from
130 resources gained as adults, as opposed to capital breeder insects, for which reproduction
131 depends on resources cumulated as juveniles (Stearns, 1992; Jönsson, 1997). It feeds at both

132 the larval and the adult stages and females lay eggs in several reproductive bouts along its
133 lifespan (Dick, 1937; Hughes, 2017). These features possibly allow females to adjust their
134 lifetime reproductive effort according to their physiological state and environment (Kasumovic,
135 Brooks, & Andrade, 2009). Usually, females can produce up to 30 eggs per day but egg
136 production often starts to decline after three weeks (Dick, 1937). Such an age-associated decline
137 in female fecundity may reflect either intrinsic reproductive senescence (i.e., chronological
138 decline in reproductive performance that does not necessary result from the cost of a previous
139 reproductive effort) and/or, based on the disposable soma theory of ageing, trade-off between
140 early and late reproduction (Williams, 1966, Kirkwood, 1977, Ricklefs & Wikelski, 2002).

141

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

157 (Fedorka, Zuk, & Mousseau, 2004; McKean & Nunnery, 2001). Haemocyte numbers decline or
158 remain unchanged with age, depending on the insect species studied (Adamo, Jensen, &
159 Younger, 2001; Doums, Moret, Benelli, & Schmid-Hempel, 2002; Horn, Leips, & Starz-
160 Gaiano, 2014; Jehan, Chogne, Rigaud, & Moret, 2020). In addition, microbial challenge also
161 induces the production of antibacterial peptides in the haemolymph (Vigneron et al., 2019), an
162 energetically costly process that may reduce survival (Moret & Schmid-Hempel, 2000).
163 However, previous work found that variation in immune parameters, as a function of age and
164 reproductive effort, does not always predict organism resistance or tolerance to an immune
165 challenge by natural pathogens (Adamo et al., 2001; Khan et al., 2017). Therefore, a direct
166 measure of immune protection, through a pathogenic exposure, is required in addition to the
167 assessment of immune parameters for a comprehensive understanding of their variation. Hence,
168 confronting putative up- or down-regulation of immune components with organism resistance
169 as a function of age and reproductive allocation will help the interpretation of variation of
170 immune parameters in terms of somatic protection or dysregulation (Khan, Prakash, & Agashe,
171 2016; Licastro et al., 2005).

172

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

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

183

184 **Material and methods**

185 INSECT CULTURE AND EXPERIMENTAL DESIGN

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

191 The following experiments had two objectives. First, we assessed the influence of age-specific
192 reproduction and the intensity of the reproductive effort on patterns of actuarial, reproductive
193 and immune senescence. For this purpose females were allowed to reproduce either early (from
194 10 to 20 days old), late (from 40 to 60 days old) or both early and late in life and then assessed
195 for their longevity, fecundity, resistance to a bacterial infection and variation in their immune
196 parameters. The early-life reproduction period corresponds to the age range during which all
197 females are sexually mature and produce a large number of eggs (up to 30 eggs) per day (Dick,
198 1937; Drnevich, Papke, Rauser, & Rutowski, 2001). The late-life reproductive period
199 corresponds to the age range during which survival is still high (Jehan et al., 2020), preventing
200 selective disappearance, and for which egg production is strongly declining. Indeed, from 40 to
201 60 days old, egg production is usually about one egg a day and negligible after 80 days old
202 (Dick, 1937; Jehan et al., 2020). Second, we assessed the immediate cost of one single
203 reproductive event performed by young and old females, and immune defences with the aim of
204 explaining the patterns of senescence previously observed.

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

208

209 EXPERIMENT 1: AGE-SPECIFIC REPRODUCTIVE EFFORT, FECUNDITY AND 210 LONGEVITY

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

219 Each reproducing female was paired with a 10 ± 2 days old virgin male for 10 days. This male
220 was then replaced by a new 10 ± 2 days old virgin male, with which the female spent 10
221 additional days. Couples were maintained in a Petri dish containing bleached flour and provided
222 *ad libitum* food and water under standard laboratory conditions. Female reproductive output
223 was estimated by counting the number of eggs laid by the female every 3 or 4 days along the
224 20 days episode of reproduction. Couples were transferred into a new Petri dish and eggs were
225 counted by sieving the flour (mesh size $600 \mu\text{m}$) (Moreau, Martinaud, Troussard, Zanchi, &
226 Moret, 2012). Body mass of the females was measured to the nearest 1 mg using an OHAUS
227 balance (discovery series, DU114C) at the date of the first episode of reproduction. When
228 females were not allowed to reproduce during the above specified reproductive episodes, they
229 were treated as those allowed to reproduce, except they were paired with 10 ± 2 days old virgin

