1 # Selection on age of female reproduction in the marula fruit fly,
2 # *Ceratitis cosyra* (Walker) (Diptera: Tephritidae), decreases total
3 # antioxidant capacity and lipid peroxidation

4

5 **Kevin Malod[a*], Petrus D. Roets[a], Carel Oosthuizen[a], Jonathan D. Blount[b], C. Ruth**
6 **Archer[c], Christopher W. Weldon[a]**

7

8 *[a]Department of Zoology and Entomology, University of Pretoria, Private bag X20, Hatfield*
9 *0028, South Africa.*
10 *[b]Centre for Ecology and Conservation, College of Life and Environmental Sciences,*
11 *University of Exeter, Penryn, UK.*
12 *[c]Institute for Evolutionary Ecology and Conservation Genomics, University of Ulm, Ulm,*
13 *Germany.*

14

15 **Corresponding author: E-mail: kevin.malod@gmail.com*

16

17 **Abstract:**

18 The oxidative damage caused to cells by reactive oxygen species (ROS) is one of several factors
19 implicated in causing ageing. Oxidative damage may also be a proximate cost of reproductive effort
20 that mediates the trade-off often observed between reproduction and survival. However, how the
21 balance between oxidative damage and antioxidant protection affects life-history strategies is not fully
22 understood. To improve our understanding, we selected on female reproductive age in the marula fruit
23 fly, *Ceratitis cosyra*, and quantified the impact of selection on female and male age-dependent
24 mortality, female fecundity, male sperm transfer, calling and mating. Against expectations, upward-
25 selected lines lived shorter lives and experienced some reductions in reproductive performance.
26 Selection affected oxidative damage to lipids and total antioxidant protection, but not in the direction
27 predicted; longer lives were associated with elevated oxidative damage, arguing against the idea that
28 accumulated oxidative damage reduces lifespan. Greater reproductive effort was also associated with
29 elevated oxidative damage, suggesting that oxidative damage may be a cost of reproduction, although
30 one that did not affect survival. Our results add to a body of data showing that the relationship between
31 lifespan, reproduction and oxidative damage is more complex than predicted by existing theories.

32

33 **Keywords:** tephritidae; oxidative stress; lifespan; reproduction; antioxidant

34

35 # 1. Introduction

36 Of the theories attempting to explain the mechanistic basis of ageing, the free radical theory of ageing
37 (FRTA) (Harman, 1956) has received the most attention. Reactive oxygen species (ROS) are central to
38 the FRTA. Oxidation-reduction reactions used by mitochondria to produce adenosine triphosphate for
39 biochemical functions are one of the main sources of ROS formation (Selman et al., 2012). ROS are
40 highly reactive and can cause cellular damage by oxidising cellular components such as proteins, lipids,
41 or nucleic acids. To prevent damage accumulation, cells have antioxidant defences that render ROS
42 inert, and can repair oxidative damage once it occurs. The FRTA predicts that when ROS levels exceed
43 the antioxidant capacity of an organism, this leads to a state of oxidative stress and oxidative damage
44 occurs. The accumulation of this damage causes cellular dysfunction and is thought to contribute
45 towards ageing. However, several studies show that the relationship between oxidative damage and
46 lifespan is oversimplified and the FRTA has fallen into disuse (Clancy and Birdsall, 2013; Pérez et al.,
47 2009; Speakman and Selman, 2011; Stuart et al., 2014). Nevertheless, oxidative damage may still play
48 a role in causing ageing (Hekimi et al., 2011; Kirkwood and Kowald, 2012).

49 More recently, ROS have been incorporated into an evolutionary framework, which suggests that ROS
50 may still affect lifespan by mediating the life-history trade-off between reproduction and lifespan
51 (Dowling and Simmons, 2009; Metcalfe and Alonso-Alvarez, 2010; Monaghan et al., 2009; Speakman
52 et al., 2015). This could occur if reproduction increases ROS production, or if reproduction utilises
53 resources that could otherwise be used to prevent or repair oxidative damage. Under either scenario,
54 reproductive effort would increase oxidative damage accumulation, and potentially reduce lifespan.
55 This idea has received some support from empirical studies (Archer et al., 2013; Blount et al., 2016;
56 Costantini, 2008; Vágási et al., 2019). However, there is a lack of direct experimental work showing
57 that laboratory selection on life-histories is accompanied by changes in oxidative damage and/or
58 antioxidant protection in the direction predicted. In addition, the few studies that investigated the topic
59 were performed on conventional model organisms such as *Drosophila melanogaster* (Arking et al.,
60 2000; Harshman and Haberer, 2000).

61 Here, we investigated whether selection on the age of female reproduction affects sex-specific patterns
62 of lifespan and reproduction. We then determined if those changes were associated with altered
63 oxidative damage and antioxidant protection. If oxidative damage contributes to variation in lifespan,
64 we predict that reduced oxidative damage is associated with longer lives. If oxidative damage is a
65 proximate cost of reproduction, then greater reproductive investment should elevate oxidative damage.
66 If reproduction reduces resources available to protect against oxidative damage, then greater
67 reproductive effort should be associated with reduced antioxidant defences. We tested these predictions
68 in the marula fruit fly, *Ceratitis cosyra* (Walker) (Diptera: Tephritidae), as an alternative to the
69 conventional biological models (e.g. *D. melanogaster*), to broaden the diversity of species investigated

and because life-history traits and ageing patterns have been well studied in tephritids (Carey, 2011; Carey and Molleman, 2010; Carey et al., 2008; Chen et al., 2013; Fanson et al., 2012; Malod et al., 2017). We found that selection on female reproductive schedules was associated with altered life-history strategies. But against expectations, lines with longer lives and elevated reproductive investment were associated with both greater oxidative damage and total antioxidant capacity.

