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

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1 **Abstract**

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

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

28 **Introduction**

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

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

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

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

67 Here, we tested for these two alternative hypotheses by mimicking bacterial infections
68 in females of the mealworm beetle, *Tenebrio molitor*. *T. molitor* is a cosmopolitan pest of
69 stored grain product that is currently raising considerable interests for its use as an alternative
70 protein source for food and feed (Vigneron et al., 2019). Females begin to reproduce from
71 their fifth day post-eclosion, and can mate many times with several males within weeks of
72 their adult life. *T. molitor* is iteroparous and is an income breeder insect (Zanchi et al., 2019):
73 it reproduces using resources gained as an adult, as opposed to a capital breeder insect, for
74 which reproduction depends on resources cumulated during the juvenile stages (Jönsson,
75 1997; Stearns, 1992). *T. molitor* feeds at both the larval and the adult stages and females lay
76 eggs in several reproductive bouts along its lifespan (Dick, 1937; Hughes, 2017). These
77 features possibly allow females of this insect to adjust their lifetime reproductive effort

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

87 In the present study, individual *T. molitor* females were immune challenged with the
88 inactivated bacterium, *Bacillus cereus*, an important natural and virulent pathogen of *T.*
89 *molitor* (Du & Laing, 2011; Jurat-Fuentes & Jackson, 2012). This challenge was made either
90 early or late in life to examine the effect of a pathogenic threat at different ages on fecundity,
91 post-reproduction survival and immunity. Indeed, since young females (i.e., below c.a. 15
92 days post-eclosion – Jehan et al., 2020) still have a high residual reproductive value, we
93 expect that an immune challenge occurring early in life should not trigger a reproductive shift
94 toward a terminal investment (Duffield et al., 2017). As the immune response is costly, we
95 even predict that early challenged females should exhibit reduced fecundity (Moreau et al.,
96 2012). By contrast, as the residual reproductive value of old females becomes low, exposure
97 to an immune challenge is expected to trigger a life history shift resulting in an increased
98 reproductive investment in line with the terminal investment hypothesis (Duffield et al.,
99 2017). Such an increased reproductive investment late in life should be accompanied by both
100 a reduced immunity and a reduced survival. If these assumptions are not fulfilled, then *T.*
101 *molitor* females may alternatively use the reproductive restraint strategy. The reproductive
102 restraint strategy shall be supported if the following results are found. First, late-life

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

107

108 **Material and Methods**

109 INSECT CULTURE AND EXPERIMENTAL DESIGN

110 Virgin adult beetles of controlled age (10 ± 2 days post-eclosion) were obtained from pupae,
111 randomly sampled from a stock culture and maintained in standard laboratory conditions (see
112 below). Prior to the experiments, all the experimental insects were maintained separately, at
113 $24 \pm 1^\circ\text{C}$, 70% RH in permanent darkness, supplied with *ad libitum* bran flour and water, and
114 supplemented once a week with apple.

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

120

121 EXPERIMENT 1: AGE-SPECIFIC IMMUNE CHALLENGE, FECUNDITY AND 122 LONGEVITY

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

127 the inactivated bacterium, *Bacillus cereus* (10^8 bacteria.mL⁻¹ in Phosphate Saline Buffer: PBS
128 10 mM, pH 7.4). A second group of 60 females received a 5- μ L injection of sterile PBS only
129 as a procedural control (see below for inactivation procedure). Immediately after their
130 respective immune treatment, females were allowed to reproduce for a 20-days period (from
131 day 10 to day 30 post-eclosion) to assess their early-life reproductive performance. To this
132 end, each female was paired with a 10 ± 2 -day old virgin male for 10 days. Then, the male
133 was replaced by a new 10 ± 2 -day old virgin male, with which the female spent 10 additional
134 days. Couples were maintained in a Petri dish containing bleached flour and provided *ad*
135 *libitum* food and water under standard laboratory conditions (see above). Female reproductive
136 output was estimated by counting the number of eggs produced by the female every 3 or 4
137 days along the 20-day episode of reproduction. For this purpose, couples were transferred into
138 a new Petri dish and eggs were searched by sieving the flour (600 μ m) and counted (Moreau
139 et al., 2012). At the end of this early reproductive episode, each female was kept alone in a
140 new Petri dish with *ad libitum* food and water for 10 days (until day 40 post-eclosion)
141 awaiting their assessment for their late-life reproductive performance.

