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1 **Living with predators at the larval stage has differential long-lasting effects on adult life**  
2 **history and physiological traits in two anopheline mosquito species**

3

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17

18 **Key words:** *Anopheles gambiae*, *Anopheles coluzzii*, Carry-over effect, Life history traits,

19 Malaria vectors, Oxidative stress, Predation stress, Teneral reserves.

20

21 **Abstract:**

- 22 1. Under predation stress, individuals increase their metabolic rate and adopt different  
23 resource allocation strategies to favor their direct survival to the detriment of growth,  
24 reproduction and self-maintenance. Species and sexes are both faced with different  
25 physiological and life history demands and should adopt different strategies which lead to  
26 carry-over effects on adult metabolic reserves and on oxidative status.
- 27 2. Malaria mosquitoes *Anopheles coluzzii* and *An. gambiae* are sibling species. In nature,  
28 their larvae are exposed to different levels of predation risk and *An. coluzzii* larvae are  
29 more capable of surviving predation threats than *An. gambiae*, because of a better ability  
30 to gauge the threat level and to produce an adapted behavioral response. Impact of this  
31 response on adult physiology is however unknown.
- 32 3. Here, we investigated the carry-over effects of larval predation stress: first, on life history  
33 traits such as development length, size and weight at emergence, fecundity and survival;  
34 second, on metabolic resources at emergence such as lipids, proteins, carbohydrates and  
35 amino acids; and third, on oxidative status with the total anti-oxidant capacity and the  
36 oxidation of proteins in the two species and in both sexes.
- 37 4. We found that on one hand, it took more time for *An. coluzzii* to develop in presence of a  
38 predator and they emerged with a smaller size but a similar weight as control individuals.  
39 On the other hand, *An. gambiae* exposed to predators developed as fast as controls but  
40 with both a smaller size and a smaller weight. Fecundity was negatively impacted in both  
41 species following exposure to predation threats. Teneral reserves were affected depending on  
42 the species and the sex. The total anti-oxidant capacity of individuals exposed to stress  
43 was lower than in the controls but a significant level of damages to proteins was detected  
44 in *An. gambiae* males only.

45

46 5. Our findings suggest that predation stress induces very different physiological responses  
47 even in closely related species and between sexes. Allocation of resources in larvae may  
48 differ and depend on the species' aptitude to develop in presence of predators and on the  
49 sex, since males and females might adopt different strategies to fulfill their fitness.

Journal Pre-proofs

**50 Introduction**

51 Non-consumptive predation effects are significant drivers of the behavioral,  
52 physiological, morphological and life history traits of individuals (Lima, 1998; Benard, 2004;  
53 Preisser, Bolnick & Benard, 2005; McCauley, Rowe & Fortin, 2011). Under predation risk,  
54 prey often exhibit reduced foraging activities, which lowers nutrient acquisition in addition to  
55 shifting resource allocation from growth, reproduction and self-maintenance to direct  
56 survivorship (Lima & Dill, 1990). This symptomatic response is thus accompanied by a  
57 reduction in protein content to the benefit of carbohydrates (McPeck, Grace & Richardson,  
58 2001; Stoks, De Block & McPeck, 2005). In parallel, the vigilance of the prey is heightened,  
59 and its metabolic rate increases (*i.e.* respiration) to fuel emergency functions more rapidly  
60 (General Stress Paradigm, Hawlena & Schmitz, 2010).

61 Predator-induced phenotypic changes can be especially pervasive during development,  
62 with strong carry-over effects on adult phenotypes (Benard & Fordyce, 2003; De Block &  
63 Stoks, 2005; Pechenik, 2006; Crean, Monro & Marshall, 2011; McCauley et al., 2011). These  
64 latent effects can be mediated either by the decreased foraging activity of prey which make  
65 them less conspicuous (Lima & Dill, 1990; Ferrari, Messier & Chivers, 2008; Roux, Diabate  
66 & Simard, 2013), by the exploitation of patches of resources that are less time-consuming for  
67 the individual but which may also be of lower quality, or by physiological adaptations that  
68 may alter the way nutrients are assimilated and allocated to growth or emergency needs (Slos  
69 & Stoks, 2008; Van Dievel, Janssens & Stoks, 2016). In insects, the decreased quality and  
70 quantity of food intakes and the overconsumption of energetic metabolites such as lipids and  
71 carbohydrates lower the amount of body stores (McPeck et al., 2001; Stoks et al., 2005;  
72 Hawlena & Schmitz, 2010). This has significant consequences for the individuals, since body  
73 reserves can be depleted after larvae achieve the energetically demanding metamorphosis  
74 stage (Pechenik, 2006 and references therein), with dramatic consequences for the subsequent

75 fitness of the adult (Boggs, 1981; Briegel, 1990; Telfer & Kunkel, 1991; Telang, Buck &  
76 Wheeler, 2002).

77         Moreover, while enhancing chances to survive and escape predator attacks, the anti-  
78 predator responses affect energy budgets by shunting energy away from several other  
79 physiological functions, including antioxidant activities (Stoks et al., 2005; Selman, Blount,  
80 Nussey & Speakman, 2012; Janssens & Stoks, 2013; Costantini, 2014). Recently, it has been  
81 shown that predation stress is a source of oxidative stress (Janssens & Stoks, 2013) and  
82 affects enzymatic kinetics (Slos & Stoks, 2008; Guerra, Zenteno-Savín, Maeda-Martínez,  
83 Abele & Philipp, 2013; Pinya, Tejada, Capó & Sureda, 2016), life history traits such as  
84 longevity, fecundity and motility (Travers, Clinchy, Zanette, Boonstra & Williams, 2010;  
85 Janssens & Stoks, 2014) and their trade-offs (Dowling & Simmons, 2009; Monaghan,  
86 Metcalfe & Torres, 2009; Metcalfe & Alonso-Alvarez, 2010; Selman et al., 2012; Costantini,  
87 2014). Yet, response to oxidative stress is highly variable between species, and it has often  
88 been challenging to draw consistent conclusions within ecological and evolutionary  
89 frameworks so far. In this context, investigations based on recently diverged incipient species  
90 can provide new insights into understanding inter- and intra- specific differences in  
91 antioxidant responses and their potential impact in promoting divergences in life history traits  
92 (Costantini, 2008).

93         The *Anopheles gambiae* complex (African malaria mosquitoes) is particularly well  
94 suited to investigations on the evolution of anti-predator responses and carry-over effects on  
95 adult life histories. Indeed, in sub-Saharan Africa, *Anopheles gambiae s.s.* and *An. coluzzii*,  
96 two cryptic species of this complex, are exposed to different predation pressures at the larval  
97 stage (Diabaté et al., 2008). In western Burkina Faso, although the adults are found in  
98 sympatry, larvae might thrive in different habitats. *Anopheles coluzzii* is dominant in  
99 permanent freshwater habitats (e.g. irrigated fields such as rice paddies), which shelter higher

100 densities of predators than temporary water habitats (e.g. puddles, quarries, ruts) where *An.*  
101 *gambiae* predominates (Sunahara, Ishizaka & Mogi, 2002; Diabaté et al., 2008; Gimonneau et  
102 al., 2012a). Furthermore, *An. coluzzii* larvae are known to be better at gauging the level of  
103 predation risk and are more capable of triggering anti-predator response compared to *An.*  
104 *gambiae* larvae (Diabaté et al., 2008; Gimonneau et al., 2010; Roux et al., 2013; Roux,  
105 Diabate & Simard, 2014).

106         So far, no studies have explored the effect of predation risk on sibling species and in  
107 both sexes, despite the fact that it can have significant consequences on life history traits for  
108 these anopheline insects and potential malaria epidemiological outcomes (Roux et al., 2015).  
109 This study was thus aimed at investigating the carry-over effects of a chronic larval predation  
110 stress on adults of the sibling species *An. gambiae* and *An. coluzzii* by measuring traits  
111 describing fecundity, size and longevity, in addition to the characterization of a range of  
112 physiological parameters (antioxidant capacities, nature and amount of metabolic reserves) in  
113 both males and females. We expected that i) predation risk would reduce nutrient acquisition  
114 and transformation at the larval stages, leading to smaller adult sizes; ii) larvae would  
115 maximize the use of lipids and carbohydrates (fight-or-flight response) at the detriment of  
116 protein and amino acid stores (Stoks et al., 2005; Hawlena & Schmitz, 2010; Van Dievel et  
117 al., 2016), thus modifying the body reserves of teneral insects; iii) these altered teneral  
118 reserves would induce a significant reduction of female fecundity (Uchida et al., 2001; Zhou,  
119 Pennington & Wells, 2004); and iv) larvae that grew under predation risk would have suffered  
120 from a higher production of oxidative molecules with long-lasting footprints on adults (*i.e.*  
121 enhanced antioxidant capacities in the adults and/or increased oxidative damages to proteins).