## 2. Materials and Methods

### 2.1 Fly husbandry

Infested mangoes from across Mpumalanga province, South Africa, were collected and pupae of *C. cosyra* retrieved. The flies emerging from these pupae were used to establish a culture that was maintained at ~ 23° C in a climate room with a 14:10 dark light photoperiod. To create optimal mating conditions, 1h of dawn and dusk was simulated by turning on 8 W fluorescent tubes (T4, Eurolux, Sandton, South Africa) placed above the fly culture 1 h before and after the main light of the room was switched on. The remaining room lights, comprising a combination of 20 W (G5, Eurolux, Sandton, South Africa) and 58 W (58W/840, Osram, Germany) fluorescent tubes were also turned on for the remainder of the light period. Adults were kept in groups of ca. 200 flies in 5 L plastic cages with unrestricted access to food (hydrolysed yeast and sugar in separate dishes) and water (water-soaked cotton wool). At 15 days after emergence, wild males from the culture were crossed with females from a laboratory culture provided by Citrus Research International (Nelspruit, South Africa). Flies for the selection regime were obtained by allowing laboratory females mated with wild males to lay eggs on a 125 mL plastic container (Plastilon, South Africa) covered with a layer of laboratory film (Parafilm M, Bemis, USA) pierced several times. Tissue paper soaked with 3 mL of guava juice (Hall's concentrate, Tiger Consumer Brands Limited, Bryanston, South Africa) was placed in the plastic container to encourage females to oviposit through the film. Eggs were then harvested and placed on 125 mL of a carrot-based diet (Citrus Research International, Nelspruit, South Africa) in a plastic container at an approximate rate of 2.5 eggs/mL of medium. The container was then placed in a 2 L plastic box with a layer of sand and a ventilated lid. After 15 days, during the pupal phase, the sand was sifted and the retrieved pupae placed in a Petri-dish (ø 65 mm) and transferred into a 5 L cage with unrestricted access to food and water for emerging adults.

### 2.2 Selection regime

Selection began three generations after laboratory females had been crossed with wild males and a strong culture had been established. We selected on the age of oviposition by only providing an oviposition substrate (a 125 mL plastic container with guava juice-soaked tissue paper) when flies were 15 days old (control, CT) or 25 days old (upward selected, US). In our laboratory, 15 days is the average

105 age when eggs are collected from this species and is also when oviposition peaked in an earlier study
106 (Manrakhan and Lux, 2006). Upward selection at 25 days rather than an older age was to ensure that
107 enough females would contribute to the subsequent generation due to a gradual decline in oviposition
108 from three weeks of age (Manrakhan and Lux, 2006). This was to maintain populations of ca. 200 flies
109 per replicate and avoid the risk of a population collapse. Selecting upwards on age of reproduction has
110 been shown to extend lifespan in *Drosophila* (Harshman and Haberer, 2000; Rose, 1984), probably
111 because only individuals that survive to reproduce late in life produce offspring. For each of the two
112 selection lines (CT and US) we established five replicate populations. We maintained the selection
113 regime for 20 generations. Lifespan and reproductive effort assays were performed for each line at
114 generation 20 ($G_{20}$). Because flies were selected on age of oviposition, selection lines inevitably differed
115 in their assay date.
116
117 **2.3 Survival**
118 Within 24 hours of emergence, 10 females and 10 males from each selection regime and replicate were
119 transferred to individual containers. Each container comprised a 125 mL plastic cup with another cup
120 nested inside with the base removed (for easy replacement of food and egg laying dishes; see below).
121 The containers were covered with insect screen secured by two rubber bands. The flies were provided
122 with filtered water through the insect screen with 200 µL pipette tips loosely capped at the wide end
123 with putty-like pressure-sensitive adhesive (Prestik, Bostik, South Africa) to minimise evaporation.
124 Sugar and hydrolysed yeast (Yeast Extract Powder, Biolab, Merck, Germany) were provided in the lids
125 of two 2 mL microcentrifuge tubes. Mortality was recorded daily, and food and water were replaced
126 when close to being depleted or if the sugar liquefied (due to its tendency to absorb water vapour). For
127 each selection regime, lifespan data were obtained for 50 females and 50 males (n = 10 per sex, per
128 replicate).
129
130 **2.4 Female reproductive effort**
131 Within a day of emergence, 50 females from each selection regime were placed into individual
132 containers as described above (n = 10 per replicate, per selection regime). An artificial egg laying dish
133 was added to each of the containers. This dish comprised a black screw-top lid (volume = 5 mL,
134 diameter = 32 mm) containing a 1:10 orange essence-water solution (Robertsons, Johannesburg, South
135 Africa) and covered with a double layer of laboratory film, pierced ten times with an entomological pin.
136 The number off eggs laid by each female was counted every five days. The total number of eggs laid
137 by females during their lifetime was calculated as the sum of all five-day oviposition intervals, and the
138 average number of eggs per day as the total number of eggs divided by lifespan. The day of peak egg
139 production was the day at the end of the five-day oviposition interval during which the maximum
140 number of eggs were obtained from a female.
141

