1   Title: **Differential oxidative costs of locomotory and genital damage in an orb-weaving**

2   **spider**

3

4   Running title: Cost of locomotory and genital damage in a spider

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17   Keywords: wound, tissue loss, genital damage, harmful male trait, oxidative status, *Larinia*

18   *jeskovi*

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22   **Word count: 1387 words** (including introduction, results, discussion, table and figure

23   legends)

24   Introduction: 481 words

25   Material and Methods: 1006 words

26   Results: 104 words

27   Discussion: 550 words

28   Table and figure legends: 252 words

29   Number of references: 27

30   Display items: 2 figures, 1 table

31 **Summary statement:**

32 Oxidative stress status of female orb-weaving spiders suggests that damage to the locomotory

33 system is physiologically costly, whereas genital mutilation by males is not.

34

35

36 **Abstract:**

37 In animals that regularly experience tissue loss, physiological responses may have evolved to

38 overcome the related costs. Changes in oxidative status may reflect such self-maintenance

39 mechanisms. Here, we investigated how markers of oxidative status varied in female orb-

40 weaving spiders (*Larinia jeskovi*) by mimicking two distinct types of tissue loss they may

41 naturally encounter: damage to their locomotory system and damage to their external genital

42 structure, as inflicted by males to females during copulation (external female genital

43 mutilation). Damage to the locomotory system resulted in a significant shift in the oxidative

44 status reflecting investment into self-maintenance. In contrast, the loss of the genital structure

45 did not result in quantitative changes of oxidative markers. The lack of response to genital

46 mutilation suggests that genital mutilation is physiologically not costly for female spiders. The

47 cost incurred to females rather arises from genital mutilation preventing the females from

48 remating with another male.

49

1. Introduction

In nature, tissue loss typically occurs because of sub-lethal predation, agonistic behaviours between conspecifics and abiotic physical damage [1]. Tissue loss may be costly when it decreases the overall performance of injured animals by limiting their ability to exploit resources (*e.g.* reduced locomotor or feeding ability) and by affecting their homeostasis (*e.g.* because of fluid loss or infection). By activating physiological processes such as healing and immune responses, injured animals may minimize these direct costs. However, investing in these self-maintenance mechanisms may limit resources available for other fitness-related functions, such as reproduction [2]. This investment trade-off may be mediated by variation in oxidative status. Antioxidant defences can neutralize the action of oxidizing species on biomolecules, thereby limiting the generation of oxidative damage in tissues and increasing the survival probability of the organism [3]. For instance, *Bicyclus anynana* butterflies solve the trade-off between longevity and fecundity under challenging conditions by increasing antioxidant defences, thereby prolonging lifespan but reducing fecundity [4]. Alternatively, to facilitate a healing and immune response following tissue loss, injured organisms may also locally reduce their antioxidant response, as oxidizing species may themselves enhance cell communication during tissue repair and eliminate pathogens [5, 6]. Moreover, in order to minimize oxidative damage on their own tissues, injured animals may simultaneously reduce their physical activity thereby reducing their overall production of oxidizing molecules [4]. Hence, the optimal maintenance response of injured animals depends on the regulation of the balance between their production of oxidizing molecules and their antioxidant response [7].

Tissue loss does not only occur because of predation or agonistic behaviours between conspecifics but also during copulation. Indeed, in a broad range of species, males can harm females while transferring sperm and seminal fluids by inflicting physical damage inside or outside females' genitalia [8]. Because sperm and seminal fluids represent resources that females may use, they can affect the physiology of inseminated females [8, 9] and may therefore affect the physiological response of females following damage during copulation. In several spider species, males mutilate the outer structures (scapus) of the female genitalia [10, 11], which makes it possible to disentangle the actual costs due to genital damage from other copulation effects. The physiological response of females following genital damage has previously been investigated with treatments mimicking genital damage by ablating a locomotory tissue [12]. However, locomotory and genital damage may not elicit a similar response. Understanding this response would clarify the mechanisms underlying sexual conflict in species where female genital mutilation occurs.

84   Here, we investigated the physiological response to locomotory and genital damage by

85   measuring different markers of oxidative status in female spiders experiencing tissue loss. To

86   mimic locomotory and genital damage, we applied experimental ablation of one leg and of the

87   scapus. If males and females coevolved to reduce the physiological costs of genital mutilation

88   [12], we expected genital mutilation to trigger a weaker physiological response than locomotory

89   tissue loss.

