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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 μ 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 ± 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 ± 0.02 vs. 10.6 ± 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 ± 0.03 vs. 10.5 ± 0.03 days; $X^2_1=0.3$; $P=0.5$). Males emerged
305 earlier than females (10.61 ± 0.02 vs. 10.71 ± 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 ± 15 vs. $3361 \pm 18 \mu\text{m}$ in *An. coluzzii* and 3428 ± 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 ± 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 ± 0.02 mg vs. 1.36 ± 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 ± 0.02 mg vs. 1.40 ± 0.02 mg, respectively; $X^2_1=1.15$;
321 $P=0.28$). Females were heavier than males (1.47 ± 0.01 vs. 1.23 ± 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 ± 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 ± 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 ± 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 ± 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 ± 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 ± 5.8 vs. 92.5 ± 5.6
368 nmol/mg , respectively; $X^2_1=7.01$; $P=0.008$; Fig. 5c). Females had significantly fewer
369 carbohydrates than males (70.3 ± 3.8 vs. 97.1 ± 6.1 nmol/mg , respectively; $X^2_1=26.5$; $P<0.001$)
370 and *An. gambiae* had a lower carbohydrate content than *An. coluzzii* (68 ± 4.1 vs. 102.8 ± 5.5
371 nmol/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 ± 6.4 vs. 90.7 ± 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 ± 5.4 vs. 62.3 ± 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 ± 5.9 vs. 98.2 ± 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 ± 0.004 vs.
399 0.11 ± 0.02 , respectively) while exposed females had a lower carbonyl proportion than control
400 females (0.05 ± 0.03 vs. 0.11 ± 0.03 , respectively).

401

402 **Discussion**

403 Behavioral and physiological responses to life-threatening stress are supposed to
404 increase immediate individual survival (Hawlana & Schmitz, 2010). During such episodes,
405 and according to the general stress paradigm (Hawlana & 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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740

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.

756

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

An. coluzzii
An. gambiae



predator



Life history traits

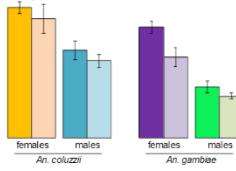
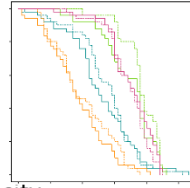
Development length
Fecundity
Survival
Size and weight

Oxidative stress

Total antioxidant capacity
Carbonyl

Metabolic reserves

Lipids
Amino acids
Proteins
Carbohydrates



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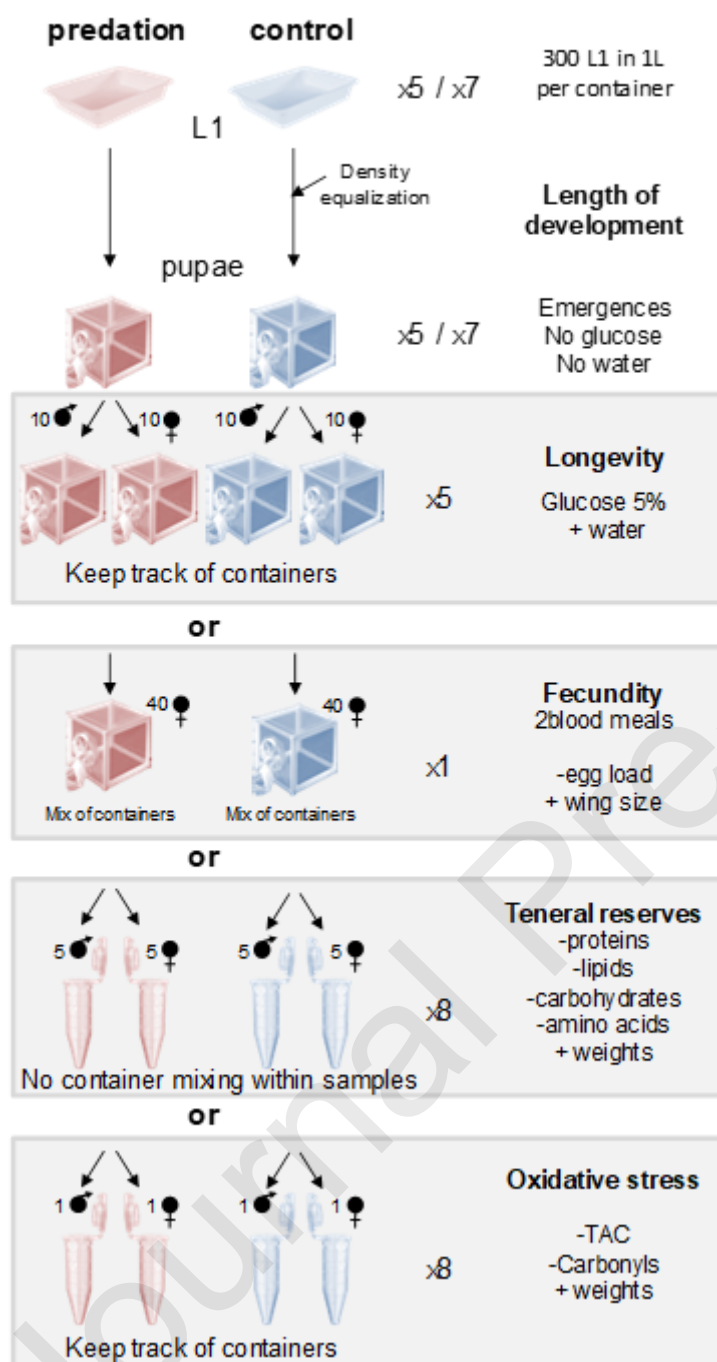
767 Highlights