142 **2.5 Male reproductive effort**

143 For each selection regime, groups of 50 males were taken from each replicate shortly after emergence
144 and kept in separate plastic cages to prevent mating. At ages 5, 15 and 25 days ($t_5$, $t_{15}$ and $t_{25}$), focal
145 males were paired with virgin females from an unselected laboratory culture one hour before the
146 simulated sunset. This species only mate at dusk and new mating does not occur after darkness (personal
147 observation). For each selection regime, age, and replicate, six pairs were assayed. The females used as
148 mates were all between 10 and 20 days old to minimise the effect of female age on male reproductive
149 measurements. The pairs were placed into cylindrical transparent plastic containers (height = 52 mm,
150 diameter = 35 mm) for easy observation. All pairs were observed until all lights are turned off (two
151 hours) to record if male calling and mating occurred. Due to the tendency of *C. cosyra* to mate for up
152 to 12 hours (personal observation), flies were left to mate overnight.
153
154 The following morning, mated females were dissected under a stereo microscope to analyse sperm
155 transfer, using methods described by Roets et al. (2018). A total of 110 females were dissected (CT: $t_5$
156 = 23, $t_{15}$ = 17, $t_{25}$ = 17; US: $t_5$ = 19, $t_{15}$ = 13, $t_{25}$ = 21). Spermathecae of females were removed and
157 placed individually into 15 µL drops of water on microscope slides. Each spermatheca was then crushed
158 with an entomological pin attached to a thin wooden dowel. The crushed spermatheca was then spread
159 by vigorous stirring for 30 seconds before covering it with a 22 × 22 mm cover slip. The slides were
160 left to dry for 2-3 days before gluing the corners of the coverslip to the slide using clear nail varnish.
161
162 The number of sperm transferred were estimated using a phase contrast microscope (BX43, Olympus
163 Corporation, Japan) and methods described in Taylor *et al.* (2000). In summary, a matrix of 25 fields
164 of view at 100 × magnification (17.36% of the coverslip area) were selected. The number of sperm
165 counted in this area was multiplied by 5.76 to estimate the total number of sperm stored per
166 spermatheca. If no sperm were found in the 25 fields checked, the whole slide was checked to confirm
167 absence of sperm.
168
169 **2.6 Lipid peroxidation and total antioxidant capacity**

170 For each selection regime, 20 females and 20 males were collected at three different ages (0, 25 and 50
171 days). Flies were individually placed in 2 mL microcentrifuge tubes and frozen and stored at - 80º C.
172 On the day of the assay, flies were removed from the freezer and weighed to determine wet mass. Flies
173 were then placed in a 1.5 mL microcentrifuge tube with 1 mL of 5 mM phosphate-buffered saline and
174 homogenised for 30 s using a homogeniser (T25 Ultra-Turrax, IKA, Germany). Malondialdehyde
175 (MDA) concentration was measured using high-performance liquid chromatography according to
176 Agarwal and Chase (2002). A standard curve was obtained using a serial dilution series of 1,1,3,3-
177 tetraetoxypropane (Helfenstein et al., 2010; Mougeot et al., 2009). In order to determine total
178 antioxidant capacity (TAC) for each individual, we used an antioxidant assay kit (Cayman, Cat no.

179 709001, USA) according to the manufacturer's recommendations. The TAC assay detects both non-
180 enzymatic (e.g. ascorbic acid, α-tocopherol) and enzymatic antioxidants (e.g. superoxide dismutase,
181 catalase) but cannot discriminate between them.

182

183 **2.7 Data analyses**

184 To determine the effect of selection and sex on survival, a Cox proportional hazards model was
185 performed using the "coxme" function from the "survival" package (Therneau, 2015) in R (v 3.5.3, The
186 R Foundation for Statistical Computing). Selection regime, sex and their interaction were fixed effects
187 in the model, while replicate population was included as a random effect. We used backwards step-wise
188 deletion of fixed effects to determine the minimal adequate model based on the lowest value for
189 Akaike's information criterion. If a significant effect was detected, the model coefficient was used to
190 determine the direction of the difference.

191 Generalised linear mixed effects models were used to analyse the other traits with Poisson (total eggs
192 and sperm transfer), gamma (day of peak egg production, eggs per day, TAC and MDA) or binomial
193 distributions (propensity of male calling or mating) with selection regime included as a fixed effect and
194 replicate population included as a random effect. Other explanatory variables in each model are detailed
195 below. Models were built using the function "glmer" from the "lme4" package (Bates et al., 2015).
196 Where appropriate, we corrected for over-dispersion by adding an observation level random effect
197 (Harrison, 2014). If a significant effect was detected, *post hoc* pairwise comparisons tests of the
198 estimated marginal means were performed using the function "emmeans" (Russell, 2020).