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

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

153

154 EXPERIMENT 2: AGE-SPECIFIC IMMUNE CHALLENGE ON ANTIBACTERIAL 155 ACTIVITY

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

165 Six groups of females were created, according to their respective age-specific immune
166 treatment and the age at which their haemolymph was sampled to assess antibacterial activity.
167 Four groups of 20 females were treated as described above (immune-challenged early, late,
168 both early and late, and not immune-challenged as control) to estimate antibacterial activity in
169 their haemolymph late in life. Females were allowed to reproduce as previously described,
170 and provided a sample of haemolymph immediately after their late reproductive episode (at
171 60 days post-eclosion). Two additional groups of 25 females were created to estimate
172 antibacterial activity early in life. Here, the sample of haemolymph was taken at 30 days post-
173 eclosion (i.e., immediately after their early reproductive episode). In one group, females were
174 immune challenged early at 10 days post-eclosion, while in the second group they were not
175 immune challenged. These two additional groups were created to test the effect of the immune

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

178

179 BACTERIAL CULTURES FOR IMMUNE CHALLENGES

180 The bacterium *Bacillus cereus* used to perform the immune challenges was obtained from the
181 Pasteur institute (CIP69.12). The bacteria were grown overnight at 28°C in liquid Broth
182 medium (10 g/L bacto-tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7). Bacteria were then
183 inactivated in 0.5% formaldehyde prepared in phosphate-buffered saline (PBS 10 mM, pH
184 7.4) for 30 minutes, rinsed three times in PBS, and their concentration adjusted to 10⁸ bacteria
185 per mL using a Neubauer improved cell counting chamber (Dubuffet et al., 2015). The
186 success of the inactivation was tested by plating a sample of the bacterial solution on sterile
187 Broth medium with 1% of bacterial agar and incubated at 28°C for 24 hours. Aliquots were
188 kept at -20 °C until use. Immune challenges were performed by injection through the pleural
189 membrane between the second and the third abdominal tergites using sterile glass capillaries
190 that had been pulled out to a fine point with an electrode puller (Narashige PC-10). Immune
191 challenge with inactivated *B. cereus* using this method has proven its efficacy to efficiently
192 stimulate the immune response of *T. molitor* (Jehan et al., 2021).

193

194 HAEMOLYMPH COLLECTION AND ANTIBACTERIAL ACTIVITY

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

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

209

210 STATISTICS

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

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

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

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

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

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

251

252 **Results**

253 LONGEVITY REMAINED UNALTERED BY AGE-SPECIFIC IMMUNE CHALLENGE

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

261

262 BODY MASS DECREASED WITH AGE

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

267

268 REPRODUCTION DECLINED WITH AGE AND AS A RESULT OF AN IMMUNE 269 CHALLENGE EARLY IN LIFE

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

274 affected female fecundity, but as two-way interaction terms with age and with life expectancy
275 (Table 1). The interaction term between the early immune challenge and age shows that the
276 early immune challenge induced a decline in female fecundity at early age only (Fig. 2, Fig.
277 3A, B). The significant interaction term between early challenge and life expectancy (Table 1)
278 shows that females with the longest life expectancy were those that exhibited the largest
279 decline in fecundity after an immune challenge early in life (Fig. 3A). The late immune
280 challenge had no significant effect on late-life female fecundity, whether they were previously
281 exposed to an early challenge or not (Table 1, Fig. 2). Female body mass significantly
282 explained variation in female fecundity, as the heaviest females laid more eggs than the
283 lightest ones (Table 1).

284

285 ANTIBACTERIAL IMMUNE RESPONSE

286 The challenge with inactivated *B. cereus* significantly induced an antibacterial immune
287 response in young (assessed at 30 days post-eclosion) and old (assessed at 60 days post-
288 eclosion) females (Table 2a, Fig. 4). The analysis revealed a significant interaction between
289 female age and the immune challenge (Table 2a), which mainly results from the fact that old
290 control females exhibited a slightly higher antibacterial activity than young control ones (Fig.
291 4). This is also the reason why antibacterial activity was marginally increased with age (Table
292 2a, Fig. 4), since the level of antibacterial activity in young and old immune-challenged
293 females was similar (Fig. 4).