90

91   **2. Material and Methods**

92   **Study animals:**

93   We collected sub-adult (one moulting stage from adulthood) females of the orb-weaving spider

94   *Larinia jeskovi*, Marusik 1986 (Araneidae) [13] in August 2015 and 2016 in the Biebrza

95   National Park, Poland (53°21'01.36''N, 22°34'37.45''E). In the laboratory, we housed females

96   individually in 250 mL plastic cups at room temperature and under a natural light cycle.

97   Females were fed with one fly (*Lucilia sericata*) every three days and watered daily. Sub-adult

98   females (one moulting stage from adulthood) were checked daily for moulting events. After

99   their final moult, adult females were used for the experimental setup.

100

101   **Experimental setup (figure 1):**

102   In order to assess the effect of tissue loss on females' oxidative status, we amputated part of

103   one of their forelegs (randomly right or left leg) or manually ablated their scapus [10]. To

104   achieve this, females were first immobilized under a mesh and then wounded under a

105   stereomicroscope. A control group was left intact but was similarly handled. In 2015, 30

106   females were randomly assigned to three treatment groups: control, tibia-amputated, scapus-

107   amputated. In 2016, we repeated the procedure with an additional wounding treatment in which

108   we removed 200 μm of the tarsus. The removal of only the tip of the leg was chosen to assess

109   the effect of a wound comparable to the scapus mutilation in terms of the amount of tissue lost.

110   Seventy-two females were randomly assigned to the four experimental amputation treatments;

111   control, mid-tibia, leg-tip and scapus. Eight hours after treatment, females were cryofixed with

112   liquid nitrogen and stored at −80 °C for later analysis. Age was calculated as the number of

113   days between the final moult and the treatment.

114

115   **Oxidative stress markers:**

116 Before measurements, all appendages (legs, pedipalps) were removed from the frozen
117 specimens on dry ice, and the body of each female was weighed to the nearest 0.01 mg
118 (Sartorius LE225D; Sartorius AG, Göttingen, Germany) before being mixed with PBS buffer.

119 In 2015, we measured two markers of oxidative status. We used the OXY-absorbent test
120 (Diacron International, Grosseto, Italy) to measure spiders' total antioxidant capacity
121 (expressed in millimole of HOCL neutralized) and the d-ROM test (Diacron International,
122 Grosseto, Italy) to measure concentrations in hydroperoxides, a marker of oxidative damage
123 deriving from the oxidation of fatty acids, proteins and nucleic acids and promoting cell death
124 (expressed in milligrams per decilitre of H_2O_2 equivalent). For both tests, we followed the
125 procedure described in [14]. Three individuals, for which d-ROM values were below the
126 detection threshold, were excluded from the analyses. This resulted in a sample size of 9 control
127 females, 10 tibia-amputated females, and 8 scapus-amputated females.

128 In 2016, we measured glutathione (GSH) levels (expressed in μmol GSH per mg
129 protein) as marker of endogenous antioxidant defences, and malondialdehyde (MDA) levels to
130 assess oxidative damage on lipids (expressed in mmol per mg protein). The thorax and the
131 abdomen were homogenized together with Triton buffer (7.5 μl for each 1 mg sample) through
132 high-speed shaking (three times for 1 min; 24 shakes/s). The resulting homogenate was
133 centrifuged (16249 g, 30 min, 4°C) before transferring the resulting supernatant to a new tube
134 and centrifuging it again (16249 g, 15 min, 4°C). The second supernatant was then used to
135 analyse total protein and MDA concentrations. MDA concentrations were determined using the
136 commercial kit MDA Microplate Assay Kit (Cat. no. CAK1011; Cohesion Bioscience; 532 and
137 600 nm). GSH levels were assessed with spectrophotometric method, which involves oxidation
138 of GSH by the sulfhydryl reagent 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB, also known as
139 Ellman's reagent) to form the yellow derivative 5′-thio-2-nitrobenzoic acid (TNB), measurable
140 at 412 nm. Total protein concentration of all samples was determined using the Bradford protein
141 assay at 595 nm. Two females died before the end of the treatment. Out of the 70 females left,
142 we were able to measure MDA levels in 62 individuals (others showing levels lower than the
143 minimal detection threshold). Consequently, 70 females were used for glutathione
144 measurements (18 control females, 18 leg-tip-amputated females, 17 tibia-amputated females,
145 and 17 scapus-amputated females) and 62 females were used for MDA measurements (17
146 control females, 15 mid-tibia-amputated females, 16 leg-tip-amputated females, and 14 scapus-
147 amputated females).