122

## 123 **Material and Methods**

### 124 **Insect collections**

125 Experiments were conducted with F1 to F3 mosquito larvae obtained from wild *An.*  
126 *gambiae* s.l. females collected while at rest in inhabited human dwellings in villages from  
127 south-western Burkina Faso (West Africa). *An. coluzzii* specimens were collected in Bama  
128 (11°23'14"N, 4°24'42"W), a village surrounded by irrigated rice fields in which females lay  
129 their eggs and where the predation density is known to be high (permanent water reservoirs).  
130 *An. gambiae* were collected in Soumousso (11°00'46"N, 4°02'45"W), a village in humid  
131 savanna where females only have access to temporary, rain-filled puddles and quarries, and  
132 where predator density is low. After individual oviposition, females were identified to the  
133 species' level by routine PCR-RFLP (Santolamazza et al., 2008). The larvae were reared in  
134 spring water under insectary conditions (27±1°C, 80±10% relative humidity, 12L:12D) and  
135 fed with Tetramin® Baby Fish Food *ad libitum*. Adults were reared in mesh cages  
136 (30x30x30cm) and provided with 5% glucose and water on imbibed cotton wool pads *ad*  
137 *libitum*.

138 Backswimmer *Anisops jaczewskii* (Hemiptera: Notonectidae) is the most abundant and  
139 widespread predatory bug in mosquito larval habitats in our study area (Diabaté et al., 2008).  
140 Predators were collected from both mosquito sampling villages. They were fed daily *ad*  
141 *libitum* with both *An. gambiae* and *An. coluzzii* larvae and maintained in the same controlled  
142 insectary conditions as the mosquito larvae. All the predators used in the experiment were  
143 adults of similar sizes.

144

## 145 **Experimental design**

146 *Exposure to predator* – 300 first instar larvae and one predator were placed together in  
147 a plastic container (Lxwxh: 28x18x5.5cm) filled with 1L of spring water under insectary  
148 conditions. The predator was free to feed upon mosquito larvae during the entire period of  
149 larval mosquito development. To standardize hunger levels, predators were starved for 48h



150 and then fed with 20 third instar mosquito larvae 12h before the beginning of the experiment.  
151 Indeed, fully starved predators were not adequate because they ate all the first instar larvae  
152 within the first 48h of exposure at the beginning of the experiment. For each experimental  
153 container, its equivalent without a predator was set up as a control. Twenty-four containers  
154 were setup, 10 containers for *An. coluzzii* (5 experimental and 5 controls) and 14 containers  
155 for *An. gambiae* (7 experimental and 7 controls) (Fig. 1). The presence of a predator and  
156 chemical cues associated with killing and consumption of mosquito larvae have been shown  
157 to induce anti-predator behaviors in *Anopheles* species (Roux et al., 2013; Roux et al., 2014).

158 Mosquito larvae were provided with Tetramin® Baby Fish Food *ad libitum* twice a  
159 day and excess food was removed each day using a Pasteur pipette to avoid water pollution.  
160 Due to predator consumption, mosquito larval densities varied over time in the experimental  
161 containers. To avoid a potential density effects on adult life history traits (Alto, Lounibos,  
162 Higgs & Juliano, 2005; Breaux, Schumacher & Juliano, 2014), we standardized the larval  
163 density by randomly removing supernumerary larvae from the control containers on one  
164 occasion when larvae reached the late second instar. Indeed, most of the direct consumptive  
165 effect of predation occurs during early development (on first and second instar larvae). Later,  
166 predation rate decreases as larvae grow bigger (third and fourth instar) and changes in density  
167 due to consumptive effect of the predator becomes negligible (see results on emergence rate).  
168 To maintain larval density equivalent among experimental treatments and over the course of  
169 the experiment, mosquito larvae were counted in both predation and control containers by  
170 pairs, and the densities made equal between these pairs by removing the exceeding numbers  
171 of larvae from the corresponding controls. At the end of larval development, the pupae were  
172 collected and placed in plastic cups at equal densities for emergence in 30x 30x30cm cages  
173 covered with a mesh. No water or glucose was provided.

174

175 **Measurement of traits**176 *Life history traits*

177 A series of life history traits susceptible of being affected by predation stress were  
178 measured; namely, mosquito development time, longevity, fecundity and adult size (Roux et  
179 al., 2015) (Fig. 1). *Development time* – This is the number of days from egg to adult  
180 emergence. Adults were collected once a day in the morning. *Longevity* – On the morning of  
181 adult emergence, forty 20x20x20cm cages were set-up (5 for each sex, predator exposure,  
182 species combination). Ten individuals that emerged from the same container were placed into  
183 one cage, and mosquitoes were provided with water and 5% glucose solution *ad libitum* (=50  
184 mosquitoes per combination, N=400 individuals). Dead individuals were counted and  
185 removed every 24h. *Fecundity* – On the morning of adult emergence, and for each species,  
186 predator exposure combination, 40 females were placed in a 30x30x30cm cage with water  
187 and 5% glucose solution *ad libitum*. At day 2 post emergence, females were starved for 12h,  
188 and were then offered a blood meal on the arm of a volunteer for 30 minutes on two  
189 consecutive days. An informed written consent was obtained from the volunteer. Females that  
190 were not fully engorged on the two occasions were discarded. Three days post-blood meal,  
191 ovaries of 35 randomly chosen females of each combination were dissected under a  
192 stereomicroscope (magnification 35x, Leica EZ4D, Wetzlar, Deutschland) and egg load,  
193 defined as the number of eggs in gravid females, was measured as a surrogate for fecundity.  
194 *Adult size* – in addition to ovaries, one wing per female was dissected, photographed and  
195 measured from the alular notch to the apical margin tip, excluding fringe scales as a proxy for  
196 adult size. Measurements were made with the ImageJ software (V1.47, Wayne Rasband  
197 National Institute of Health, USA).

198 *Physiological traits*

199 On the morning of adult emergence, mosquitoes which were not used for the life  
200 history trait study were frozen at -80 °C, and kept in the freezer until analysis. We measured  
201 teneral metabolic reserves (including the whole protein and lipid contents and non-  
202 constitutive carbohydrates and amino acids), total anti-oxidant capacity (TAC), and oxidative  
203 damages on proteins (protein carbonyls). For analysis, pools of five randomly chosen  
204 mosquitoes of the same sex and belonging to the same container (=same species, predator  
205 exposure combination) were weighed (Mettler-Toledo MX-5 microbalance, Columbus, OH,  
206 USA; d=0.1µg). These five-pooled mosquitoes were considered as a unique sample for the  
207 following analyses (Fig. 1).

208 *Protein assays* - Protein assays were conducted as described by Foray et al. (2012).  
209 Briefly, for each combination (species x sex x predator exposure), the pools of five  
210 mosquitoes were homogenized in 180µL of phosphate buffer for 90s at 25Hz using a bead-  
211 beating device (Retsch™ MM301, Retsch GbmH, Haan, Germany). Following low-spin  
212 centrifugation (180g, 4 °C) to remove insect debris, a volume of 5µL of the supernatant was  
213 used to measure body protein content using Bradford's technique (Bradford, 1976).  
214 Absorbance of the samples was read at 595nm. The quantity of proteins was calculated from a  
215 bovine serum albumin (CAS number: 9048-46-8) calibration curve. The remaining 175µL of  
216 each sample were then used for the following physiological assays.