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

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

300

301 **Discussion**

302 Challenging the immune system of breeding *T. molitor* females early or late in life did not
303 induce measurable effects on their longevity in our experimental conditions. Reduced
304 longevity was expected if the immune response imposed a substantial survival cost or if the
305 challenged females shifted toward a terminal reproductive investment. The immune function
306 is known to be costly for organisms (Moret & Schmid-Hempel, 2000) and could cause self-
307 damage (named immunopathology) (Sadd & Siva-Jothy, 2006), which is expected to reduce
308 longevity (Pursall & Rolff, 2011; Urabe et al., 1994). Previous studies found that the immune
309 response causes self-damages in *T. molitor* (Khan et al., 2017; Sadd & Siva-Jothy, 2006) and
310 that females exposed to an early life immune challenge suffered from a reduced longevity
311 (Khan et al., 2017). Our results, contrasting with those of these previous studies, might have
312 at least two main causes in addition to many other small differences. First, Khan et al. (2017)
313 did not allow females to reproduce, whereas our experiment allowed all the females to
314 reproduce early and late in life. It was previously found that reproduction significantly
315 stimulates immune activity in *T. molitor* females (Jehan et al., 2021), which may have
316 buffered the effect of the immune challenge in our experiment. Second, Khan et al. (2017)
317 kept their insects at a suboptimal temperature condition (30°C) compared to the optimum
318 range of temperature of 25-28°C usually used to grow *T. molitor* (Punzo, 1975; Ribeiro et al.,
319 2018) and that we have used in the present study. Actually, in the study by Khan et al. (2017),
320 control (non-immune challenged) females of *T. molitor* showed a 90th percentile survival time
321 of 83.1 days, whereas it was 112 days in our study, a difference suggesting that in their study,
322 females were maintained under stressful conditions, allowing the expression of the cost of an

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

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

336 It should be noted that fecundity of old females was about half that of young ones
337 among controls. This is consistent with previous observations, showing that despite being
338 continuously sexually receptive during adulthood, *T. molitor* female fecundity declines after
339 about two weeks but does not necessary stop (Dick, 1937; Drnevich et al., 2001; Jehan et al.,
340 2020). This might be due to senescence. The age-specific decline of female body mass, which
341 positively predicted female fecundity, may support this view. However, ageing females are
342 also expected to exhibit reduced somatic protection, such as a reduced immune activity. This
343 was clearly not the case here, although our measure of immune activity was restricted to the
344 ability to produce an antibacterial immune response. Antimicrobial activity found in early
345 challenged females is still high late in life. This suggests that old females are still protected by
346 the antibacterial activity induced 50 days earlier, which is, at least, twice as long as previously
347 observed in this insect species (Dhinaut et al., 2018; Haine et al., 2008) and is in line with

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

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

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

374 As noted earlier, our study comforts the results of a previous study which found no
375 evidence of a terminal investment with age in *T. molitor* females (Jehan et al., 2021).
376 However, this previous study did not involve cues, such as an immune challenge, that would
377 represent a terminal investment trigger (Duffield et al., 2017). Here, we have used *B. cereus*,
378 recognized as an important natural pathogen of *T. molitor* (Du & Laing, 2011; Jurat-Fuentes
379 & Jackson, 2012), to challenge the females' immune system. Such an immune challenge was
380 expected to mimic a systemic infection by the bacterium, representing a significant cue
381 predicting imminent death. Accordingly, experimental infections with this bacterium can kill
382 90% of *T. molitor* females in less than three days (Dhinaut et al., 2018; Jehan et al., 2021).
383 Here, the use of *B. cereus* immune challenge did not trigger terminal investment either in
384 young or old females. However, we cannot dismiss that the way the immune challenge was
385 performed might have been too artificial to accurately mimic a deadly infection by *B. cereus*.
386 *B. cereus* infects *T. molitor* mostly orally and more accidentally by wounding through the
387 cuticle. The host immune response can greatly vary according to the route of infection
388 (Behrens et al., 2014), as host adaptation can be contingent on the infection route taken by
389 pathogens (Martins et al., 2013). Future studies may need to perform oral immune challenges
390 to mimic the trigger that would induce terminal investment. Nevertheless, *Bacilli* bacteria that
391 include numerous entomopathogens such as *B. cereus*, appear to have been of paramount
392 importance in the evolution of *T. molitor* immunity, especially through the evolution of
393 immune priming (Dubuffet et al., 2015). This is shown by the fact that they induce highly
394 persistent antibacterial immune responses compared to other bacterial pathogens (Dhinaut et
395 al., 2018). The very long-lasting antibacterial response resulting from the *B. cereus* challenge

