

1 **IMMUNE FUNCTION AND THE DECISION TO DEPLOY WEAPONS DURING FIGHTS IN THE**
2 **BEADLET ANEMONE *ACTINIA EQUINA***

3 **Running title:** Immunity and weapon use during fights

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24 **Summary statement**

25 Here, we demonstrate how strategic fighting decisions affect an individual's subsequent
26 ability to mount an immune response.

27

28 **Abstract**

29 The ability to mitigate the costs of engaging in a fight will depend on an individual's
30 physiological state. However, the experience of fighting itself may in turn affect an
31 individual's state, especially if the fight results in injury. Previous studies found a correlation
32 between immune state and fighting success, but the causal direction of this relationship
33 remains unclear. Does immune state determine fighting success? Or does fighting itself
34 influence subsequent immune state? Using the beadlet anemone *Actinia equina*, we
35 disentangle the cause and effect of this relationship, measuring immune response once pre-
36 fight and twice post-fight. Contrary to previous findings, pre-fight immune response did not
37 predict fighting success, but rather predicted whether an individual used its weapons during
38 the fight. Furthermore, weapon use and contest outcome significantly affected post-fight
39 immune response. Individuals that used their weapons maintained a stable immune
40 response following the fight, while those that fought non-injurious did not. Furthermore,
41 although winners suffered a similar reduction in immune response to losers immediately
42 post-fight, winners began to recover pre-fight levels within 24 hours. Our findings indicate
43 that immune state can influence strategic fighting decisions and moreover that fight
44 outcome and the agonistic behaviours expressed can significantly affect subsequent
45 immunity.

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47 **Keywords:** *Actinia equina*, contest behaviour, fighting success, immune response, injuries,
48 weapon use

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52 **1. Introduction**

53 The costs of engaging in a fight can be substantial, and in fact often outweigh the potential
54 benefits. It is for this very reason, that the majority of fights are settled using non-injurious
55 behaviours such as agonistic displays. But even non-injurious behaviours can be
56 physiologically costly to produce, for example leg displays used by the sierra dome spider
57 *Neriene litigiosa* require 3.5x more energy than resting (Decarvalho et al., 2004), while ‘trials
58 of strength’ such as wrestling in the house cricket *Acheta domesticus* demand 40x more
59 oxygen than a resting state (Hack, 1997). Furthermore, when fights cannot be settled via
60 non-injurious means, opponents escalate into the use of agonistic attacks in an effort to
61 force their opponent to retreat. Attacks can result in injuries to the recipient and, as
62 recently reviewed, to the attacker itself (Lane and Briffa, 2017). As animals have finite
63 energy stores, participation in a contest could reduce capacities for general maintenance,
64 including the ability to mount an efficient immune response.

65 The ability to maintain a normal immune system and mount an adequate reaction to
66 infection requires a great deal of resources. Even a mild infection can bring about huge
67 shifts in resource allocation, increasing both nutritional and metabolic demands (Lochmiller
68 and Deerenberg, 2000). It is thus generally assumed that only individuals of high quality are
69 capable of maintaining a normal immune system while also investing in other costly traits.
70 As a result, costly sexually selected traits including elaborate ornaments, courtship
71 behaviours (Folstad and Karter, 1992; Gilbert and Uetz, 2016) and even sperm quality
72 (Simmons, 2011) can provide an honest signal of an individual’s condition to potential mates
73 (i.e. the immunocompetence handicap hypothesis (e.g. Folstad and Karter, 1992; Gilbert and
74 Uetz, 2016; Simmons, 2011)). In addition to sexual signals, immunity has also been found to
75 covary with fighting ability and social status. However, despite recently explained
76 similarities between courtship and fighting (Mowles and Ord, 2012), the links between
77 immunity and agonistic behaviour have yet to be resolved. Previous work has indicated that
78 in general, immunity is positively correlated with fighting success (although see Vaananen et
79 al., 2006 for an exception), with winners exhibiting higher general immunity (Dijkstra et al.,
80 2011; Filby et al., 2010; Koskimäki et al., 2004; Rantala and Kortet, 2004; Steiger et al., 2012)

81 and an increased ability to respond to infection in comparison to losers (Hawley et al., 2007;
82 Kelly, 2014; Zuk and Johnsen, 2000). These findings suggest that immunity may be a
83 predictor of fighting success, such that individuals possessing higher immune abilities are
84 better able to win fights. However, in all of these studies, immunity was only measured
85 once, either before or after a fight and thus the evidence gathered to date does not allow us
86 to disentangle the causal direction of the relationship between immunity and fighting
87 success. Does pre-fight immunity predict fighting success or does fighting success affect
88 subsequent immunity?