148

149 **Statistical analyses:**

150 To test the effects of treatment on antioxidant capacity, hydroperoxide and MDA levels, we

151 built linear models with antioxidant capacity, hydroperoxide or MDA levels as dependent

152 variables, and treatment, age, body mass and protein concentration (to correct for concentration

153 differences between samples) as independent variables. To test the effect of treatment on

154 glutathione levels, we built a linear model with glutathione corrected for body mass as

155 dependent variable (to reach normality) and treatment and age as independent variables. We

156 checked linearity assumptions graphically as well as with a Bartlett test for homoscedasticity

157 and Shapiro test for normality of the residuals. The model for antioxidant capacity required the

158 exclusion of one outlier (from control treatment) to match linearity assumptions. There was no

159 significant correlation among variables (table S1, Suppl. Mat.) leading to no collinearity among

160 explanatory variables. In all models, quantitative variables were centred and standardized [15].

161 For each model, we considered all plausible candidate models and ranked them

162 according to their AICc value [16, 17]. To evaluate the contribution of each predictor to the

163 model prediction, we calculated its sum of Akaike weights and used "full model averaging" to

164 calculate parameter estimates β [17]. Since the sum of weights may provide a poor evaluation

165 of the predictors' importance [18], we calculated the 85% confidence interval for each

166 parameter estimate [19]. Parameter estimates whose confidence interval did not include zero

167 were considered as having a significant effect. The evaluation of predictors' contribution results

168 in parameter estimates for which the first level of a factor is set as a reference. Thus, the results

169 of the amputation treatments are presented as mid-tibia, leg-tip and scapus amputation

170 treatments compared to the control treatment as the reference. All analyses were performed in

171 R software [20]. The MuMIn v1.40.4 [21] and Plotrix 3.7-6 [22] packages were used for

172 procedures of model selection and model averaging and for calculating the standard errors of

173 the means in figure 2, respectively.

3. Results

175 Spiders did not differ in body mass and age between treatments (Suppl. Mat., figure S1). In

176 2015, females amputated at the mid-tibia showed higher antioxidant capacity and lower

177 hydroperoxide levels than control females (table 1). Similarly, in 2016, both mid-tibia and leg-

178 tip amputations were associated with lower MDA levels compared to control females.

179 However, glutathione levels were similar in all treatment groups (table 1). Scapus-amputated

180 females did not show any alteration of their oxidative status relative to control females both in

181 2015 and 2016 (figure 2, table 1). As a result, leg-amputated spiders segregated from scape-

182 amputated and control spiders in oxidative space (figure 2).

183

4. Discussion

184

185 Leg amputation led to a shift in the oxidative status of female spiders irrespective of amputation

186 extent. In contrast, scapus amputation did not affect their oxidative status. These results were

187 consistent across different markers of oxidative damage measured in different individuals in

188 two distinct experiments. Hence, our study suggests that a physical harm inflicted to the

189 locomotory system of female spiders affects their oxidative balance, whereas a damage to their

190 external genitalia does not.

191   In agreement with our predictions, tissue loss due to leg amputation induced a shift in

192 females' oxidative balance, as amputated females showed higher antioxidant capacity than

193 intact females. However, glutathione levels were similar in all treatment groups. This pattern

194 suggests that amputated females invested in self-maintenance mechanisms by upregulating

195 their production of some endogenous antioxidant defences, which did not involve glutathione

196 or involved glutathione in combination with other antioxidant compounds. This upregulation

197 was not associated with stable oxidative damage in amputated females but with lower oxidative

198 damage. Low oxidative damage in amputated females is likely related to their probable lower

199 locomotory activity following leg amputation leading to lower production of oxidizing

200 molecules [4]. Moreover, low physical activity in amputated females may help them to save

201 resources that can be allocated to maintenance mechanisms, such as antioxidant defences.

202 Finally, the fact that both markers of oxidative damage decreased in amputated individuals can

203 be explained by the biochemical proximity between both markers, as MDA results from the

204 decomposition of hydroperoxides [23].

205   In contrast to leg amputation, the removal of external genital structure did not affect the

206 females' oxidative status, which was comparable to that of intact females. It seems possible that

207 the extent of the harm was not sufficient to induce a detectable physiological response.

208 However, the amputation of the leg tip, an injury comparable to scapus mutilation in terms of

209 tissue loss, affected the females' oxidative balance. This difference may be due to the negative

210 effect of locomotory tissue loss on the overall physical activity of spiders, which is unlikely to

211 occur following genital mutilation [4]. Since locomotory and genital damage trigger different

212 physiological responses, we argue that ablating a locomotory tissue to mimic the effects of

213 genital damage is not relevant [12], and only genitally-mutilated females should be used to

214 examine the effects of such mutilation.