217 *Carbohydrate and amino acid assays* – In order to examine the effects of predation on  
218 the variation of the different metabolic families (amino acids, sugars) of each individual, the  
219 gas chromatography–mass spectrometry (GC-MS) platform was used, as described in  
220 Khodayari et al. (2013). A volume of 450µL of methanol-chloroform (ratio 2:1,  
221 volume:volume) was added to the remaining 175µL of mosquito homogenate obtained from  
222 protein assays and were homogenized using a bead-beating device at 25Hz for 90s. Then,  
223 125µL of ultrapure water was added to the samples that were then further centrifuged at

224 4000g, at 4 °C, for 10min. An aliquot of the supernatant (90 µL), which contained polar  
225 metabolites, was transferred to new microtubes, and the aliquots were vacuum-dried (Speed  
226 Vac Concentrator, MiVac, Genevac Ltd., Ipswich, United Kingdom). Dried samples were re-  
227 suspended in 30µL of 20mg/mL methoxyamine-hydrochloride in pyridine prior to incubation  
228 under orbital shaking at 40°C for 60min, as described in Dahirel et al. (2019). Following  
229 incubation, 30µL of N-methyl-bis(trifluoroacetamine) (BSTFA) were added, and  
230 derivatisation was conducted at 40°C for 60min under agitation. Then, 1µL of each sample  
231 was injected using the split mode (25:1) in the GC injector set at 260°C. The oven, equipped  
232 with a 30m fused silica column (TR5 MS, I.D. 0.25mm, 95% dimethyl siloxane, 5% phenyl  
233 polysilphenylene-siloxane) was programmed to remain at 70°C for 4min and then increased at  
234 a rate of 5°C/min until it reached 300°C for 10min. Helium was used as carrier gas at  
235 1mL/min. The temperatures of mass spectrometry transfer line and of the ion source were set  
236 at 260°C. Detection was conducted by electronic impact method (70eV). Chromatograms  
237 were deconvoluted using XCalibur v2.0.7. Calibration curves were run for 62 pure reference  
238 compounds at concentrations of 1, 2, 5, 10, 20, 50, 100, 200, 500, 750, 1000 and 1500 µM.  
239 The same carbohydrates and amino acids were detected in all samples, and were then pooled  
240 to run statistical comparisons among metabolic families. Both detailed quantitative and  
241 qualitative metabolomic data will be further analysed and used for addressing distinct  
242 research questions.

243 *Lipid assays* - The triglyceride assays were performed on the samples prepared for  
244 GC-MS analyses, and as recommended by the manufacturer of the triglyceride colorimetric  
245 assays (Triglycerides, kit reference CC02200, LTA srl, Italy). Briefly, a volume of 110µL of  
246 chloroform was collected for each sample which was subsequently dried under a fume hood  
247 for 24h. Then, the lipid droplet was re-dissolved into 150µL of Triton-BSA buffer (750mg  
248 BSA, 50µL triton 100X, 25mL ultrapure water) and incubated for 10min at 60°C immediately

249 before the assays. A volume of 10  $\mu$ L of the supernatant was used to measure triglycerides.

250 Absorbance of the samples was read at 510 nm.

251 *Oxidative stress* – Mosquitoes were homogenized individually according to the  
252 manufacturer's instructions (Cayman Chemical: Total antioxidant capacity, item No. 709001  
253 and Protein carbonyl, item No. 10005020); the total antioxidant capacity and the oxidized  
254 proteins (protein carbonyls) were quantified on different mosquitoes. The total antioxidant  
255 capacity (TAC) measures the combination of the entire antioxidant system of the sample  
256 including enzymes and different molecules such as macromolecules and vitamins from endo-  
257 or exogenous (diet) origins and thus represents the cumulative antioxidant capacity of the  
258 sample/organism. The TAC is measured and compared to an equivalent of trolox (vitamin E  
259 analogue) antioxidant activity.

260 The protein carbonyl is a marker of protein oxidation. Protein carbonyls are measured  
261 through a reaction with dinitrophenylhydrazine (DNHP) which form hydrazone detectable  
262 through spectrophotometric analyzes. The result is expressed as a proportion of oxidized  
263 proteins on the total protein content.

264 For each kit, we analyzed 8 individuals per sex, species and predator exposure  
265 combination (N=128).

266

## 267 **Statistical analyses**

268 *Life history traits* - During experiments, the origin of each mosquito (*i.e.* larval  
269 container) was tracked to be included as random factor in all analyses. Cox's mixed model  
270 was used to analyze larval development time and adult survivorship with predator exposure  
271 (yes or no), sex, species and their interactions coded as fixed effects. Mosquito weight and  
272 female wing size were analyzed separately with a linear-mixed model (LMM) with predator  
273 exposure, species, sex (in weight analysis only) and their interactions as fixed effect. Egg load

274 was analyzed using a LMM with wing size, predator exposure and their interactions as fixed  
275 effects. As body mass and wing size were not measured on the same individuals (see Fig 1),  
276 they were not considered as covariates in longevity and fecundity analyses. To check for the  
277 reliability of our method to equalize larval densities between predator exposed and control  
278 containers, we analyzed the number of emerged adults with a Wilcoxon paired test.

279 *Physiological traits* – Proteins, lipids, total antioxidant capacity, carbohydrates and  
280 amino acids were analyzed separately with a LMM with predator exposure, species, sex and  
281 their interactions as fixed effect. Protein carbonyls were considered as a fraction of total  
282 protein content and were analysed using a quasi-binomial generalized linear-mixed model  
283 (GLMM). Predator exposure, species, sex and their interactions were considered fixed effects.

284 When necessary, raw data were power transformed to meet normality and/or  
285 homogeneity of variance assumptions (box-cox transformation with the "powerTransform"  
286 function in the "car" package). For model selection, we used the stepwise removal of terms,  
287 followed by likelihood ratio tests (LRT). Term removals that significantly reduced  
288 explanatory power ( $P < 0.05$ ) were retained in the minimal adequate model (Crawley, 2007).  
289 All analyses were conducted using the R software (v. 2.12.1).

290

## 291 **Results**

### 292 **Mosquito development, size and weight**

293 Among larvae exposed to predation, *An. coluzzii* survived better than *An. gambiae*  
294 with 772 out of 1500 larvae for *An. coluzzii* (51.5%, mean±se survivors/container: 154±12)  
295 and 693 out of 2100 larvae for *An. gambiae* (33%, mean±se survivors/container: 99±6)  
296 ( $X^2_1=122$ ;  $P < 0.001$ ). After correction of densities, 756 control larvae of *An. coluzzii* (50.4%,  
297 mean±se survivors/container: 151±8) and 821 control larvae of *An. gambiae* (39.1%, mean±se

298 survivors/container:  $117 \pm 2$ ) were obtained, which was not statistically different from exposed  
299 larvae containers ( $W=9$ ;  $P=0.8$  and  $W=3$ ;  $P=0.08$ , respectively).

300 The mean ( $\pm$ s.e.) larval developmental time of survivors was significantly affected by  
301 the interaction between predator exposure and species ( $X^2_1=8$ ;  $P=0.004$ ). Exposed larvae of  
302 *An. coluzzii* had a longer period of development than their controls ( $11.1 \pm 0.02$  vs.  $10.6 \pm 0.02$   
303 days;  $X^2_1=10.5$ ;  $P=0.001$ ) while no significant difference was found between exposed and  
304 control larvae of *An. gambiae* ( $10.5 \pm 0.03$  vs.  $10.5 \pm 0.03$  days;  $X^2_1=0.3$ ;  $P=0.5$ ). Males emerged  
305 earlier than females ( $10.61 \pm 0.02$  vs.  $10.71 \pm 0.02$  days;  $X^2_1=11.3$ ;  $P<0.001$ ; Fig. 2a), regardless  
306 of predator exposure (*i.e.* no sex X predator exposure interaction;  $X^2_1=0.81$ ;  $P=0.3$ ). There  
307 was no significant effect for other interactions (species X sex:  $X^2_1=0.65$ ;  $P=0.4$  and species X  
308 sex X predator exposure:  $X^2_1=0.55$ ;  $P=0.4$ ).