89 Fighting may affect immunity for several different reasons. Firstly, as mentioned
90 earlier, fighting is energetically costly and may trade-off with immunity over resource
91 allocation. Secondly, exposure to stress can cause changes in immune response (Yin et al.,
92 1995; Demers and Bayne, 1997; Ortuño et al., 2001; Padgett and Glaser, 2003) and thus
93 entering into a direct contest with another individual may modulate an individual's immune
94 response regardless of the behaviours expressed within the contest or indeed the outcome
95 of the interaction. Finally, if a contest escalates into the use of agonistic attacks, injuries
96 may result for either one or both of the contestants, as a result of injuries inflicted by the
97 opponent and of damage caused by an individual's own agonistic behaviour (see Lane and
98 Briffa, 2017). Injuries not only require the reallocation of energy and resources in order to
99 heal, but may also increase an individual's susceptibility to pathogens, especially when the
100 injury incurred is an open wound. Injuries thus require an acute upregulation of immune
101 response in order to both protect an individual from infection and to heal the wound
102 (Lochmiller and Deerenberg, 2000). Consequently, changes in immunity triggered by a
103 particular fight may depend upon the level of agonistic behaviour expressed by each
104 opponent as well as the outcome of the fight.

105 Here, we explore the effects of fighting behaviour on the immune response of the
106 beadlet sea anemone *Actinia equina*. *A. equina* are one of the simplest organisms to engage
107 in contests. *A. equina* possess weapons in the form of specialised stinging structures called
108 acrorhagi which are full of stinging cells (nematocytes) and used solely to attack conspecifics
109 (Williams, 1978; Brace et al., 1979; Bigger, 1982). *A. equina* provide an interesting system
110 for studying immune response in relation to fighting as they incur injury not only by
111 receiving attacks but also by inflicting attacks (Lane and Briffa, 2017; also see figure 1).

112 During an attack, pieces of the attacker's acrorhagial epithelium peel off and stick to the
113 recipient. This act causes localised necrosis on the recipient, but also has the unavoidable
114 effect of ripping holes in the acrorhagi of the attacker, such that both individuals may be
115 susceptible to infection. It has recently been demonstrated that the mucus produced by *A.*
116 *equina* as a protective coating against desiccation and other environmental stressors
117 contains antimicrobial properties (Stabili et al., 2015). One such property is a lysozyme-like
118 enzyme which acts as an antibacterial agent, degrading the cell walls of gram-positive
119 bacteria such as *Micrococcus lysodeikticus*. Lysozyme is one of the best characterised
120 enzymes in self-defence against bacteria and has previously been shown to play a key role in
121 controlling the inflammation and development of subcutaneous infection, for example in
122 mice (Ganz et al., 2003). Here, in order to examine the effects of fighting and injury
123 (including self-inflicted injuries) on immune response in *A. equina*, we measure lysozyme-
124 like activity at three different time points, once before a fight and twice afterwards. We
125 then calculate the change in lysozyme-like activity over the three measurements with
126 respect to both fight outcome and injury state. Thus, we quantify the cost of self-inflicted
127 injuries for the first time in any fighting animal. A comparison of post-fight lysozyme
128 activities between individuals that inflict and receive epithelial peels will reveal the relative
129 costs of injuries received from an opponent and injuries that are self-inflicted. If inflicting
130 peels is costly in terms of immune response, individuals in poor condition should be less
131 likely to use their weapons to inflict peels compared with those in good condition. In this
132 case, pre-fight immune status should predict the chance of using acrorhagi in a subsequent
133 fight. Fundamental contest theory assumes that for dangerous fighting to be adaptive, the
134 benefits of any agonistic behaviour must outweigh the costs but this assumption has yet to
135 be tested in terms of self-inflicted injuries.

136

137 **2. Materials and methods**

138 (a) Anemone collection and staging contests

139 *Actinia equina* ($N=97$) were collected intertidally from Portwrinkle (Cornwall UK; grid
140 reference: SX 357539) between November 2016 and June 2017 and taken back to the lab
141 within 1-2 hours of collection. As in previous studies of agonistic interactions in *A. equina*

142 only anemones of the red/brown colour morph were collected (the red/brown colour
143 morph has previously been shown to exhibit higher levels of aggression than the
144 green/orange morph – Manuel, 1988). In the lab anemones were housed individually in
145 plastic tanks (23 x 16 x 17.5cm) containing 700ml of filtered, aerated seawater (the
146 seawater was pumped from Mount Batten, Plymouth UK; grid reference: SX 4871 5319) and
147 maintained at 15°C ± 0.5°C. Throughout the experiment, anemones were fed *ad libitum* on
148 aquaria marine flakes every 2-3 days and seawater was changed every 7 days.