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

233

234 EXPERIMENT 2: INFLUENCE OF AGE AND REPRODUCTION ON RESISTANCE TO 235 A BACTERIAL INFECTION

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

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

254

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

257 Potential variation in resistance to the infection by *B. cereus* was further examined by testing
258 the effect of female age and reproductive effort on various immune effectors, before and after
259 an immune challenge. For this purpose, we took a 5- μ L sample of haemolymph in females of
260 60 days old, coming from groups that varied in their reproduction (virgin, early reproduction
261 only, late reproduction only and both early and late reproduction – Fig. S1). This sample taken
262 at the end of their reproductive treatment, was used to test for the concentration of the main
263 haemocytes of *T. molitor* and its antibacterial activity while the females were unchallenged
264 (corresponding to basal levels of these immune parameters). Immediately after this first sample
265 of haemolymph, the females were immune challenged through a single injection of a 5- μ L
266 suspension of inactivated *B. cereus* at a concentration of 10^8 cells per mL of phosphate buffer
267 saline solution (PBS 10 mM, pH7.4). A second 5- μ L sample of haemolymph from each female
268 was collected 3 days after this challenge, corresponding to the peak of the immune response
269 (Haine, Moret, Siva-Jothy, & Rolff, 2008; Haine, Pollitt, Moret, Siva-Jothy, & Rolff, 2008) for
270 the concentration of the main haemocytes and the antibacterial activity of their haemolymph
271 while they were immune challenged.

272 In addition to these groups of females that were assessed late in life, two additional groups of
273 15 to 25 females (kept virgin or allowed to reproduce in early life only - Fig. S1) were assessed
274 at 30 days old (immediately after the early sequence of reproduction). These additional groups
275 were used to assess the age-specific effect of reproduction on immune parameters, when
276 compared to the groups of old (60 days old) females that were kept virgin and those that
277 reproduced late in life only. In all groups, animals were sacrificed after the second haemolymph
278 sample.

279

280 BACTERIAL CULTURES FOR IMMUNE CHALLENGES AND INFECTIONS

281 The bacterium *Bacillus cereus* used in this study to perform immune challenges and infections
282 was obtained from the Pasteur institute (CIP69.12). The bacterium was grown overnight at
283 28°C in liquid Broth medium (10 g bacto-tryptone, 5 g yeast extract, 10 g NaCl in 1000 mL of
284 distilled water, pH 7). Bacteria used to perform immune challenges were inactivated in 0.5%
285 formaldehyde prepared in PBS for 30 minutes, rinsed three times in PBS, and their
286 concentration adjusted to 10⁸ bacteria per mL using a Neubauer improved cell counting
287 chamber. The success of the inactivation was tested by plating a sample of the bacterial solution
288 on sterile Broth medium with 1% of bacterial agar and incubated at 28°C for 24 hours. Aliquots
289 were kept at -20 °C until use. Immune challenges were performed by injection through the
290 pleural membrane between the second and the third abdominal tergites using sterile glass
291 capillaries that had been pulled out to a fine point with an electrode puller (Narashige PC-10).
292 For infection, overnight bacterial cultures (20 mL) were centrifuged at 3500 g at 4°C for 30
293 min. The supernatant was discarded and the bacteria pellet was used for infection.

294

295 HAEMOLYMPH COLLECTION AND IMMUNE PARAMETERS

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

305 an N-phenylthiourea-coated microcentrifuge tube (P7629; Sigma-Aldrich, St Louis, MO,
306 USA) flash-frozen in liquid nitrogen and then stored at -80 °C for later examination of its
307 antibacterial activity.