309 Predator exposure also influenced mean ( $\pm$ s.e.) adult body size in the two species.  
310 Females that emerged from larvae exposed to predators had significantly shorter wings than  
311 their control counterparts ( $3229 \pm 15$  vs.  $3361 \pm 18 \mu\text{m}$  in *An. coluzzii* and  $3428 \pm 23$  vs.  
312  $3521 \pm 21 \mu\text{m}$  in *An. gambiae*,  $X^2_1=13.32$ ;  $P<0.001$ ; Fig. 2b). Females of *An. coluzzii* were  
313 smaller than those of *An. gambiae* ( $3296 \pm 14$  vs.  $3475 \pm 17 \mu\text{m}$ , respectively,  $X^2_1=26.2$ ;  
314  $P<0.001$ ). There was no significant interaction between species and predator exposure  
315 ( $X^2_1=0.5$ ;  $P=0.4$ ).

316 Mosquito mean ( $\pm$ s.e.) body weight was significantly affected by the interaction  
317 between predator exposure and species ( $X^2_1=4.3$ ;  $P=0.03$ ), thus *An. gambiae* adults from  
318 exposed larvae were lighter than their controls ( $1.27 \pm 0.02$ mg vs.  $1.36 \pm 0.02$ mg, respectively;  
319  $X^2_1=13.5$ ;  $P<0.001$ ; Fig. 2c), while no significant differences were detected between exposed  
320 and control groups for *An. coluzzii* ( $1.37 \pm 0.02$ mg vs.  $1.40 \pm 0.02$ mg, respectively;  $X^2_1=1.15$ ;  
321  $P=0.28$ ). Females were heavier than males ( $1.47 \pm 0.01$  vs.  $1.23 \pm 0.01$ mg, respectively;  
322  $X^2_1=186$ ;  $P<0.001$ ). There was no other significant interaction (sex X species:  $X^2_1=2.3$ ;

323  $P=0.12$ ; sex:predator exposure:  $X^2_1=0.6$ ;  $P=0.43$  and species X sex X predator exposure:  
324  $X^2_1=0.05$ ;  $P=0.8$ )

325

### 326 **Mosquito fecundity**

327 After two blood meals, all females were gravid. The mean egg load ( $\pm$ s.e.) was  
328 negatively affected by exposure to predators (159 $\pm$ 5 eggs in predator-exposed vs. 180 $\pm$ 6 eggs  
329 in control;  $X^2_1=4.7$ ;  $P=0.02$ ; fig. 2d). *Anopheles coluzzii* had more eggs than *An. gambiae*  
330 (205 $\pm$ 4 vs. 136 $\pm$ 4 eggs, respectively;  $X^2_1=59$ ;  $P<0.001$ ). Finally, egg load was positively  
331 associated with wing size ( $X^2_1=14$ ;  $P<0.001$ ; Fig. 3). There was no significant interaction  
332 (predator exposure X wing size:  $X^2_1= 3.6$ ;  $P=0.057$ ; predator exposure X species:  $X^2_1=0.4$ ;  
333  $P=0.5$ ; species X wing size:  $X^2_1=0.2$ ;  $P=0.6$ ; predator exposure X species X wing size:  
334  $X^2_1=0.7$ ;  $P=0.4$ ).

335

### 336 **Mosquito longevity**

337 Adults emerged from predator-exposed or control larvae had the same longevity  
338 (30 $\pm$ 0.7 vs. 28.5 $\pm$ 0.8 days, N=20 cages each;  $X^2_1= 0.1$ ;  $P=0.7$ ) and no interaction was found  
339 between predator exposure and sex, or between predator exposure and species ( $X^2_1= 0.62$ ;  
340  $P=0.43$ ,  $X^2_1= 0.15$ ;  $P=0.69$ , respectively). However, the interaction between sexes and species  
341 was significant ( $X^2_1= 14.4$ ;  $P<0.001$ ; Fig. 4): males surpassed females at surviving in *An.*  
342 *coluzzii* (26.7 $\pm$ 1 vs. 19.3 $\pm$ 0.9 days, N=100 and 99, respectively;  $X^2_1= 22.2$ ;  $P<0.001$ ), whereas  
343 males of *An. gambiae* only slightly survived better than females (36.3 $\pm$ 0.7 vs. 34.7 $\pm$ 0.7 days,  
344 N=100 and 100, respectively;  $X^2_1= 4.3$ ;  $P=0.037$ ).

345

### 346 **Metabolic resources**



347 Mean ( $\pm$ s.e.) protein content was similar in the two species ( $31.8\pm 0.7$  vs.  
348  $32.6\pm 0.5\mu\text{g}/\text{mg}$  in *An. coluzzii* and *An. gambiae*, respectively;  $X^2_1=1.4$ ;  $P=0.2$ ; Fig. 5a).  
349 However, there was a significant interaction between sex and predator exposure ( $X^2_1=10.9$ ;  
350  $P<0.001$ ), predator-exposed females had lower protein content than their control counterparts  
351 ( $30\pm 0.7$  vs.  $34\pm 0.7\mu\text{g}/\text{mg}$ , respectively;  $X^2_1=124$ ;  $P<0.001$ ), while no significant difference  
352 was found between predator-exposed and control males ( $33\pm 0.7$  vs.  $31.7\pm 0.9\mu\text{g}/\text{mg}$ ,  
353 respectively;  $X^2_1=1.05$ ;  $P=0.3$ ). There was no significant effect for other interactions (species  
354 X sex:  $X^2_1=0.9$ ;  $P=0.3$ ; predator exposure X species:  $X^2_1=0.7$ ;  $P=0.3$  and predator exposure X  
355 species X sex:  $X^2_1=2.8$ ;  $P=0.09$ ).

356 Mean ( $\pm$ s.e.) lipid content was significantly affected by the interaction between  
357 species, sex and predator exposure ( $X^2_1=8.6$ ;  $P=0.003$ ; Fig. 5b). Species subset analysis  
358 showed that in *An. coluzzii*, predator exposed mosquitoes had almost twice more lipids than  
359 controls ( $9.6\pm 0.6$  vs.  $4.9\pm 1.2\mu\text{g}/\text{mg}$ , respectively;  $X^2_1=14.4$ ;  $P<0.001$ ) and that sex had no  
360 effect (males:  $8.2\pm 1\mu\text{g}/\text{mg}$  vs. females:  $6.2\pm 1.2\mu\text{g}/\text{mg}$ ,  $X^2_1=2.3$ ;  $P=0.12$ ). In *An. gambiae*,  
361 lipid content was significantly affected by the interaction between predator exposure and sex  
362 ( $X^2_1=7.3$ ;  $P=0.006$ ). Females exposed to predation stress as larvae had a lower lipid content  
363 than control ( $5.2\pm 0.7$  vs.  $7.5\pm 0.9\mu\text{g}/\text{mg}$ , respectively;  $X^2_1=21$ ;  $P=0.02$ ) while no significant  
364 difference was detected in males (predator exposed:  $6.7\pm 0.3\mu\text{g}/\text{ml}$  vs. control:  $5.3\pm 1.2\mu\text{g}/\text{mg}$ ;  
365  $X^2_1=8$ ;  $P=0.2$ ).

366 The mean ( $\pm$ s.e.) carbohydrate content was negatively affected by predator exposure.  
367 Exposed individuals had a lower carbohydrate content than controls ( $76.3\pm 5.8$  vs.  $92.5\pm 5.6$   
368  $\text{nmol}/\text{mg}$ , respectively;  $X^2_1=7.01$ ;  $P=0.008$ ; Fig. 5c). Females had significantly fewer  
369 carbohydrates than males ( $70.3\pm 3.8$  vs.  $97.1\pm 6.1$   $\text{nmol}/\text{mg}$ , respectively;  $X^2_1=26.5$ ;  $P<0.001$ )  
370 and *An. gambiae* had a lower carbohydrate content than *An. coluzzii* ( $68\pm 4.1$  vs.  $102.8\pm 5.5$   
371  $\text{nmol}/\text{mg}$ , respectively;  $X^2_1=16.2$ ;  $P<0.001$ ). There was no significant effect of interactions

372 (species X sex:  $X^2_1=1.3$ ;  $P=0.2$ ; predator exposure X species:  $X^2_1=2.4$ ;  $P=0.1$ ; predator  
 373 exposure X sex:  $X^2_1=0.04$ ;  $P=0.8$  and predator exposure X species X sex:  $X^2_1=0.64$ ;  $P=0.4$ ).