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

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

411

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416

417 **Authors' contributions**

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

421

422 **Data accessibility**

423 Data will be available from the Dryad Digital Repository.

424

425 **References**

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- 608

609 **Table 1.** Mixed linear model investigating variance in female egg production as a function of
 610 their age, their experience of an immune challenge occurring early and/or late in life, body
 611 mass measured before each reproductive episode and residual life expectancy calculated at
 612 each reproductive episode. Female identity was included as a random factor. Values $P \leq 0.05$
 613 are given in bold.
 614

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

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

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

617
 618

619

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

627

628

629 Source of variation	β	s.e.	Wald χ^2	d.f.	P
630 (a) Young and old females					
631 Age ^a	1.87	0.81	3.64	1	0.056
632 Challenge ^b	3.54	0.71	32.67	1	< 0.001
633 Age* Challenge	-2.07	0.88	5.58	1	0.018
634					
635					
636					
637					
638 (b) Old females					
639 Early Challenge ^b	1.43	0.43	7.14	1	0.008
640 Late Challenge ^b	1.51	0.43	8.65	1	0.003
641 Early Challenge* Late Challenge	-1.37	0.56	6.02	1	0.014
642					
643					

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

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

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650

651 **Figure 1.** Mean body mass (\pm s.e.) of females at the age at which they started their early (at
 652 10 days post-eclosion) and their late (at 40 days post-eclosion) reproductive episodes.
 653 Numbers at the bottom of the bars are sample size.

654

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

660

661 **Figure 3.** Relationship between fecundity (number of eggs) and age-specific life expectancy
 662 in days of (A) young and (B) old females that have been exposed to an early immune
 663 challenge. In (A), the relationships between fecundity and life expectancy were $y = 0.85 x$
 664 days of life expectancy + 38.32, $r^2 = 0.34$, $P < 0.0001$ and $y = 0.40 x$ days of life expectancy +
 665 34.04, $r^2 = 0.15$, $P = 0.0017$ among controlled and early-challenged females, respectively. In
 666 (B), the relationships between fecundity and life expectancy were $y = 0.47 x$ days of life
 667 expectancy + 5.21, $r^2 = 0.21$, $P = 0.0033$ and $y = 0.50 x$ days of life expectancy - 0.62, $r^2 =$
 668 0.20, $P < 0.0084$ among controlled and early-challenged females, respectively.

669

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

676 **Supplementary information**

677

678 **Table S1.** Mixed effects Cox regression analysis for *T. molitor* female longevity according to
679 early challenge ('early reproduction': yes/no), late challenge ('Late reproduction': yes/no) and
680 their interaction as independent categorical covariates in a full factorial design. Female body
681 mass ('Mass') was also included as an independent continuous covariate, which was tested as
682 a main effect only. To account for female individual heterogeneity, female identity was
683 included as a random effect in the model. We limited model reduction to the interaction terms
684 when found not significant, whereas main effects were always kept in the final model (N =
685 120).