308 Antimicrobial activity of the haemolymph was measured using the inhibition zone assay
309 described by Moret (2006). Briefly, haemolymph samples were thawed on ice, and 2 µL of
310 the sample solution was used to measure antibacterial activity on zone inhibition plates
311 seeded with *Arthrobacter globiformis* from the Pasteur Institute (CIP105365). An overnight
312 culture of the bacterium was added to Broth medium containing 1 % agar to achieve a final
313 concentration of 10^5 cells.mL⁻¹. Six millilitres of this seeded medium were subsequently
314 poured per Petri dish. After solidification, sample wells were made using a Pasteur pipette
315 fitted with a ball pump. Two microlitres of sample were deposited in each well. Plates were
316 then incubated overnight at 28°C and the diameter of each zone of inhibition was measured.

317

318 STATISTICS

319 All the statistical approaches are summarized in Table 1.

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

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

335 We analysed the differences in female longevity from Experiment 1 as a function of their
336 reproductive treatment using a mixed effects Cox model (Therneau, 2012) including early-life
337 reproduction (yes/no), late-life reproduction (yes/no) and their interaction as independent
338 categorical covariates. This allowed us to test, in a full factorial design, whether the impact of
339 late reproduction could be dependent on late reproduction, which would be revealed as a
340 significant interaction term, since the effects of early reproduction and late reproduction on late
341 survival might not be a priori additive. We also included female initial body mass as an
342 independent continuous covariate, which was tested as a main effect only. To account for
343 potential variation in frailty among female individuals, their identity was included as random
344 effect (Table 1).

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

355 independent covariates in a full factorial design. Note that old females that reproduced both
356 early and late in life were not used in this analysis. Female initial body mass was included as
357 an independent continuous covariates, which was tested as a main effect only (Table 1). In this
358 experiment, survival was assayed for 14 days. Therefore, beetles that survived beyond this
359 period were censored. These analyses also took into account potential inter-individual variation
360 by including female identity as random effect (Table 1). All Cox regression analyses were
361 performed using R version 3.6.3 (R Core Team, 2020) and the ‘coxme’ package version 2.2-
362 16. Model reduction was limited to non-significant interaction terms whereas non-significant
363 main effects were kept in the final model. Proportional assumption was checked using `cox.zph()`
364 function in the R survival package.

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

379

380 **Results**

381 AGE-SPECIFIC REPRODUCTIVE EFFORT AND LATE-LIFE HISTORY TRAITS

382 *Longevity*

383 Early and late-life reproduction had no effect on female longevity either as main effects
384 (Table S1, Mixed effects Cox Regression analysis: early reproduction $z = 0.03$ $df = 1$ $P =$
385 0.980 , late reproduction $z = 1.22$, $df = 1$, $P = 0.220$) or as an interaction (effect before being
386 removed from the last model: $z = -0.24$ $df = 1$ $P = 0.810$), while accounting for inter-
387 individual variation by including female identity as random effects. Only the initial body
388 mass of females explained variation in longevity, with the heaviest females surviving slightly
389 longer than the lightest ($z = -2.34$, $df = 1$, $P = 0.019$, Odd ratio = 0.98). Furthermore, as age-
390 specific survival within the 70 first days post-emergence, which covers the two reproductive
391 periods during which females were allowed to reproduce, was very high (ranging from 100%
392 to 96 % - Fig. S2). Therefore, any later difference in life histories could not be attributed to
393 selective disappearance of frail individuals during this period of female lifespan.

394

395 *Fecundity*

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

400

401 Considering only the reproduction late in life, the number of eggs laid by females was lower
402 when they had first reproduced early in life than when they had not (Table S3, GLM $\chi^2_{1,53} =$
403 9.87 $P = 0.002$, Fig. 2). Female initial body mass did not explain late fecundity (initial body
404 mass $\chi^2_{1,53} = 0.53$ $P = 0.465$). However, late post-reproduction survival (in days) was

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

408 Finally, among the females that reproduced twice, there was a positive correlation between
409 the number of eggs laid early and late in life (Table S4, GLM $\chi^2_{1,27} = 9.15$ P = 0.002, Fig. 3).
410 Female initial body mass did not explain variation in this relationship ($\chi^2_{1,27} = 0.05$ P =
411 0.820).