374 The mean ( $\pm$ s.e.) amino acid content was negatively affected by predator exposure.  
 375 Exposed individuals had a lower amino acid content than controls ( $74.2\pm 6.4$  vs.  $90.7\pm 6.5$   
 376 nmol/mg, respectively;  $X^2_1=5$ ;  $P=0.02$ ; Fig: 5d). Females had significantly more amino acids  
 377 than males ( $106.6\pm 5.4$  vs.  $62.3\pm 4.6$  nmol/mg, respectively;  $X^2_1=51$ ;  $P<0.001$ ), and *An.*  
 378 *gambiae* had a lower amino acid content than *An. coluzzii* ( $68.5\pm 5.9$  vs.  $98.2\pm 6$  nmol/mg,  
 379 respectively;  $X^2_1=17.9$ ;  $P<0.001$ ). There was no significant effect of interactions (species X  
 380 sex:  $X^2_1=0.5$ ;  $P=0.5$ ; predator exposure X species:  $X^2_1=0.3$ ;  $P=0.5$ ; predator exposure X sex:  
 381  $X^2_1=1.88$ ;  $P=0.2$  and predator exposure X species X sex:  $X^2_1=1.04$ ;  $P=0.3$ ).

382

### 383 Oxidative status

384 Individuals exposed to predation stress had a lower mean ( $\pm$ s.e.) total anti-oxidant  
 385 capacity than controls ( $20.6\pm 0.8\mu\text{M}/\text{mg}$  vs.  $23.8\pm 0.7\mu\text{M}/\text{mg}$ , respectively;  $X^2_1=7.1$ ;  $P=0.007$ ;  
 386 Fig. 6a). *An. gambiae* had a lower total anti-oxidant capacity than *An. coluzzii*  
 387 ( $20.5\pm 0.6\mu\text{M}/\text{mg}$  vs.  $23.8\pm 0.9\mu\text{M}/\text{mg}$ , respectively;  $X^2_1=7.5$ ;  $P=0.006$ ). There was no  
 388 significant effect of sex (males:  $22.1\pm 1\mu\text{M}/\text{mg}$  vs. females:  $22.1\pm 0.7\mu\text{M}/\text{mg}$ ;  $X^2_1=0.0002$ ;  
 389  $P=0.98$ ) or interaction (predator exposure X sex X species:  $X^2_1=0.1$ ;  $P=0.7$ ; predator exposure  
 390 X sex:  $X^2_1=0.4$ ;  $P=0.5$ ; predator exposure X species:  $X^2_1=0.08$ ;  $P=0.8$ ; sex X species:  
 391  $X^2_1=0.06$ ;  $P=0.8$ ).

392 The mean ( $\pm 95\%$ CI) carbonyl proportion was significantly affected by the interaction  
 393 between species, sex and predator exposure ( $X^2_1=5.6$ ;  $P=0.017$ ; Fig. 6b). Species subset  
 394 analysis showed that in *An. coluzzii*, neither predator exposure nor sex or their interaction had  
 395 a significant effect on carbonyl proportion ( $X^2_1=1.24$ ,  $P=0.3$ ;  $X^2_1=0.5$ ,  $P=0.5$  and  $X^2_1=0.5$ ,  
 396  $P=0.5$ , respectively). In *An. gambiae*, the proportion of carbonyl was significantly affected by

397 the interaction between predator exposure and sex. Male exposed to predation stress during  
398 their larval development had a higher carbonyl proportion than control males ( $0.15 \pm 0.004$  vs.  
399  $0.11 \pm 0.02$ , respectively) while exposed females had a lower carbonyl proportion than control  
400 females ( $0.05 \pm 0.03$  vs.  $0.11 \pm 0.03$ , respectively).

401

## 402 **Discussion**

403 Behavioral and physiological responses to life-threatening stress are supposed to  
404 increase immediate individual survival (Hawlena & Schmitz, 2010). During such episodes,  
405 and according to the general stress paradigm (Hawlena & Schmitz, 2010), both nutrition and  
406 respiration can be altered, lowering resource acquisition and increasing the chances of  
407 oxidative stress.

408 In the *An. gambiae* complex, both *An. coluzzii* and *An. gambiae s.s.* larvae exposed to  
409 a predation risk adopted a less conspicuous behavior to avoid their detection by predators, but  
410 did not reduce the time spent to feed (Roux et al., 2013). In this study, predator exposed *An.*  
411 *coluzzii* larvae, which are adapted to high predator densities, took more time to develop than  
412 their control relatives. Larval survival during prolonged exposure to predation risk is possible  
413 through an anti-predator behavior proportional to the threat level (Roux et al., 2014). In our  
414 experimental conditions, it allows individuals to reach the same weight at emergence as  
415 controls but with a smaller wing size. This finding is in line with earlier studies conducted on  
416 *Aedes notoscriptus* and *Culex pipiens* exposed to fishes and backswimmer, respectively  
417 (Beketov & Liess, 2007; van Uitregt, Hurst & Wilson, 2012). A disequilibrium in allometric  
418 relationship between body mass and wing size was also observed in *Aedes albopictus* exposed  
419 to stressful conditions (Reiskind & Zarrabi, 2012) showing that wing size is not an  
420 appropriate proxy of mosquito weight for larval stressful environments.

421 Interestingly, *An. gambiae* larvae exposed to predators were characterized by a similar  
422 developmental duration than their respective controls, which resulted in both smaller body  
423 mass and wing size at emergence. This strategy of fast growth (compared to *An. coluzzii*),  
424 which supposedly decreases the time of exposure to predation threat, might reveal the lower  
425 level of adaptation of these larvae to the presence of predators (Diabaté et al., 2008;  
426 Gimonneau et al., 2012b; Roux et al., 2013; Roux et al., 2014). Since both species do not  
427 seem to change their feeding rates (Roux et al., 2013), it is likely that these strategies are  
428 resource consuming and/or alter nutrient assimilation or allocation which would explain  
429 differences in weight or in wing size (growth-predation risk trade-off, McPeck et al., 2001;  
430 Stoks et al., 2005).

431 Compared to controls, a reduced fecundity was observed in both *An. gambiae* and *An.*  
432 *coluzzii* exposed to predator as larvae, thus suggesting that female reduced size cannot be the  
433 only explanation for this pattern. In *An. gambiae* females, the fast development under  
434 predation threat and/or the induced stress have resulted in a reduction of all of the possible  
435 energy sources (proteins: -10%, amino acids: -27%, lipids: -31% and carbohydrates: -31%). In  
436 *An. coluzzii* females however, only protein stocks were depleted (-14%) while lipid reserves  
437 were increased (+73%). These quantities were analyzed at adult emergence, and the observed  
438 difference in terms of fecundity, despite the two blood meals and glucose provided *ad libitum*  
439 during 7 days, suggests that female fecundity also greatly depends on their preexisting  
440 resources acquired during larval development to produce the first batch of eggs (Briegel,  
441 1990; Briegel, Hefti & DiMarco, 2002; Zhou et al., 2004). This dependence between larval  
442 resources and fecundity was already demonstrated in *Aedes aegypti*, in which a large amount  
443 of larval derived lipid and carbohydrates reserves are used to produce the first egg batch  
444 (Zhou et al., 2004). Amino acids are also known to be essential to the initiation of ovarian

445 development and their concentration in hemolymph is positively correlated to the number of  
446 eggs produced (Uchida et al., 2001).

447 Carbohydrates are known to be the metabolites that fuel flight in most insects  
448 (Sacktor, 1955; Nayar & Sauerman, 1971), flying capacities being highly important in  
449 *Anopheles* species for mating. Indeed, males form swarms at dusk in which they are in  
450 competition for females and a longer swarming activity increases the male's competitiveness  
451 (Yuval, Holliday-Hanson & Washing, 1994). Swarming behavior can consume more than  
452 50% of the individual's carbohydrate reserves (Yuval et al., 1994; Maiga et al., 2014), and  
453 males with low levels of carbohydrates do not participate in mating (Yuval et al., 1994;  
454 Yuval, Kaspi, Shloush & Warburg, 1998). Even if carbohydrates reserves can be replenished  
455 while feeding on sugar (mainly flower nectars), having a large amount of energy available  
456 directly at emergence may allow males to actively participate to swarming as soon as they are  
457 mature (about 24h after emergence). All together, it may explain why males had more  
458 carbohydrates than females at emergence.