199 Because zero values prevent use of the gamma family, if the number of eggs laid per day for a female
200 was zero, this value was replaced with the smallest value of the dataset for this trait and divided by ten.
201 To determine the effect of selection and age on the total number of sperm transferred to spermathecae,
202 the number of sperm transferred per day, and the propensity of males to call and mate, selection regime
203 and age, as well as their interaction were included as fixed effects.

204 If the value of the detected TAC for an individual was zero, it was replaced with the smallest TAC value
205 in the dataset and divided by ten. For TAC and lipid peroxidation, the fixed effects added to the model
206 were selection regime, age, and sex. Body mass was included as a covariate to account for variation in
207 fly size. Interactions between all terms were included to the model, except for the covariate and random
208 effect. If a significant effect of the covariate was detected, the coefficient and the significance of the
209 slope were inspected. For graphical representation of TAC and lipid peroxidation, the means predicted
210 by the models were plotted.

211

212 # 3. Results

213 **3.1 Survival**

214 Selection regime was the only variable retained in the minimal adequate model ($\chi 2 = 16.64$, df = 1, $p <$
215 $0.001$), with US flies having a significantly higher mortality risk (coefficient = 1.13, $p < 0.001$) than
216 CT flies (Fig. 1). On average, US flies lived for 147.2 ± 6.85 days and CT flies for 210.0 ± 7.4 days
217 (mean ± SE). The longer lifespan of CT was the consequence of an extended period when no mortality
218 was recorded in either sex (females: 61 days; males: 73 days).

219

220 **3.2 Female reproductive effort**

221 We observed fewer eggs being laid by US females than CT females (Fig. 2a), but this difference was
222 not statistically significant ($\chi 2 = 3.35$, df = 1, $p = 0.067$). The day of peak egg production was
223 significantly later for US females ($\chi 2 = 8.46$, df = 1, $p = 0.004$) (Fig. 2b). The selection regime had a
224 significant effect on the number of eggs laid per day ($\chi 2 = 8.11$, df = 1, $p = 0.004$), with females from
225 US lines producing on average 0.42 ± 0.07 eggs per day in comparison with 0.96 ± 0.18 for CT line
226 females.

227

228 **3.3 Male reproductive effort**

229 All dissected females had sperm stored in their spermathecae. Both, selection regime and male age
230 affected the number of sperm transferred, but there was no significant interaction between them ($\chi 2 =$
231 $0.76$, df = 2, $p = 0.683$). Over the three tested ages, males from the CT lines consistently transferred
232 more sperm (coefficient = 0.589, $p < 0.001$) than those from the US lines ($\chi 2 = 4.72$, df = 1, $p = 0.029$)
233 (Fig. 3). In both lines, sperm transfer changed with age ($\chi 2 = 18.28$, df = 2, $p < 0.001$) and was lowest
234 when males were 5 days old (5 vs 15 days: coefficient = - 0.581, $p = 0.003$; 5 vs 25 days: coefficient =
235 - 0.847, $p < 0.001$), but there was no difference between sperm transferred at 15 and 25 days (coefficient
236 = - 0.266, $p = 0.301$) (Fig. 3). The proportion of males calling or mating were not affected by the
237 selection regime (Calling: $\chi 2 = 1.16$, df = 1, $p = 0.282$; Mating: $\chi 2 = 0.037$ , df = 1 , $p = 0.847$) or male
238 age (Calling: $\chi 2 = 0.67$, df = 2, $p = 0.716$; Mating: $\chi 2 = 3.44$, df = 2, $p = 0.179$). In addition, there was
239 no significant interaction between the selection regime and male age (Calling: $\chi 2 = 0.52$, df = 2, $p =$
240 $0.769$; Mating: $\chi 2 = 4.22$, df = 2, $p = 0.112$). Across selection regimes and ages, 52% of the males
241 exhibited courtship behaviour and 68% mated successfully.

242

243 **3.4 Total antioxidant capacity and lipid peroxidation**

244 There was a significant interaction between selection, age, and sex affecting TAC (Table 1). In newly
245 emerged flies, US females and males had TAC that was 6-fold lower than CT flies of the same age, but
246 by 50 days it was reversed with TAC being approximately double that of US flies (Table S1; Fig. 4).
247 At 25 days of age, US males had lower TAC than CT and US females. TAC of US females (Fig. 4) was
248 similar at all ages (Table S1), while the TAC of the US males was the lowest at 25 days of age (Table

249 S1; Fig. 4). In contrast, there was a significant decrease of TAC with age in both sexes of the CT lines
250 (Table S1; Fig. 4).
251
252 Lipid peroxidation was affected by an interaction between selection regime, age, and sex (Table 1), and
253 there was a significant positive effect of body mass (Table 1; coefficient = 0.107, p = 0.006). Overall,
254 oxidative damage was lower in US flies (Table S2; Fig. 4). Females from the US lines had significantly
255 lower lipid peroxidation than those of the CT lines at 0 and 25 days, but not at 50 days where it was
256 similar (Fig. 4). In males, oxidative damage to lipids was lower in US flies only on the day of emergence
257 (Table S2) (Fig. 4). The changes in age-specific level of lipid peroxidation differed between selection
258 regimes. In the US lines, oxidative damage to lipids remained constant, whereas in the CT lines damage
259 decreased with age (Table S2; Fig. 4).
260

## 261 4. Discussion

262 Selecting upwards on age of female reproduction produced individuals with reduced survival and
263 reproductive capacity, but a later peak in egg production. This latter observation is important as it
264 reveals that selection regime was successful in altering how females schedule their reproductive effort.
265 Fertility was negatively impacted in males, as they transferred less sperm to female's spermathecae.
266 Male courtship and mating success, as well as female lifetime fecundity were the only traits that did not
267 respond to the selection regime. In addition, lifespan was positively correlated with the number of sperm
268 transferred and negatively correlated with the peak of egg production; showing that the value of these
269 traits are a good indicator to determine if a fly belongs to the upward-selected or control lines (Appendix
270 A, Fig. S1).