412

413 *Post-infection survival*

414 Female resistance to a bacterial infection occurring late in life (at 60 days old) was not
415 affected by reproduction either early in life (Table S5, Mixed Effects Cox regression analysis,
416 $z = -1.26$ df = 1 P = 0.210), late in life ($z = 2.08$ df = 1 P = 0.089) or their interaction (effect
417 before being removed from the last model: $z = -0.41$ df = 1 P = 0.680), while accounting for
418 inter individual variation by including female identity as random effects. Heavier females
419 exhibited greater resistance to infection than light ones ($z = -2.28$, df = 1, P = 0.023, Odd ratio
420 = 0.98).

421

422 *Immune parameters*

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

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

430 described in this insect species (Urbański et al., 2018). While early reproduction did not
431 change the granulocyte count, late reproduction resulted in a decline of this type of haemocyte
432 (Table 2, Fig. 4b). However, granulocyte count was insensitive to the immune challenge
433 either as a main effect or as an interaction with early or late reproduction (Table 2, Fig. 4b).
434 Plasmatocyte count, which varied between 29 and 69 % of the total haemocyte count, was
435 affected by the interaction between early and late reproduction (Table 2). While plasmatocyte
436 count tended to increase when females had reproduced only once (early reproduction only or
437 late reproduction only), it was at the lowest value in females that had reproduced both early
438 and late in life (Fig. 4c). The immune challenge increased the plasmatocyte count irrespective
439 of early or late reproduction (Table 2, Fig. 4c).
440 The prohaemocyte count (about 0.5% of the total haemocyte count) was unaffected by early
441 reproduction whereas it was reduced by late reproduction (Table 2, Fig. 4d). The immune
442 challenge induced a decline in the count of this haemocyte type independently of early or late
443 reproduction (Table 2, Fig. 4d).
444 Reproduction, either early or late, had no effect on the haemolymph antibacterial activity,
445 which was stimulated by the immune challenge only (Table 2, Fig. 4e).

446

447 EFFECT OF AGE AND REPRODUCTION ON IMMUNOCOMPETENCE

448 *Post-infection survival*

449 Old *T. molitor* females had less resistance to infection by *B. cereus* than young females (Table
450 S6, Mixed Effects Cox Regression analysis: $z = 8.12$, $df = 1$, $P = 0.002$, Odd ratio = 1.03, Fig.
451 5). Whether the females reproduced or not before being exposed to the infection had no effect
452 on their resistance ($z = 2.73$, $df = 1$, $P = 0.073$). The interaction term between age and
453 reproduction did not explain any variation of resistance to the infection (not retained by the
454 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

479 **Discussion**

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

484 *T. molitor* females are sexually mature from the fifth day post-emergence. While they are
485 continuously receptive during their adulthood, egg production may decline from the third
486 week onward of adult life (Dick, 1937). Such an age-associated decline in female fecundity
487 may reflect either intrinsic reproductive senescence and/or trade-off between early and late
488 reproduction (Ricklefs & Wikelski, 2002). In our study, the assessment of female age-specific
489 fecundity demonstrated clear evidence for reproductive senescence in *T. molitor* females, as
490 the number of eggs laid for the first time by older females (from 40 to 60 days old) is nearly
491 half that laid by younger females (from 10 to 30 days old), all else being equal (Fig. 1). This
492 decline in egg production is similar to results identified in other insect species, such as the
493 fruit fly, *Drosophila melanogaster* (Churchill, Dytham, & Thom, 2019), and the cockroach,
494 *Nauphoeta cinerea* (Moore & Sharma, 2005). In addition, older female fecundity is further
495 reduced when they had reproduced early in life, demonstrating the occurrence of a trade-off
496 between early and late reproduction, which accelerated reproductive senescence. However,
497 our results failed to provide evidence for such a trade-off at the individual level, as females
498 with the highest fecundity early in life were also those with the highest fecundity late in life.
499 This relationship cannot be attributed to selective disappearance of frail individuals since,
500 within this period of time, age-specific mortality is very low. Interestingly, initial body mass,
501 which was found to be a good indicator of body condition and potential female fecundity
502 (Zanchi et al., 2019), did not explain variation in the female trade-off between early and late
503 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 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

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

556

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

565 Haemocyte counts, and especially variation of the different haemocyte types, may partly
566 explain variation in female post-infection survivorship. Nonetheless, an important insight
567 from our data is that measuring total-haemocyte count, without taking into account the
568 variation of the different types of haemocytes, appears insufficient to characterize female
569 immunocompetence according to age and reproductive effort. Plasmatocytes are considered
570 as the second most important haemocytes, after granulocytes, mainly involved in
571 phagocytosis, encapsulation and wound healing (Theopold, Schmidt, Söderhäll, & Dushay,
572 2004; Vigneron et al., 2019). Their recruitment was induced by the immune challenge and
573 reproduction, putatively explaining variation in total-haemocyte count as a function of these
574 factors. Such a reproduction-dependent increase of plasmatocytes after an immune challenge
575 suggests that females are likely wounded during mating and/or oviposition, which may have
576 primed their immune system, resulting in an increased concentration of plasmatocytes.