215   External female genital mutilation by males is a common feature of the mating system

216 of several spider species that allows males to secure paternity [10, 11]. As a harmful male

217   adaptation, the mutilation of female genitalia may be subject to a sexual conflict [8]. However,

218   the costs and benefits for females are unclear. Our findings suggest that female spiders do not

219   experience oxidative changes due to genital mutilation, suggesting no costs in terms of self-

220   maintenance. The absence of physiological costs might therefore be the result of selection on

221   males and females to reduce the costs associated with a harm that would ultimately result in

222   lower reproductive performance in both females and males [9, 12, 24]. Our results therefore

223   corroborate the oxidative shielding hypothesis, postulating that oxidative damage in

224   reproductive females is minimized to avoid deleterious effects on reproductive performance

225   [25]. Overall, our study suggests that males benefit from mutilating female genitalia without

226   impairing female's fecundity, which sheds new light on the evolution of external female genital

227   mutilation in animals [26].

228

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291   (http://dx.doi.org/...)

292

293 **Table 1:** Predictor's sum of weights, parameter significance and 85% confidence interval (CI)

294 after full model averaging on the set of candidate models, assessing the effect of body mass,

295 experimental amputation treatment, age and protein concentration on levels of antioxidant

296 defence (estimated by the total antioxidant capacity test and GSH levels) and oxidative damage

297 (estimated by hydroperoxide levels and MDA levels) in the spider *Larinia jeskovi*.

Predictor	$\sum w_i$	Parameter	Significance	85% CI
Total antioxidant capacity				
		Intercept	+	278.00; 297.49
Body mass	1	Body mass	+	19.04; 31.54
Protein concentration	0.30	Protein concentration	N.S.	-10.83; 1.81
Treatment	0.22	Mid tibia	+	1.72; 30.29
		Scapus	N.S.	-0.59; 30.71
Age	0.20	Age	N.S.	-4.52; 8.31
Hydroperoxides				
		Intercept	+	3.24; 4.38
Age	0.58	Age	+	0.09; 0.85
Body mass	1	Body mass	+	1.01; 1.78
Protein concentration	0.23	Protein concentration	N.S.	-0.21; 0.61
Treatment	0.22	Mid tibia	-	-1.93; -0.17
		Scapus	N.S.	-1.68; 0.30
GSH/Body mass				
		Intercept	+	7.720; 9.994
Treatment	0.05	Leg tip	N.S.	-4.707; 1.205
		Mid tibia	N.S.	-3.492; 2.526
		Scapus	N.S.	-3.033; 2.962
Age	0.35	Age	N.S.	-0.339; 1.765
MDA				
		Intercept	+	0.395; 0.634
Body mass	0.24	Body mass	N.S.	-0.066; 0.045
Treatment	0.83	Leg tip	-	-0.370; -0.086
		Mid tibia	-	-0.419; -0.128
		Scapus	N.S.	-0.210; 0.084
Protein concentration	0.24	Protein concentration	N.S.	-0.045; 0.065
Age	0.32	Age	N.S.	-0.088; 0.019

298 Notes: Parameter estimates after model averaging of treatment (in grey) are compared to the

299 reference level "control". An estimate whose 85% CI does not include zero is considered

300   significant: N.S. non-significant parameter, + positive significant parameter, − negative

301   significant parameter.

302

303

304   **Figure legends:**

305   **Figure 1:** *Larinia jeskovi* female in ventral view (A). The two black rectangles indicate the leg

306   and the genital areas. Close up picture of the leg with the experimental amputation treatments

307   at the mid-tibia and leg-tip represented by the dashed lines (B). Close up picture of the external

308   female genitalia with the experimental amputation treatment applied at the basis of the scapus

309   represented by the dashed line (C).

310

311

312   **Figure 2**: Mean values (± SE) of A: total antioxidant capacity and oxidative damage

313   (hydroperoxide concentration), B: endogeneous antioxidant defence (glutathione

314   concentration) and oxidative damage on lipids (MDA concentration) for each experimental

315   amputation treatment: control (blue), mid-tibia (red), legtip (orange) and scapus (yellow) in the

316   spider *Larinia jeskovi*. Markers of antioxidant defences are represented on the x-axes and

317   markers of oxidative damage on the y-axes. Levels of antioxidant defences and oxidative

318   damage markers are corrected for body mass.