- 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

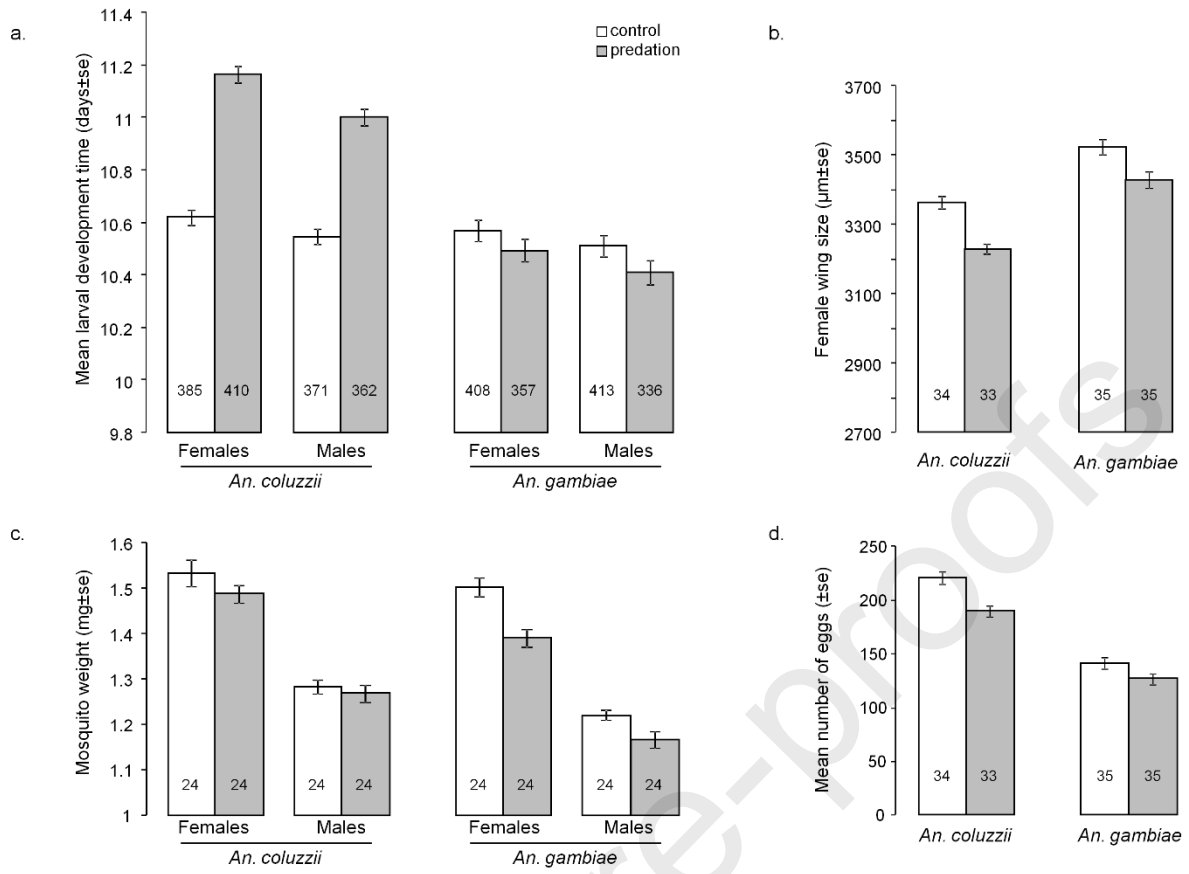