149 After a 7 day acclimatisation period, anemones were dislodged from their position in
150 the tank and provided with stones to attach to. On the afternoon of the next day, anemones
151 were randomly paired and placed into the centre of a clean plastic tank containing 700ml of
152 clean aerated, filtered seawater. Anemones were positioned such that their body columns
153 were touching in order to stimulate agonistic behaviour. This contact marked the beginning
154 of the fight and fights were considered concluded when one anemone (the loser) either: (a.)
155 moved an approximate distance of one pedal disc away from its opponent (estimated
156 visually) or (b.) retracted its tentacles completely for at least 10 minutes. After this point
157 anemones were separated and returned to their tanks. At the end of the contest individuals
158 were checked for the presence of acrorhagial peels. Individuals were then classified into the
159 following groups: - No injuries; received injuries (i.e. peels attached to the column); inflicted
160 injuries (i.e. holes present in acrorhagi). These groups were further broken down into
161 winners and losers for analysis. All fights were recorded using a Canon LEGRIA HF R706 High
162 Definition Camcorder.

163 All pairwise encounters that resulted in a fight were included in the analysis outlined below.
164 Pairs that failed to fight were removed from the study.

165

166 (b) Mucus collection and lysozyme-like assays

167 Mucus collection and lysozyme-like assays were carried out following the methods of Stabili
168 et al. 2015, which allows samples to be collected non-invasively and thus at several time
169 points in the same individuals. Lysozyme-like activity (our experimental design does not
170 allow characterization of a specific lysozyme, so cell lysis is attributed to a lysozyme-like
171 enzyme) was measured at 3 different time points, once on the morning before the fight and

172 twice post-fight (1 hour and 24 hours after the fight). In order to observe how lysozyme
173 activity varies over time in *A.equina* naturally, mucus was also collected and analysed for a
174 subset of control anemones ($n = 23$) that did not participate in staged fights. At these time
175 points, anemones were removed from their tanks, washed with sterile seawater and placed
176 individually into clean dry petri dishes where they were left for 30 minutes to stimulate
177 mucus secretion (Stabili et al., 2015). After 30 minutes, the excreted mucus was collected
178 and centrifuged at 12,000 x g for 30 minutes at 4°C. The supernatant was then transferred
179 into a fresh microcentrifuge tube and frozen at -20°C until use.

180 Lysozyme-like activity was measured using a standard intra-agar lysis assay in which
181 7mL of 0.04M sodium phosphate agarose (pH 6.0) was inoculated with 700µl of 5mg/mL of
182 freeze-dried *Micrococcus lysodeikticus* (Sigma-Aldrich, Gillingham, Dorset, UK) and then
183 spread across a Petri dish. Once dry, six wells of 6.3mm in diameter were punched into the
184 agarose using a core borer and each filled with 30µl of mucus. The dishes were then
185 incubated at 37°C for 22 hours, after which the diameter of the lysed area around each well
186 was measured to the nearest 0.1mm using callipers. All samples were carried out in
187 duplicate and an average lysis diameter calculated from the two measures.

188 All experimental procedures adhered to the ethical guidelines of the Association for
189 the study of Animal Behaviour (ASAB). After use in this study all anemones were returned to
190 the collection site at Portwrinkle. No licences or permits were required for this study.

191

192 (c) Statistical analysis

193 To investigate the change in lysozyme activity over time in anemones that did not fight, we
194 conducted a repeated measures linear mixed effects model on the control anemones using
195 the lmer function of the R package lme4 (Bates et al., 2015). We included lysozyme activity
196 as the response variable, occasion (1, 2 and 3) as a fixed effect and anemone ID as a random
197 intercept effect.

198 To test the effect of pre-fight immune response on fight outcome and injury state,
199 we conducted two generalised linear mixed effects models (GLMMs) with a binomial error
200 family (logit link function) using the glmer function of R package lme4 (Bates et al., 2015),

201 with pre-fight lysozyme-like activity as a fixed effect and fight outcome and injury state as
202 response variables, respectively. As relative size difference (RSD) between opponents has
203 previously been shown to impact contest dynamics in *A. equina* (Rudin and Briffa 2011), RSD
204 was calculated (as described in Rudin and Briffa 2011) and included as a covariate in the
205 models. To control for the effect of including both individuals from a single fight in the
206 model, fight ID was included as a random effect. Next, to compare lysozyme-like activity at
207 the three sampling occasions between fight outcomes (win, lose or draw) and injury states
208 (received injuries from opponent, received no injuries or received self-inflicted injuries) we
209 conducted two separate repeated measures linear mixed effects models (using the lmer
210 function of the lme4 package) with lysozyme activity as the response variable and either
211 fight outcome or injury state as a fixed effect. Occasion (pre-fight, 1 hr post-fight, 24 hrs
212 post-fight) was included as a fixed effect in both models, along with body size as a covariate
213 to account for any correlation between body size and lysozyme activity. Anemone ID and
214 fight ID were included as nested random effects. To allow us to investigate the effects of
215 receiving and inflicting injury on lysozyme activity, we conducted a further model in which
216 only individuals that had been involved in injurious fights were included. In order to explore
217 significant effects revealed by these models at a finer scale, we conducted post-hoc linear
218 contrasts using the R package lsmeans (Lenth, 2016).