577 Indeed, priming was previously found associated with enhanced circulation of haemocytes
578 (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

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

611
612 To summarize, we confirmed that *T. molitor* females exhibit age-related decline in fecundity
613 and that this reproductive senescence could be accelerated by a significant cost of early
614 reproduction. Females did not increase their reproductive effort with age at the expense of
615 their late post-reproduction survival. Although we found that females become less able to
616 survive a bacterial infection with age, reproductive effort (even late in life) did not negatively
617 impact resistance. While some cellular immune parameters, such as prohaemocytes, declined
618 with age and reproductive effort, others, like plasmatocytes and antibacterial activity,
619 remained at high levels or still exhibited a strong response potential to an immune challenge
620 late in life, irrespective of reproduction. Therefore, while we found some evidence of immune
621 senescence, some components of immunity remained relatively high late in life. Together,
622 these results suggest that *T. molitor* female late life reproductive effort is not achieved at the
623 expense of post-reproduction survival and somatic functions, which is not consistent with the
624 terminal investment hypothesis (Williams, 1966; Clutton-Brock, 1984; Forslund & Pärt,
625 1995; Isaac & Johnson, 2005). Conversely, these results, in addition to the observation of a
626 life history priority scheme (Rigby & Moret, 2000), in which longevity is favoured at the
627 expense of reproduction, suggest that *T. molitor* reproduction late in life is more consistent
628 with the reproductive restraint hypothesis (McNamara et al., 2009). Therefore, our study

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

633 Life history priority schemes, in which life history traits might be favoured at the expense of
634 others, may have important implications for the evolution of reproductive allocation
635 strategies. Life-history theory predicts that costs of reproduction in long-lived species with
636 low mortality should impact future reproduction rather than survival, while the opposite is
637 expected for short-lived species (Hamel et al., 2010). Here, we found evidence that *T. molitor*
638 females exhibit comparable life history tactics than that of long-lived animals, despite being
639 relatively short-lived. Here, we propose that a priority scheme favouring longevity at the
640 expense of reproduction could be frequent in income breeder insects such as *T. molitor*, in
641 comparison to capital breeder insects. Indeed, longevity may provide the opportunity for
642 income breeder insects to optimise their reproductive effort overtime according to their
643 resource access. This is supported by the fact that *T. molitor* adult females kept in rich food
644 conditions can maintain higher fecundity over time than females kept with low food,
645 irrespective of the nutritive environment they were exposed to as larvae (Zanchi et al., 2019).

646 Further studies are needed to formally test this priority scheme hypothesis between longevity
647 and reproduction in capital and income breeder insects, as it would highlight different life
648 history strategies with regards to senescence. More over, the fact a relatively short-lived
649 insect could evolve life history tactics similar to that of long-lived large mammals change our
650 consideration about the relative importance of longevity (at least its absolute value) in the
651 evolution of life history strategies.

652

653

654 **Acknowledgements**

655 This study was supported by the CNRS and a grant from the ANR (ANR-14-CE02-0009) to

656 Y.M. The authors declare no conflict of interest.

657

658

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>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>
<i>Between subjects</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>															
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		

1 **Figure Legends**

2

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

6

7 **Fig. 2.** Relationship between female late fecundity (number of eggs) and female post-late
8 reproduction survival (in days) as a function of whether they were allowed to reproduce early
9 in life (filled circles and continuous line, $y = 0.23 \times$ post-late reproduction survival days +
10 35.82, $r = 0.27$) or not (opened circles and dashed line, $y = 0.40 \times$ post-late reproduction
11 survival days + 48.32, $r = 0.42$). Shades correspond to the 95% confidence intervals for the
12 relationships.