459 Carbohydrate reserves were similar in both controls and exposed *An. coluzzii* males  
460 while they were lowered in exposed *An. gambiae* males (-29%). Consumption of short-term  
461 energy metabolites, including carbohydrates, increases under stressful conditions in order to  
462 support responses like fight-or-flight strategy (McPeck et al., 2001; Hawlena & Schmitz,  
463 2010; Van Dievel et al., 2016). Here, *An. coluzzii*, which spends more time in less  
464 conspicuous locations than *An. gambiae* (Roux et al., 2014), might also have evolved a lower  
465 metabolite rate that would reduce the consumption of body carbohydrates. Conversely, *An.*  
466 *gambiae* which is less adapted than *An. coluzzii* to the presence of predators (Diabaté et al.,  
467 2008) needs to be ready to develop a fight-or-flight response and consequently could consume  
468 more energy.

469 According to the general stress paradigm (Hawlena & Schmitz, 2010), exposure to  
470 predation stress increases metabolic rates and oxygen consumption to make the body ready  
471 for a fight-or-flight response (emergency functions) which consequently accentuates the  
472 alteration of teneral reserve accumulation, and may increase the risk of oxidative stress. Our  
473 results show a decrease in the total antioxidant capacity in the two sexes of the two mosquito  
474 species exposed to predation risk. Such a decrease or absence of variation in antioxidant  
475 capacity during chronic stress was already observed in damselflies (Slos & Stoks, 2008;  
476 Janssens & Stoks, 2013), Corixids (Trekels, Van de Meutter, Bervoets & Stoks, 2012) and  
477 amphipods (Jermacz et al., 2020a; Jermacz, Nowakowska, Kletkiewicz & Kobak, 2020b), and  
478 was described as a mechanism to reduce the cost of production of antioxidant response which  
479 cannot be sustained for long. Three scenarios could explain these results. In the first, larvae  
480 could reduce their metabolic rate due to the habituation to predation threat cues (Ferrari,  
481 Elvidge, Jackson, Chivers & Brown, 2010; Roux et al., 2014) and/or to reduce costs due to the  
482 production of reactive oxygen species (ROS) and antioxidant molecules (Jermacz et al.,  
483 2020a). Late instar larvae which reset their activity would be more vulnerable to predator  
484 attacks unless their larger sizes dissuaded predators. In the second scenario, larvae could  
485 reconfigure their physiology to conserve both an increased metabolism and their ability to  
486 develop a fight-or-flight response, while reducing costs related to a higher ROS level  
487 (Jermacz et al., 2020a; Jermacz et al., 2020b). In the third scenario, individuals could adjust  
488 their levels of defense to maintain a certain level of damages (Janssens & Stoks, 2013). In all  
489 these scenarios, the capacity to repair or replace damaged molecules should also be  
490 considered since it could be energetically ‘cheaper’ than trying to avoid oxidation (Halliwell  
491 & Gutteridge, 2007).

492 In our study, late instar larvae still produce anti-predator behavior by staying on the  
493 wall of the container (pers. obs.), we however did not control for metabolic rate. Moreover,

494 we did not look at other potential damages on lipids and DNA. In addition, we chose to look  
495 at protein damages during the emergence of adults, just after metamorphose during which a  
496 lot of molecules are lysed and could be eliminated or repaired if oxidized (Campero, De  
497 Block, Ollevier & Stoks, 2008). This choice to investigate carry-over effects is relevant but  
498 may mask oxidative/antioxidant mechanisms at work during larval development.

499 Finally, none of the changes recorded in metabolic reserves or in oxidative status  
500 affects longevity. Nevertheless, some carry-over effects of predation on longevity are  
501 detectable in more challenging situations such as under low food regimen (Roux et al., 2015).

502

503 To summarize, as expected, larval predation stress affected negatively some life  
504 history traits and all the nutritional reserves analyzed. This highlights a potential prioritization  
505 of nutrient assimilation to fulfill some fitness traits or to fuel a fight-or-flight anti-predator  
506 behavior according to the species' degree of adaptation to the level of predation threat. We  
507 expected a higher level of Total Antioxidant Capacity but the contrary was observed, without  
508 an increased oxidation of proteins (except for *An. gambiae* males). This result could be an  
509 adaptation to chronic stress to decrease the cost of long-term exposure. Overall, our results  
510 revealed that *An. coluzzii* may suffer less from predation stress, or at least managed differently  
511 nutrient assimilation than *An. gambiae* does under predation threat, which could be due to  
512 their respective ecology.

513 To conclude, during the last decade, evolutionary ecologists have attempted to  
514 conceptualize the links between oxidative stress, resource allocations and life history traits in  
515 the oxidative stress hypothesis of life history (Dowling & Simmons, 2009; Monaghan et al.,  
516 2009; Selman et al., 2012; Speakman et al., 2015; Costantini, 2019). However, despite  
517 growing evidence that oxidative stress might play a significant role in physiological adaptive  
518 responses to environmental stressors and in evolutionary trajectories, questions like "How do

519 stress responses vary between species or sex?" remain unresolved. Our results provide  
520 evidence that even between closely related species and between sexes, physiological  
521 responses, life histories and oxidative status can be very different and complex.

522

### 523 **CRediT authorship contribution statement**

524 **Olivier Roux:** Conceptualization, Methodology, Data collection, Formal analyses and  
525 Writing- original draft; **Karine Mouline:** Methodology, Data collection; **Frederic Simard:**  
526 Conceptualization and Funding acquisition; **David Renault:** Data collection and Writing-  
527 original draft preparation. All authors contributed critically to the drafts and gave final  
528 approval for publication.

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537

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539



541 **References**

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741 **Figure legends**

742

743 **Figure 1:** Experimental work flow from larval predation exposure to adult collection for life  
744 history trait measurements and metabolite dosages.

745

746 **Figure 2:** Effect of larval predation threat on life history traits. **a.** Mean length of larval  
747 development; **b.** Mean female wing size; **c.** Mean adult weight; **d.** Mean number of produced  
748 eggs. The numbers in the bars indicate the number of mosquito samples. For the record, to  
749 avoid density effect, the number of larvae in control groups was adjusted according to the  
750 mean number of surviving larvae in predation settings. Consequently, sample size does not  
751 reflect control survivorship.

752

753 **Figure 3:** Effect of species and female size (wing size) on female egg load.

754

755 **Figure 4:** Carry-over effect of larval predation threat on adult longevity.

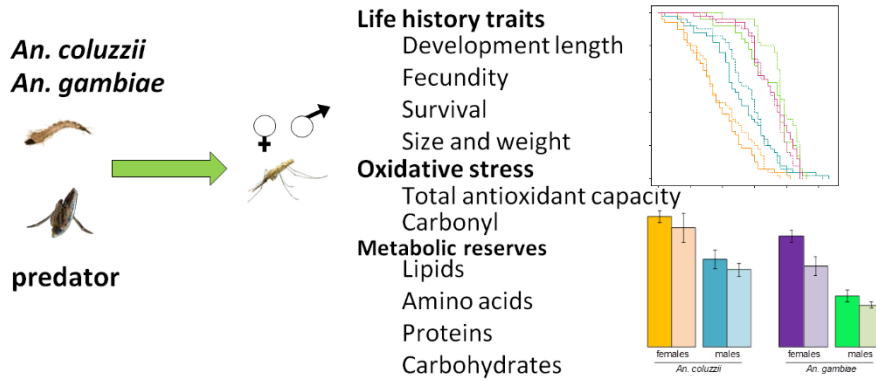
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757 **Figure 5:** Carry-over effect of larval predation threat on metabolic content at emergence. **a.**  
758 Mean protein content; **b.** Mean lipid content; **c.** Mean carbohydrate content; **d.** Mean amino  
759 acid content. The numbers in the bars indicate the number of assays performed.

760

761 **Figure 6:** Carry-over effect of larval predation threat on adult oxidative status. **a.** Mean total  
762 anti-oxidant capacity expressed as Trolox equivalent; **b.** Mean proportion of carbonyl  
763 (oxidized proteins) on total content of proteins. The numbers in the bars indicate the number  
764 of mosquito samples.