271 These results are surprising and contrast with existing literature on how selection on female
272 reproductive scheduling should impact life-histories, where selection upwards on age of female
273 reproduction extends lifespan. Here, we found the opposite; upwards selection on age of reproduction
274 was associated with reduced survival. However, it is worth noting that the unselected control flies
275 exhibited sixty to seventy days with zero mortality in either sex. This may be the main factor driving
276 the survivorship difference between control and upward-selected flies. This pattern could reflect the
277 study sample size, which is too small to allow accurate parameterisation of a survival curve using
278 parametric survival models (Promislow et al., 1999; Shouman and Witten, 1995). However, forty
279 individuals is sufficient to provide reasonable results using Cox models (the approach employed here)
280 (Johnson et al., 1982). Some authors have suggested that it may be worth abandoning analyses of
281 mortality rates and instead use summary statistics such as life-expectancy at birth, which should not
282 depend heavily on sample size (Promislow et al., 1999). Furthermore, extended periods of high
283 survivorship are not unusual and have been observed in various species (Cypser et al., 2006; Krainacker

284 et al., 1987; Norry et al., 2006; Roets et al., 2018) as well as in earlier generations of our selected lines
285 (unpublished data). For example, female Ceratitis capitata reared on plum experience negligible
286 mortality for twenty days, which is approximately 40 % of the total lifetime of these flies (Krainacker
287 et al., 1987). Therefore, while the period if zero mortality we observed is likely in part a statistical
288 artefact, it seems reasonable to assume that mortality early in the life of our control group is low and
289 that sizeable difference in longevity between control (147 days) and selected (210 days) flies is
290 meaningful.

291 The reduced survival and sperm transfer in the upward-selected lines might reflect that in various
292 species, including insects, older parental ages have less fit offspring due to deterioration of the parental
293 germline with age (Monaghan and Metcalfe, 2019). Accordingly, selecting for older parents might
294 result in reduced offspring fitness that accumulates over multiple generations. While we might expect
295 selection to counteract these effects (a parent that sires high-quality offspring late-in-life would have an
296 important fitness advantage), this would only happen if there was a genotype in the starting population
297 that could produce high quality offspring late in life. In addition, control flies experienced a no-mortality
298 period of fifty days, which could be seen as the main factor driving the survivorship difference between
299 control and upward-selected flies. Although we cannot say what caused these unusual results, selection
300 did alter life-histories in such a way that we can make and test predictions about correlated changes in
301 antioxidant protection and oxidative damage.

302 Selection lines varied in oxidative damage to lipids and TAC. Crucially, upward-selected flies began
303 their adult life with lower total antioxidant protection, had the best protection at the oldest age, and
304 suffered less from oxidative damage at all ages. Moreover, our lines did not show any age-associated
305 changes in either trait. Low antioxidant protection and oxidative damage in upward-selected flies
306 suggest that ROS production was decreased in these flies in comparison with control individuals. With
307 upward-selected flies performing relatively poorly in comparison with control flies, it would be
308 worthwhile studying bioenergetics efficiency of mitochondria as a potential mechanism for lower
309 oxidative damage in upward-selected *C. cosyra* (López-Lluch et al., 2006). Control flies had higher
310 lipid peroxidation and much better total antioxidant protection at emergence, but experienced age-
311 associated declines in both traits over age, which occurred at a younger age in males than in females for
312 oxidative damage. These observations contrast with studies in *D. melanogaster*, where selection for
313 long lifespan (Arking et al., 2000) or late-life reproduction (Harshman and Haberer, 2000) led to
314 extended lifespan, and increased antioxidant protection or oxidative stress resistance respectively.
315 These results contrast with the FRTA because first, the selection regime associated with the highest
316 survival experienced greater oxidative damage, and second, oxidative damage did not increase with age
317 in either sex but actively declined over age in males. Our results are in line with the growing literature

318 that undermine the FRTA and show no direct relationship between lifespan and oxidative damage
319 (Speakman and Selman, 2011).

320 Higher total and daily fecundity, as well as earlier reproductive investment in males and females from
321 control lines were associated with higher oxidative damage and greater antioxidant protection. For
322 example, greater antioxidant protection is observed in response to conditions inducing higher ROS
323 production (Towarnicki et al., 2020). This may suggest that here, reproduction is associated with greater
324 ROS production, and in turn oxidative damage, and supports the suggestion that ROS production can
325 be a cost of reproduction. However, there was no trade-off between survival and reproduction
326 suggesting that while oxidative damage may be elevated following reproduction, it does not have costly
327 survival impacts. Similar results were reported in *Teleogryllus commodus*, where reproduction was
328 associated with greater oxidative damage in males, but not reduced lifespan (Archer et al., 2015).