13

14 **Fig. 3.** Relationship between the number of eggs laid early in life (from 10 to 30 days old)
15 and the number of eggs laid late in life (from 40 to 60 days old) by *Tenebrio molitor* females
16 allowed to reproduce both early and late in life ($y = 0.34 \times$ number of eggs laid early in life +
17 1.88, $r = 0.52$). Shade corresponds to the 95% confidence intervals of the relationship.

18

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

26

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

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

30

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

38

39 **Supporting Information**

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

50

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

53

54 **Table S1.** Mixed effects Cox regression analysis for *T. molitor* female longevity according to
55 early reproduction ('early reproduction': yes/no), late reproduction ('Late reproduction':
56 yes/no) and their interaction as independent categorical covariates in a full factorial design.
57 Female initial body mass ('Mass') was also included as an independent continuous covariate,
58 which was tested as a main effect only. To account for female individual heterogeneity, female
59 identity was included as a random effect in the model. We limited model reduction to the
60 interaction terms when revealed not significant, whereas main effects were always kept in the
61 final model. Data for this analysis are from Experiment 1 and involve females that did not
62 reproduced at all, those that reproduced early in life only, those that reproduce late in life only
63 and those that reproduced both early and late in life (N = 120).

64

65 **Table S2.** Generalised linear model (GLM) for *T. molitor* female fecundity (number of eggs
66 laid during a 20 days period) when reproducing for the first time according to age ('Age') that
67 is either early in life (from 10 to 30 days old) or late in life (from 40 to 60 days old) and female
68 initial body mass ('Mass': measured at 10 days old). Data for this analysis are from Experiment
69 1 and involve females that reproduced early in life only and those that reproduced late in life
70 only (N = 59).

71

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

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

78

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

83

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

96

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

101 design. Female initial body mass (measured at 10 days old) was included as an independent
102 continuous covariate, which was tested a main effect only. To account for female individual
103 heterogeneity, female identity was included as a random effect in the model. As survival was
104 assayed for 14 days post-infection, beetles that survived beyond this period were censored. We
105 limited model reduction to the interaction terms when revealed not significant, whereas main
106 effects were always kept in the final model. Data for this analysis are from Experiment 2 and
107 involve 30 days old infected females that were either or not allowed to reproduce early in life,
108 and 60 days old infected females that were either or not allowed to reproduce late in life (N=
109 95).