686

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

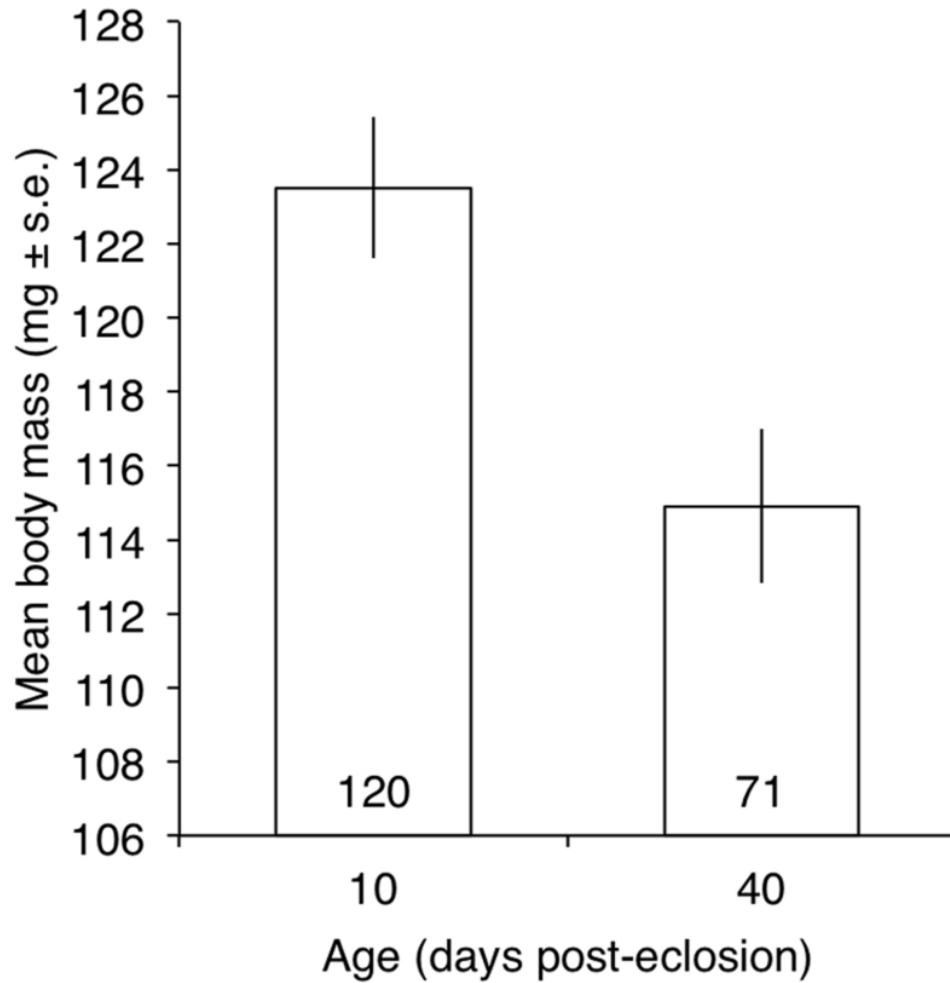


Figure 1. Mean body mass (\pm s.e.) of females at the age at which they started their early (at 10 days post-eclosion) and their late (at 40 days post-eclosion) reproductive episodes. Numbers at the bottom of the bars are sample size.

70x70mm (300 x 300 DPI)