765



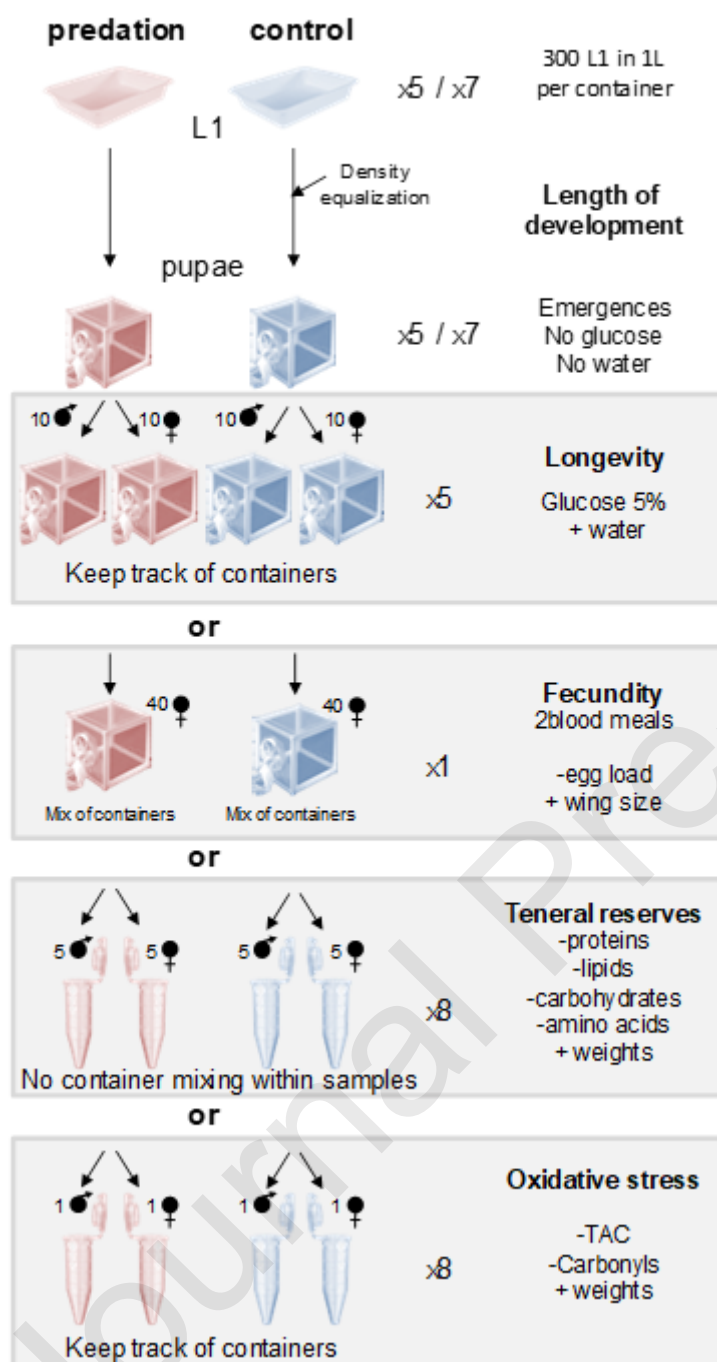
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767 Highlights

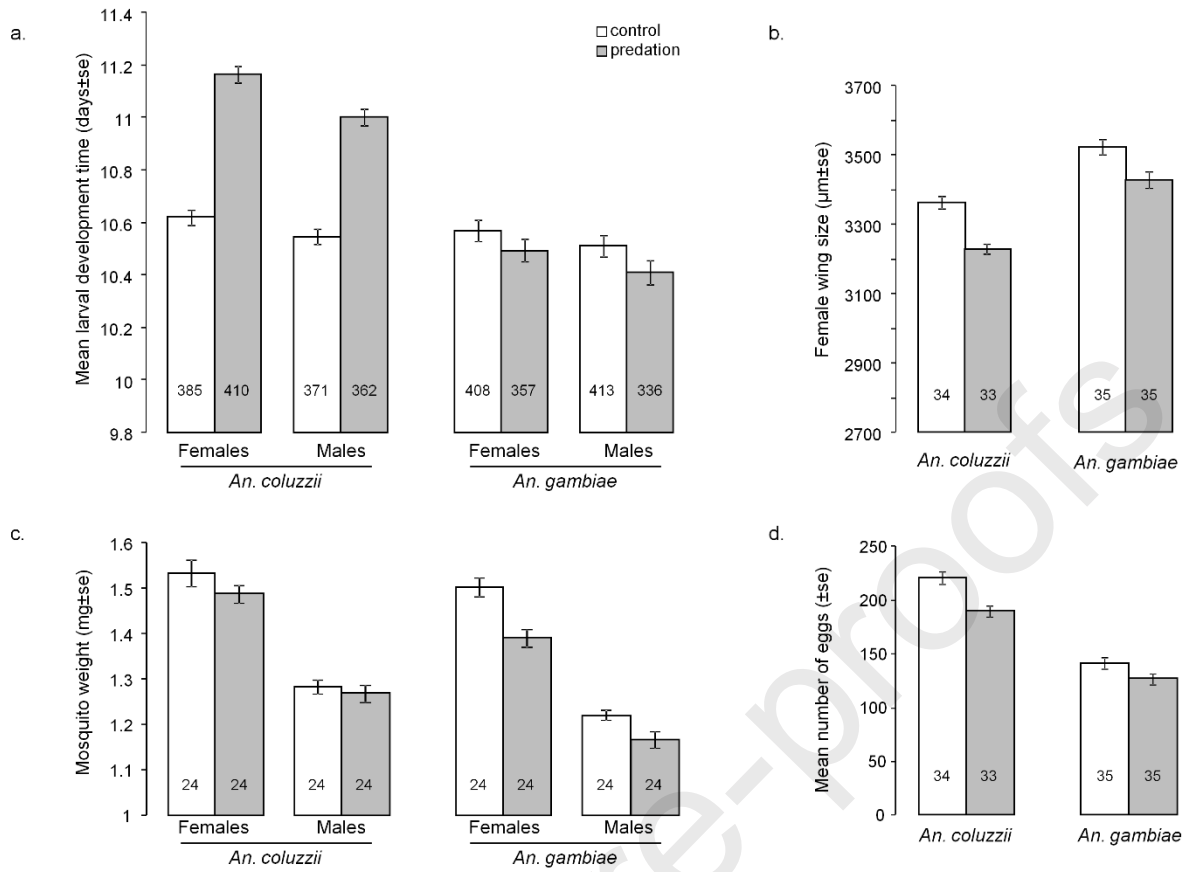
- 768 - Life history traits and all the nutritional reserves were negatively affected
- 769 - *An. coluzzii* suffer less from stress than *An. gambiae*
- 770 - Differential nutrient assimilation to fulfill fitness or to fuel fight-or-flight response
- 771 - Adaptation to chronic stress to decrease the cost of long-term exposure