329 In conclusion, selecting upwards on age of female reproduction in *C. cosyra* led to a decreased life
330 expectancy, lower reproductive capacity in males and delayed reproductive investment in females. This
331 was associated with lower oxidative damage to lipids and total antioxidant protection. Our results do
332 not support the predictions of the FRTA as the highest survival was observed with flies sustaining the
333 most oxidative damage but also experiencing the most important drop in total antioxidant protection as
334 they aged. In addition, our observations support the idea that reproduction may be a source of oxidative
335 damage but that this higher damage did not result in reduced lifespan.

336


337 **Acknowledgements**

338 We thank Melia Burdon for assistance with the biochemical assays, and Henriek Bosua for helping with
339 dissections. We also thank Aruna Manrakhan and Rooikie Beck who collected the fruits to establish the
340 laboratory culture of *C. cosyra*. Two anonymous reviewers provided comments to improve an earlier
341 version of the manuscript. We thank Fabien Démares for helping with the design of the graphical
342 abstract.


343

## 344 CRediT author statement

345 **KM:** Formal analysis, Data Curation, Writing – Original Draft, Validation, Visualization. **PDR:**
346 Investigation, Data Curation, Writing Review & Editing. **CO:** Investigation, Data Curation, Writing –
347 Review & Editing. **JDB:** Methodology, Resources, Writing – Review & Editing, Supervision. **CRA:**
348 Conceptualization, Methodology, Formal analysis, Writing – Review & Editing, Validation,
349 Supervision, Funding acquisition. **CWW:** Conceptualization, Methodology, Validation, Resources,
350 Writing – Review & Editing, Supervision, Project Administration, Funding acquisition.

351

352 ## Funding

353 KM, PDR and CO were supported by a National Research Foundation Competitive Programme for
354 Rated Researchers awarded to CWW, CRA and JDB (No: 93686).

355

356 ## Declaration of interests

357 The authors have no competing interests to declare.

358

359 ## Data availability

360 Data will be made available in the online repository of the University of Pretoria upon manuscript
361 acceptance.

362

363 ## References

364 Agarwal, R., Chase, S.D., 2002. Rapid, fluorimetric–liquid chromatographic determination of
365 malondialdehyde in biological samples. J. Chromatogr B 775, 121-126.

366 Archer, C.R., Hempenstall, S., Royle, N., Selman, C., Willis, S., Rapkin, J., Blount, J.D., Hunt, J., 2015.
367 Testing the effects of DL-alpha-tocopherol supplementation on oxidative damage, total antioxidant
368 protection and the sex-specific responses of reproductive effort and lifespan to dietary manipulation in
369 Australian field crickets (*Teleogryllus commodus*). Antioxidants 4, 768-792.

370 Archer, C.R., Sakaluk, S.K., Selman, C., Royle, N., Hunt, J., 2013. Oxidative stress and the evolution
371 of sex differences in life span and ageing in the decorated cricket, *Gryllodes sigillatus*. Evolution 67,
372 620-634.

373 Arking, R., Burde, V., Graves, K., Hari, R., Feldman, E., Zeevi, A., Soliman, S., Saraiya, A., Buck, S.,
374 Vettraino, J., Sathrasala, K., Wehr, N., Levine, R.L., 2000. Forward and reverse selection for longevity
375 in *Drosophila* is characterized by alteration of antioxidant gene expression and oxidative damage
376 patterns☆. Exp Gerontol 35, 167-185.

377 Bates, D., Mächler, M., Bolker, B., Walker, S., 2015. Fitting linear mixed-effects models using lme4. J
378 Stat Softw 67, 48.

379 Blount, J.D., Vitikainen, E.I.K., Stott, I., Cant, M.A., 2016. Oxidative shielding and the cost of
380 reproduction. Biol Rev 91, 483-497.

381 Carey, J.R., 2011. Biodemography of the Mediterranean fruit fly: Aging, longevity and adaptation in
382 the wild. Exp. Gerontol. 46, 404-411.