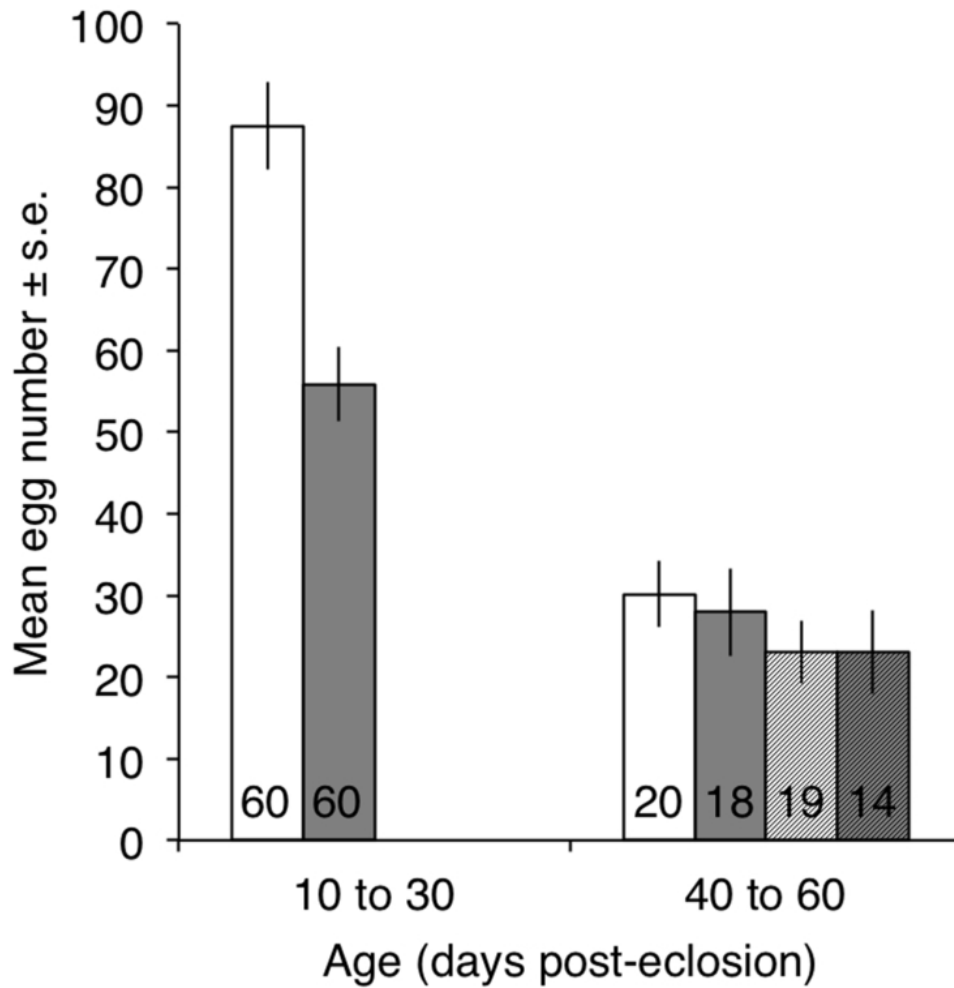


Figure 2. Mean number of eggs (\pm s.e.) produced by females early in life (between day 10 and day 30 post-eclosion) and late in life (between day 40 and day 60 post-eclosion) as function of their life time immune treatment: control (white bars), immune challenged early (grey bars), immune challenged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

64x65mm (300 x 300 DPI)

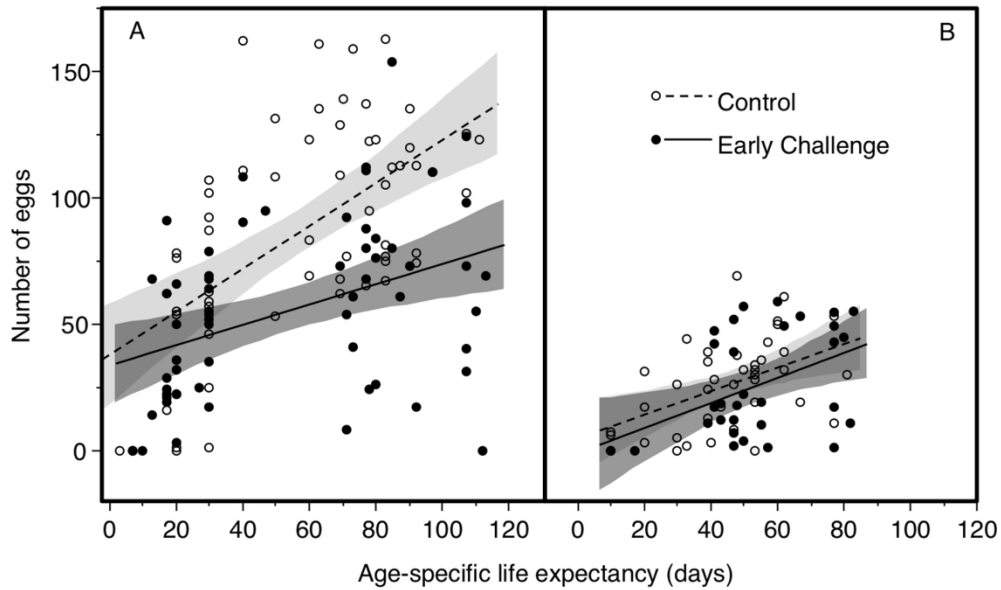


Figure 3. Relationship between fecundity (number of eggs) and age-specific life expectancy in days of (A) young and (B) old females that have been exposed to an early immune challenge. In (A), the relationships between fecundity and life expectancy were $y = 0.85 \times \text{days of life expectancy}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

109x68mm (600 x 600 DPI)