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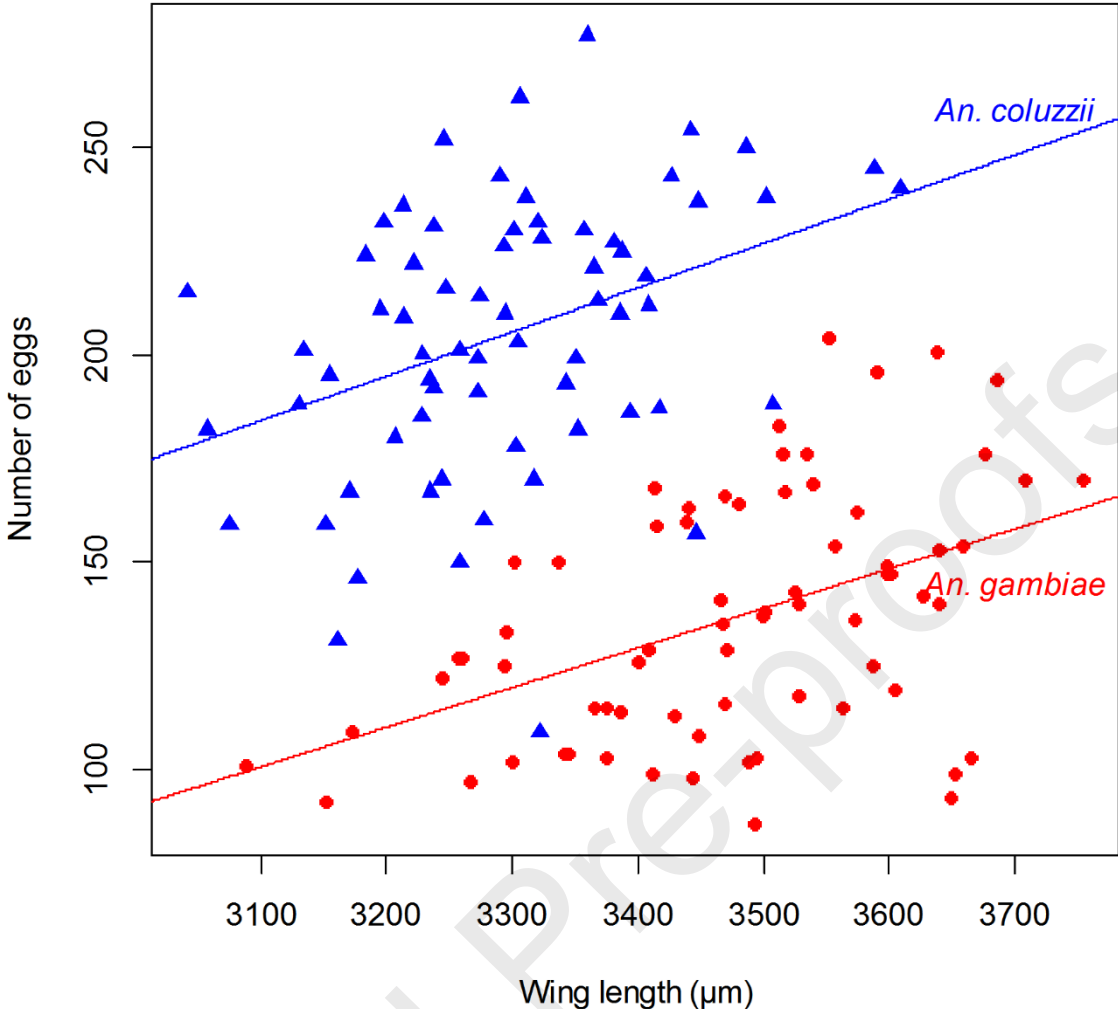
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**An. coluzzii / An. gambiae**

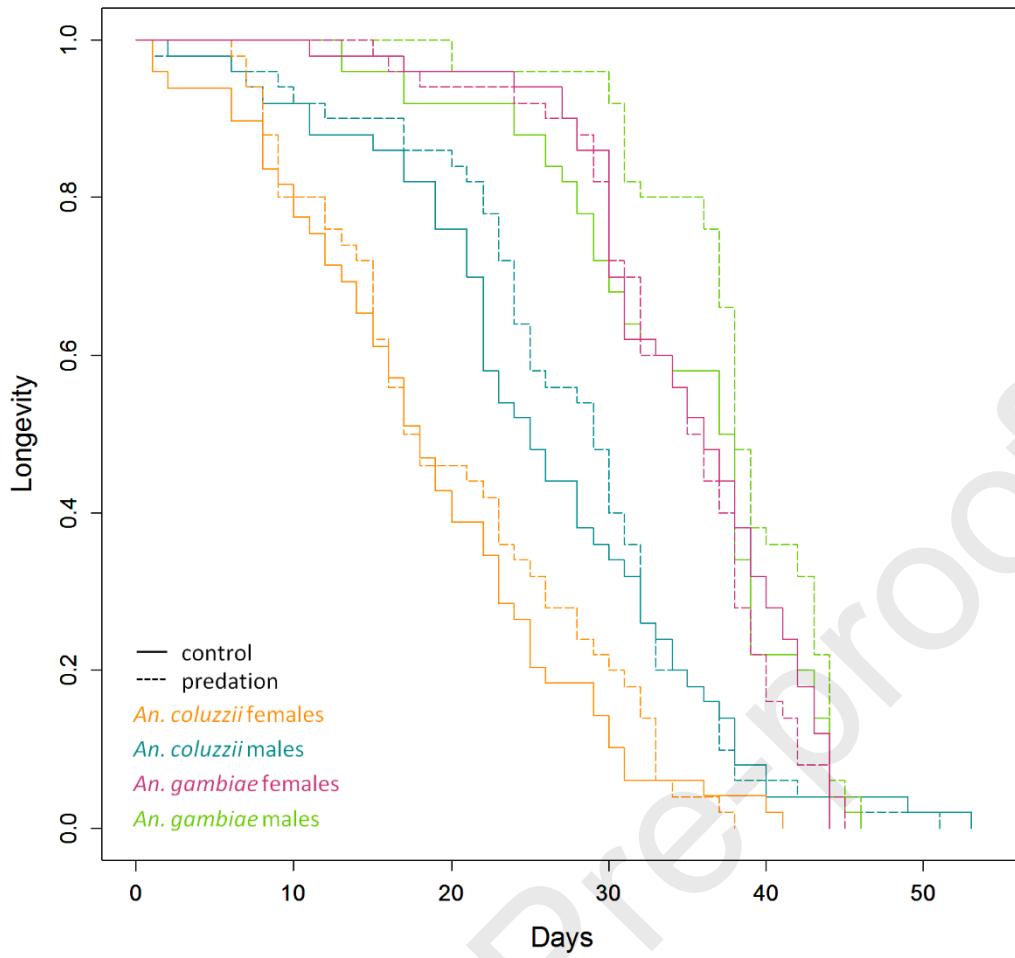
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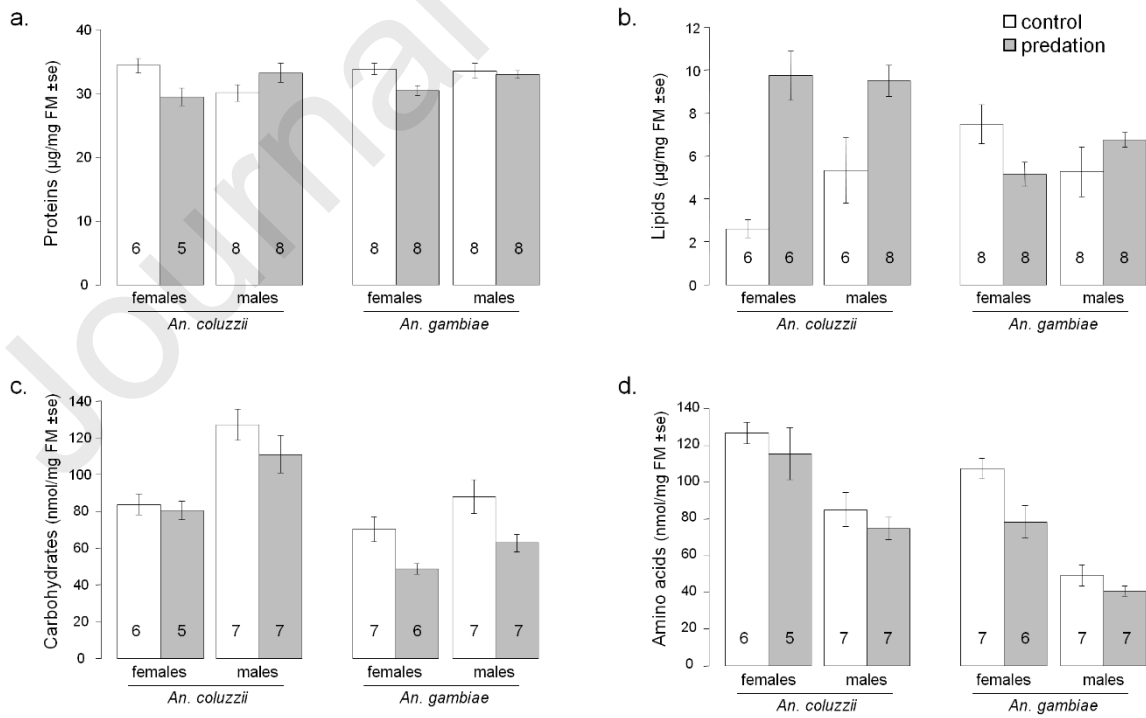
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