383 Carey, J.R., Molleman, F., 2010. Reproductive aging in tephritid fruit flies. Ann N Y Acad Sci 1204,
384 139-148.

385 Carey, J.R., Papadopoulos, N.T., Müller, H.-G., Katsoyannos, B.I., Kouloussis, N.A., Wang, J.-L.,
386 Wachter, K., Yu, W., Liedo, P., 2008. Age structure changes and extraordinary lifespan in wild medfly
387 populations. Aging Cell 7, 426-437.
388 Chen, E.H., Wei, D., Wei, D.D., Yuan, J.J., 2013. The effect of dietary restriction on longevity,
389 fecundity, and antioxidant responses in the oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Diptera:
390 Tephritidae). J. Insect Physiol. 59, 1008-1016.
391 Clancy, D., Birdsall, J., 2013. Flies, worms and the free radical theory of ageing. Ageing Res. Rev. 12,
392 404-412.
393 Costantini, D., 2008. Oxidative stress in ecology and evolution: lessons from avian studies. Ecol. Lett.
394 11, 1238-1251.
395 Cypser, J.R., Tedesco, P., Johnson, T.E., 2006. Hormesis and aging in *Caenorhabditis elegans*. Exp.
396 Gerontol. 41, 935-939.
397 Dowling, D.K., Simmons, L., 2009. Reactive oxygen species as universal constraints in life-history
398 evolution. ProC.R. Soc. Biol. Sci. Ser. B 276, 1737-1745.
399 Fanson, B.G., Fanson, K.V., Taylor, P.W., 2012. Cost of reproduction in the Queensland fruit fly: Y-
400 model versus lethal protein hypothesis. Proc Biol Sci 279, 4893-4900.
401 Harman, D., 1956. Aging: a theory based on free radical and radiation chemistry. J. Gerontol. 11, 298-
402 300.
403 Harrison, X.A., 2014. Using observation-level random effects to model overdispersion in count data in
404 ecology and evolution. PeerJ 2, e616-e616.
405 Harshman, L.G., Haberer, B.A., 2000. Oxidative stress resistance: a robust correlated response to
406 selection in extended longevity lines of *Drosophila melanogaster*? J Gerontol 55, B415-B417.
407 Hekimi, S., Lapointe, J., Wen, Y., 2011. Taking a "good" look at the free radicals in the aging process.
408 Trends Cell Biol. 21, 569-576.
409 Helfenstein, F., Losdat, S., Møller, A.P., Blount, J.D., Richner, H., 2010. Sperm of colourful males are
410 better protected against oxidative stress. Ecol. Lett. 13, 213-222.
411 Johnson, M.E., Tolley, H.D., Bryson, M.C., Goldman, A.S., 1982. Covariate analysis of survival data:
412 a small-sample study of Cox's model. Biometrics 38, 685-698.
413 Kirkwood, T.B.L., Kowald, A., 2012. The free-radical theory of ageing - older, wiser and still alive:
414 modelling positional effects of the primary targets of ROS reveals new support. Bioessays 34, 692-700.
415 Krainacker, D.A., Carey, J.R., Vargas, R.I., 1987. Effect of larval host on life history traits of the
416 mediterranean fruit fly, *Ceratitis capitata*. Oecologia 73, 583-590.
417 López-Lluch, G., Hunt, N., Jones, B., Zhu, M., Jamieson, H., Hilmer, S., Cascajo, M.V., Allard, J.,
418 Ingram, D.K., Navas, P., de Cabo, R., 2006. Calorie restriction induces mitochondrial biogenesis and
419 bioenergetic efficiency. Proc Natl Acad Sci U S A 103, 1768-1773.

420 Malod, K., Archer, C.R., Hunt, J., Nicolson, S.W., Weldon, C.W., 2017. Effects of macronutrient intake
421 on the lifespan and fecundity of the marula fruit fly, *Ceratitis cosyra* (Tephritidae): Extreme lifespan in
422 a host specialist. Ecol. Evol. 7, 9808-9817.
423 Manrakhan, A., Lux, S.A., 2006. Contribution of natural food sources to reproductive behaviour,
424 fecundity and longevity of *Ceratitis cosyra*, *C. fasciventris* and *C. capitata* (Diptera: Tephritidae). Bull.
425 Entomol. Res. 96, 259-268.
426 Metcalfe, N.B., Alonso-Alvarez, C., 2010. Oxidative stress as a life-history constraint: the role of
427 reactive oxygen species in shaping phenotypes from conception to death. Funct. Ecol. 24, 984-996.
428 Monaghan, P., Metcalfe, N.B., 2019. The deteriorating soma and the indispensable germline: gamete
429 senescence and offspring fitness. Proc Biol Sci 286, 20192187.
430 Monaghan, P., Metcalfe, N.B., Torres, R., 2009. Oxidative stress as a mediator of life history trade-
431 offs: mechanisms, measurements and interpretation. Ecol. Lett. 12, 75-92.
432 Mougeot, F., Martínez-Padilla, J., Webster, L.M.I., Blount, J.D., Pérez-Rodríguez, L., Piertney, S.B.,
433 2009. Honest sexual signalling mediated by parasite and testosterone effects on oxidative balance. Proc
434 Biol Sci 276, 1093-1100.
435 Norry, F.M., Sambucetti, P., Scannapieco, A.C., Loeschcke, V., 2006. Altitudinal patterns for longevity,
436 fecundity and senescence in *Drosophila buzzatii*. Genetica 128, 81-93.
437 Pérez, V.I., Bokov, A., Van Remmen, H., Mele, J., Ran, Q., Ikeno, Y., Richardson, A., 2009. Is the
438 oxidative stress theory of aging dead? Biochim. Biophys. Acta. 1790, 1005-1014.
439 Promislow, D.E.L., Tatar, M., Pletcher, S., Carey, J.R., 1999. Below-threshold mortality: implications
440 for studies in evolution, ecology and demography. J Evol Biol 12, 314-328.
441 Roets, P.D., Bosua, H., Archer, C.R., Weldon, C.W., 2018. Life history and demographic traits of the
442 marula fruit fly, *Ceratitis cosyra* (Walker) (Diptera: Tephritidae): potential consequences of host
443 specialisation. Physiol. Entomol. 43, 259-267.
444 Rose, M.R., 1984. Laboratory evolution of postponed senescence in *Drosophila melanogaster*.
445 Evolution 38, 1004-1010.
446 Russell, L., 2020. emmeans: estimated marginal means, aka least-squares means. R package version
447 1.4.6.
448 Selman, C., Blount, J.D., Nussey, D.H., Speakman, J.R., 2012. Oxidative damage, ageing, and life-
449 history evolution: where now? Trends Ecol. Evol. 27, 570-577.
450 Shouman, R., Witten, M., 1995. Survival estimates and sample size: what can we conclude? J Gerontol
451 50A, B177-B185.
452 Speakman, J.R., Blount, J.D., Bronikowski, A.M., Buffenstein, R., Isaksson, C., Kirkwood, T.B.L.,
453 Monaghan, P., Ozanne, S.E., Beaulieu, M., Briga, M., Carr, S.K., Christensen, L.L., Cocheme, H.M.,
454 Cram, D.L., Dantzer, B., Harper, J.M., Jurk, D., King, A., Noguera, J.C., Salin, K., Sild, E., Simons,
455 M.J.P., Smith, S., Stier, A., Tobler, M., Vitikainen, E., Peaker, M., Selman, C., 2015. Oxidative stress
456 and life histories: unresolved issues and current needs. Ecol. Evol. 5, 5745-5757.