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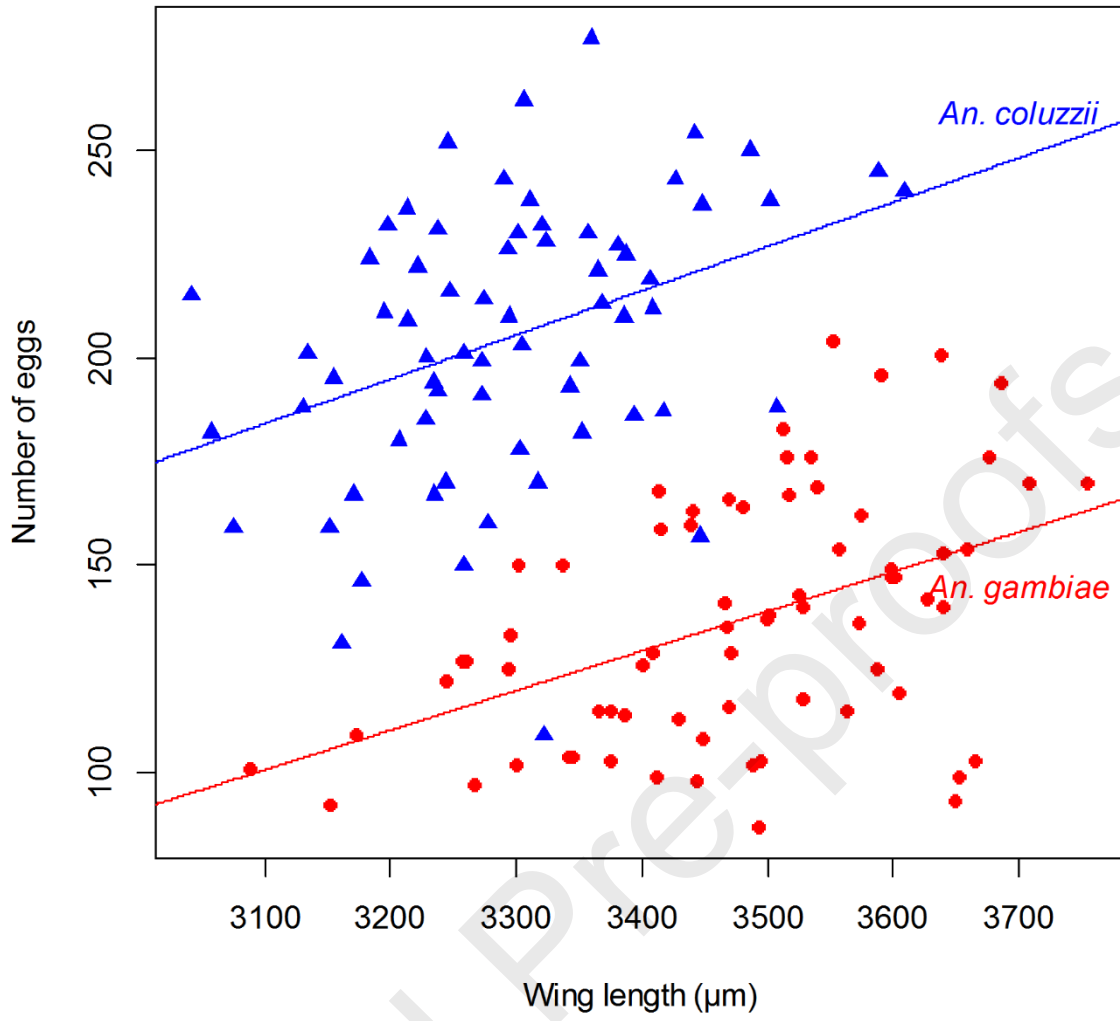
Journal Pre-proofs