110

Supporting information

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

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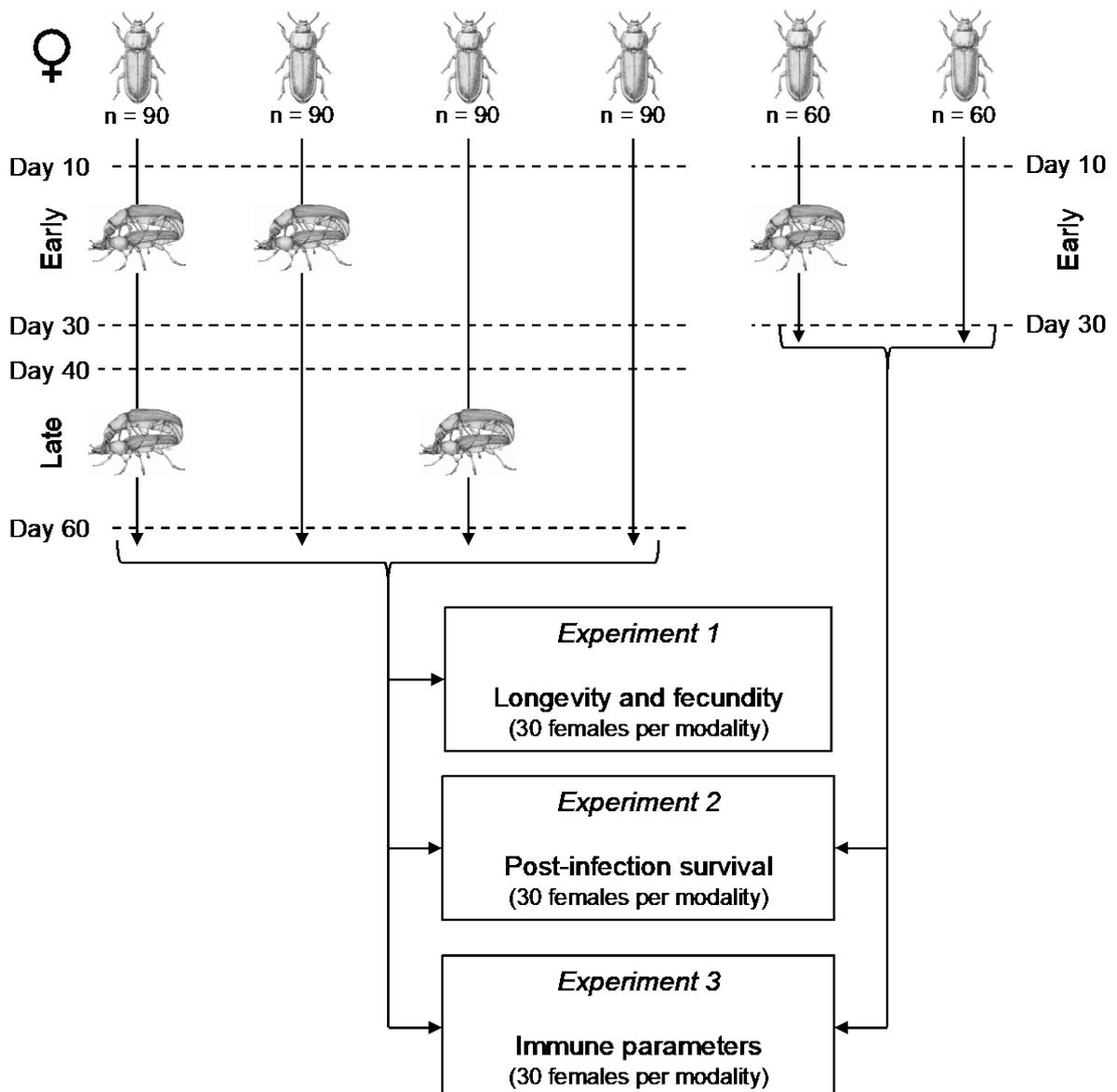


Fig. S1. Common experimental design to the three experiments studying individual female fecundity, longevity (experiment 1), post-infection survival (experiment 2) and changes in immune parameters (experiment 3) as a function of their age-specific reproduction treatment. Females were either allowed to 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).

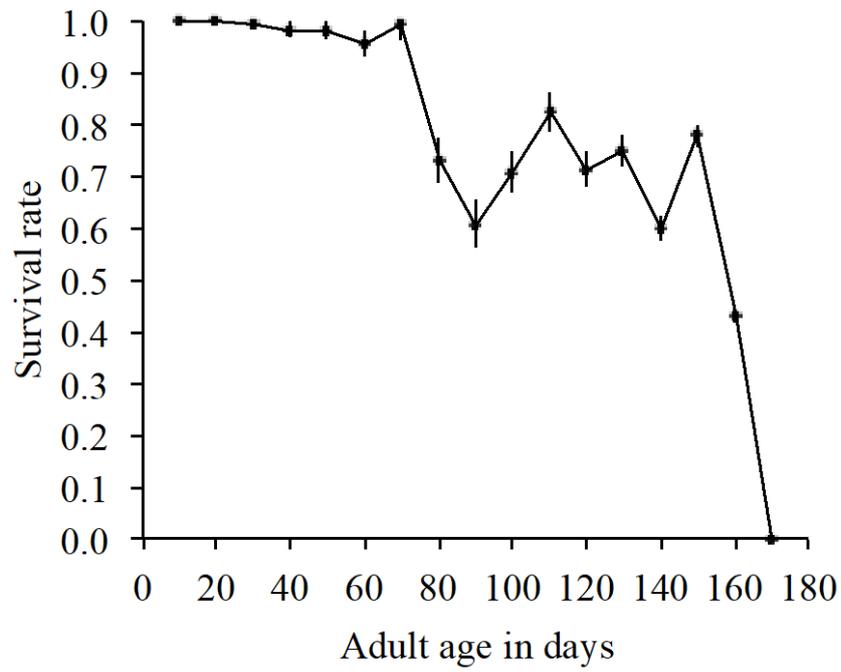


Fig. S2. Adult age-specific survival rates (\pm standard error) of *Tenebrio molitor* females. Data are from experiment 1 (N = 120).

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).