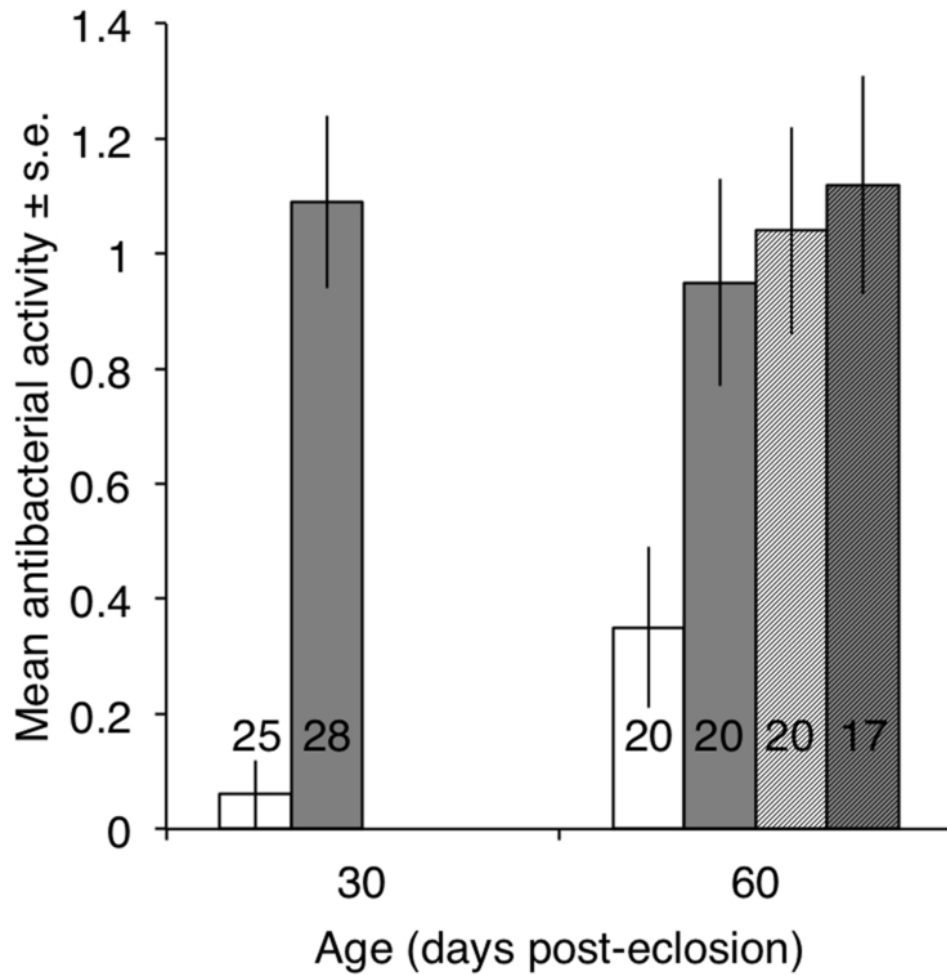


Figure 4. Mean antibacterial activity (\log_{10} (mm) \pm s.e.) in the haemolymph of young (30 days post-eclosion) and old (60 days post-eclosion) females as function of their life time immune treatment: control (white bars), immune challenged early (at 10 post-eclosion, grey bars), immune challenged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

65x66mm (300 x 300 DPI)

Supplementary Information for:

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

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

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

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

Proportional hazard assumption was checked as a non-significant relationship between residuals and time using the `cox.zph()` function in the R survival package (global test $\chi^2_{65.9} = 3.70$ P = 1, the test is also non significant for each of the covariates).

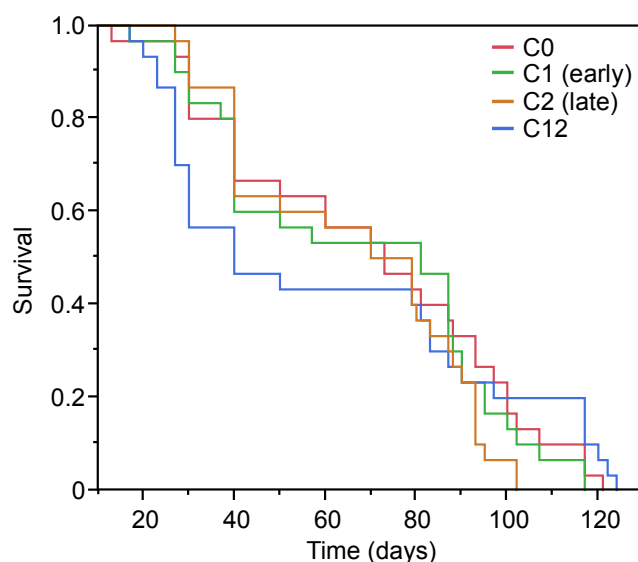


Fig. S1. Survival of *Tenebrio molitor* females as a function of their life-time immune treatment: C0 control, C1 early-life, C2 late-life and C12 early and late immune challenges with the inactivated bacterium *Bacillus cereus*.