An. coluzzii / *An. gambiae*

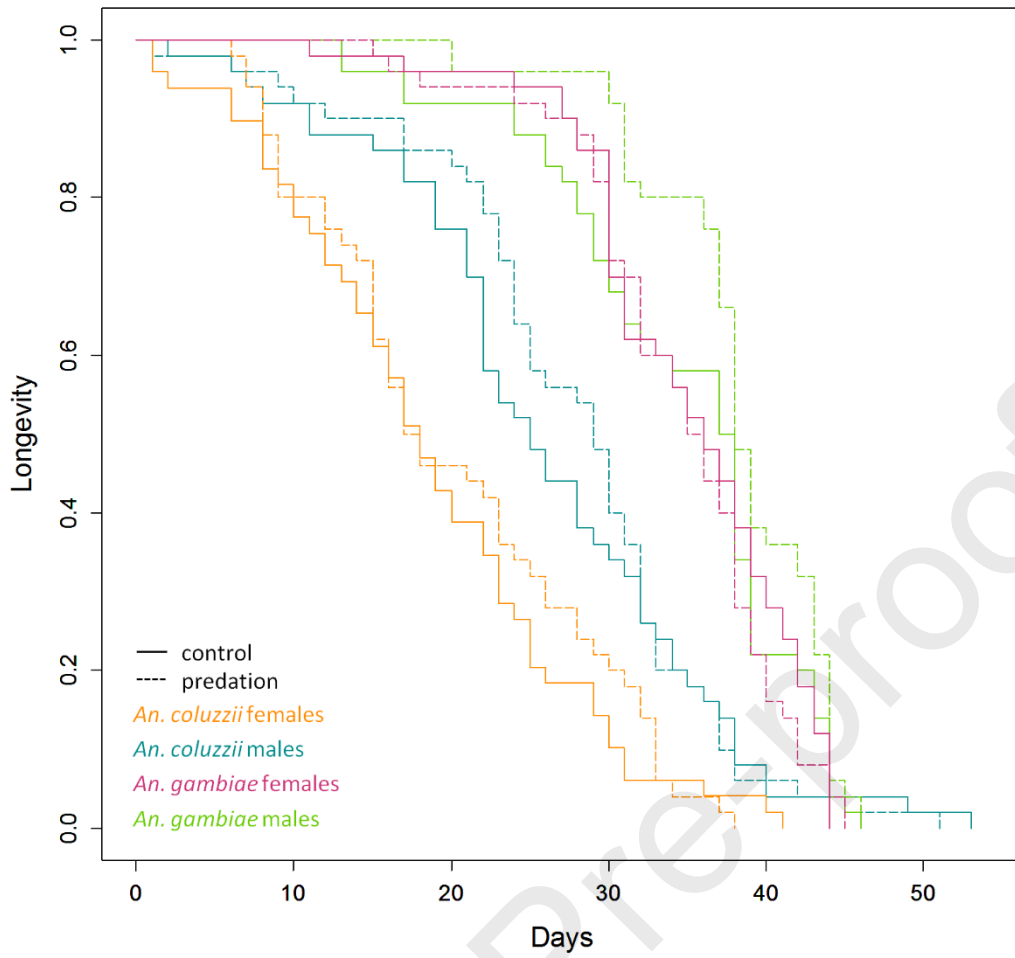
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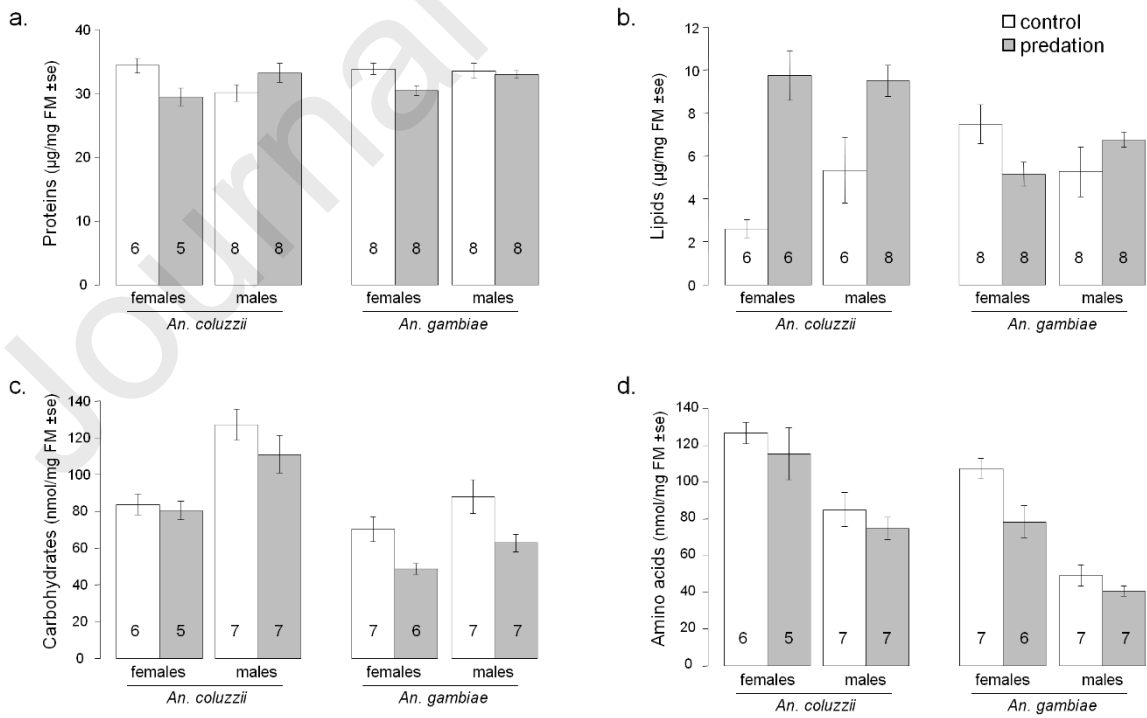
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