319

Figures:
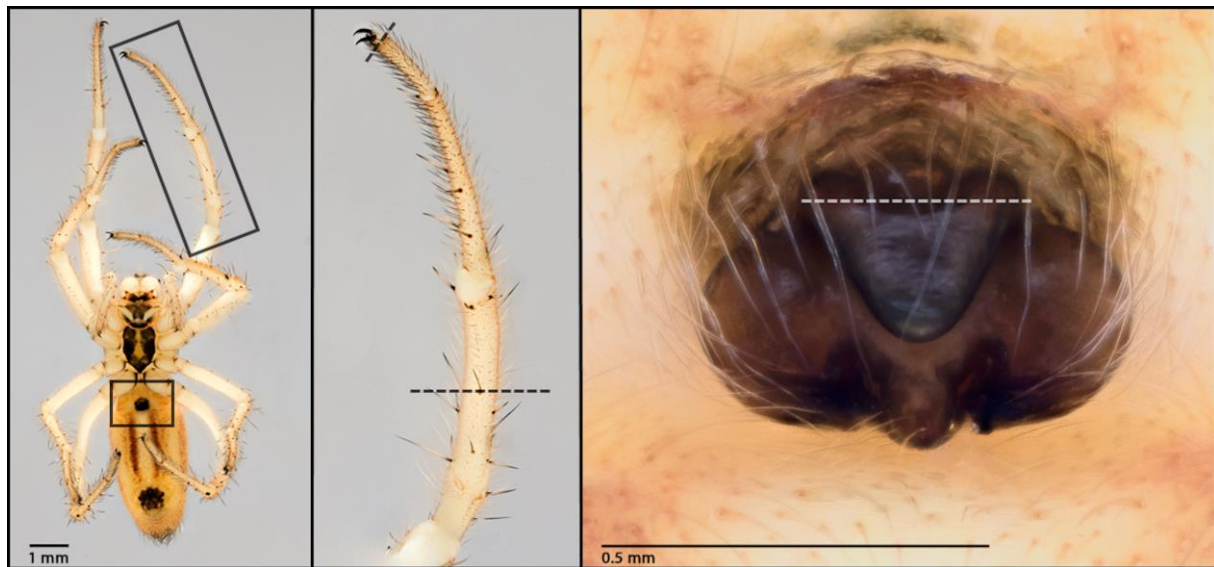
Figure 1:

Figure 2:
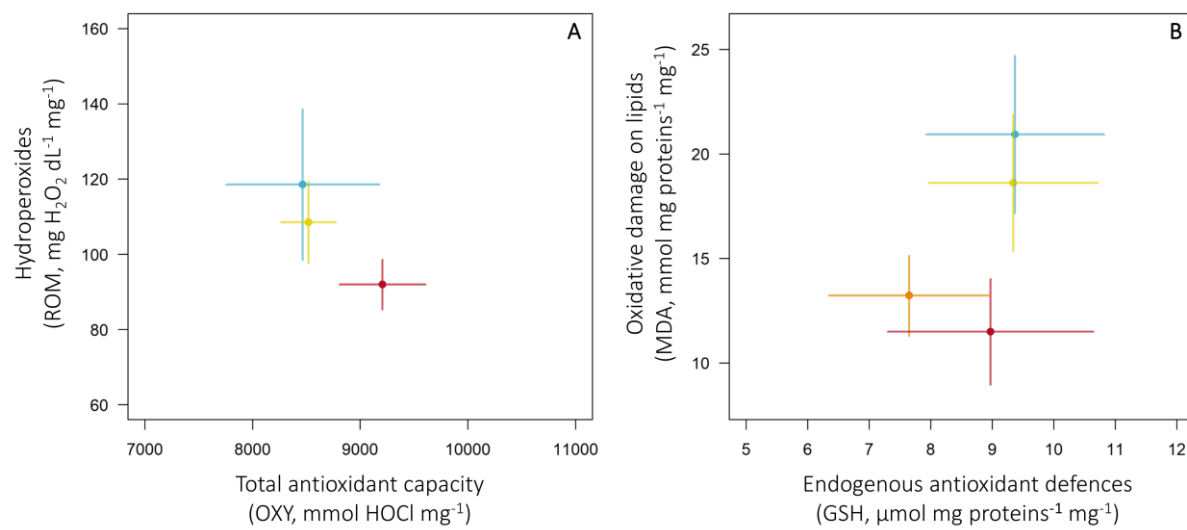
327 **Ethical statement:**

328 All spiders were handled with care and cold-euthanized.

329

330 **Data accessibility:**

331 Data are available from the Dryad Digital Repository: [27]

332

333 **Author contributions:**

334 P.M., G.U. and M.B. designed the study and contributed through discussions. P.M. collected

335 animals and carried out experimental treatments. P.M. and N.T. carried out physiological

336 measurements. P.M. and M.B. carried out statistical analyses. All authors contributed through

337 writing.

338

339 **Competing interests:**

340 No competing interests declared.

341