457 Speakman, J.R., Selman, C., 2011. The free-radical damage theory: Accumulating evidence against a
458 simple link of oxidative stress to ageing in lifespan. Bioessays 33, 255-259.
459 Stuart, J.A., Maddalena, L.A., Merilovich, M., Robb, E.L., 2014. A midlife crisis for the mitochondrial
460 free radical theory of aging. Longev. Healthspan. 3, 4.
461 Taylor, P.W., Kaspi, R., Yuval, B., 2000. Copula duration and sperm storage in Mediterranean fruit
462 flies from a wild population. Physiol. Entomol. 25, 94-99.
463 Therneau, T.M., 2015. A Package for Survival Analysis in S. version 2.38.
464 Towarnicki, S.G., Kok, L.M., Ballard, J.W.O., 2020. Yin and Yang of mitochondrial ROS in
465 *Drosophila*. J Insect Physiol 122, 104022.
466 Vágási, C.I., Vincze, O., Pătraş, L., Osváth, G., Pénzes, J., Haussmann, M.F., Barta, Z., Pap, P.L., 2019.
467 Longevity and life history coevolve with oxidative stress in birds. Funct. Ecol. 33, 152-161.
468

469 **Table 1. Analysis of total antioxidant capacity (TAC) and lipid peroxidation using a generalised**
470 **linear mixed model with gamma distribution.**

| Explanatory variables | Response traits | $\chi2$ | df | p |
|---|---|---|---|---|
| Selection | TAC | 47.85 | 1 | < **0.001** |
| | Lipid peroxidation | 35.66 | 1 | < **0.001** |
| Age | TAC | 164.18 | 2 | < **0.001** |
| | Lipid peroxidation | 49.23 | 2 | < **0.001** |
| Sex | TAC | 0.25 | 1 | 0.615 |
| | Lipid peroxidation | 0.18 | 1 | 0.674 |
| Body mass | TAC | 0.01 | 1 | 0.905 |
| | Lipid peroxidation | 7.43 | 1 | **0.006** |
| Selection x Age | TAC | 89.78 | 2 | < **0.001** |
| | Lipid peroxidation | 21.18 | 2 | < **0.001** |
| Selection x Sex | TAC | 0.49 | 1 | 0.484 |
| | Lipid peroxidation | 0.17 | 1 | 0.677 |
| Age x Sex | TAC | 65.59 | 2 | < **0.001** |
| | Lipid peroxidation | 11.39 | 2 | **0.003** |
| Selection x Age x Sex | TAC | 48.65 | 2 | < **0.001** |
| | Lipid peroxidation | 7.37 | 2 | **0.025** |

471

472

473 **Figure 1. Survival curves of *C. cosyra* issued from control and upward-selected lines.** Shading
474 indicates 95 % interval. Upward-selection was performed by allowing females to oviposit only at 25
475 days after adult emergence, whereas eggs were collected from controls at 15 days. Each curve represents
476 the sum of 50 virgin females and males kept individually in a container.

477 **Figure 2. Reproductive effort of females from control (CT) and upward-selected (US) lines.** The
478 values displayed are the average total number of eggs (a) and average day of peak of egg production
479 (b) of virgin females individually kept in a container. Each bar for panel a) represents 50 individuals,
480 bars for panel b) represent 46 (CT) and 40 (US) individuals, respectively. Error bars indicate the
481 standard error of the mean.

482 **Figure 3. Reproductive effort of males from control (CT) and upward-selected (US) lines at three**
483 **different ages.** Bars show the average total number of sperm transferred by males to females'
484 spermathecae. At ages 5, 15 and 25, males were individually paired with a virgin female of 10 to 20
485 days of age from an unselected laboratory culture. Each bar represents 13 to 23 individuals. Error bars
486 indicate the standard error of the mean.

487 **Figure 4. Total antioxidant capacity (TAC) and lipid peroxidation in females and males from**
488 **control (CT) and upward-selected (US) lines at three different ages.** Values displayed were
489 predicted from the model used for statistical analyses where body mass was included as a covariate.
490 Each bar represents 20 virgin individuals. Error bars indicate the standard error of the predicted means.