219

220 **3. Results**

221 (a) Change in lysozyme-like activity over time

222 There was a significant effect of occasion on the lysozyme-like activity of control anemones
223 ($X^2_1 = 10.91$, $P = 0.004$). Post-hoc linear contrasts revealed that the lysozyme activity of
224 control individuals declined significantly between occasions 1 and 3 ($P = 0.003$).

225

226 (b) Lysozyme-like activity and fighting success

227 Pre-fight lysozyme-like activity had no effect on the eventual fight outcome ($X^2_1 = 0.032$, $P =$
228 0.98). However, comparison of lysozyme activity across fight outcomes and over time

229 revealed a significant two-way interaction between occasion and outcome ($X^2_1 = 10.53$, $P =$
230 0.03). Post-hoc analyses revealed that the lysozyme activity of losers declined significantly
231 across all three occasions (comparisons across occasions: - 1-2: $P = 0.007$; 2-3: $P = 0.03$; 1-3:
232 $P < 0.0001$) whilst the lysozyme activity of winners only differed significantly between
233 occasions 1 and 2 ($P = 0.004$), increasing again between measures 2 and 3. Individuals that
234 drew showed a similar decline in lysozyme activity to losers, but to a lesser extent, with
235 lysozyme activity only differing significantly between occasions 1 and 3 ($P = 0.04$), reflecting
236 the temporal pattern seen in control anemones (figure 2a).

237

238 (c) Lysozyme-like activity and injury state

239 There was a significant effect of pre-fight lysozyme-like activity on injury state ($X^2_1 = 5.32$, P
240 = 0.02) indicating that individuals that inflicted injuries during a fight expressed lower pre-
241 fight levels of lysozyme-like activity than those that did not use their weapons. When
242 including all fights (non-injurious and injurious) in the analysis, we found no interaction
243 between injury state and occasion on lysozyme activity ($X^2_1 = 7.53$, $P = 0.11$). However, when
244 we analysed only individuals involved in injurious fights, we found a significant two-way
245 interaction between occasion and injury state ($X^2_1 = 6.06$, $P = 0.048$). Using post-hoc
246 analyses we found that the lysozyme activity of individuals that received injuries declined
247 significantly between occasions 1 and 3 ($P = 0.004$), whereas for individuals that inflicted
248 injuries this post-fight decline was absent. Thus anemones that inflicted injuries during
249 fights had lower levels of lysozyme-like activity prior to fighting and maintained these pre-
250 fight levels after the fight (figure 2b).

251

252 4. Discussion

253 Immune response has previously been shown to correlate with fighting success, with
254 winners generally exhibiting higher immunity than losers (Dijkstra et al., 2011; Filby et al.,
255 2010; Koskimäki et al., 2004; Rantala and Kortet, 2004; Steiger et al., 2012). However, while
256 these previous studies demonstrate a clear link between immunity and winning, immunity
257 was only measured at one time point in each of these experiments (either before or after a

258 fight), and thus the causal direction of the relationship has so far been unclear. Does
259 immune state affect the ability to win fights, or does immunity change as a consequence of
260 fighting? The results of our study indicate that contrary to what has been suggested in
261 previous work, pre-fight immunity does not predict fighting success in the beadlet sea
262 anemone *Actinia equina*. In contrast, pre-fight immune state does appear to influence how
263 animals will fight, specifically the decision to use weapons during a contest. Furthermore
264 our results show that fighting itself can directly influence an individual's immune response
265 and moreover that the extent of this effect is dependent on both fight outcome and the
266 agonistic behaviours expressed during the fight.

267 We found no evidence to suggest that pre-fight lysozyme-like activity (referred to
268 hereafter as lysozyme activity) significantly affected fighting success in *A. equina*. However,
269 pre-fight lysozyme activity did appear to influence whether or not an individual used its
270 weapons (acrorhagi) to inflict damage on its opponent during the fight. As aggression is
271 linked to fighting success (in *A. equina* for example, individuals that inflict the most damage
272 are most likely to win fights – Rudin and Briffa, 2011), and previous studies have found a
273 positive correlation between fighting success and immunity (Dijkstra et al., 2011; Filby et al.,
274 2010; Koskimäki et al., 2004; Rantala and Kortet, 2004; Steiger et al., 2012), we would
275 expect to find a positive correlation between immunity and aggression, but in fact we found
276 the opposite. Individuals that attacked their opponent exhibited lower levels of pre-fight
277 lysozyme activity in comparison to individuals that did not. At first glance these results seem
278 counterintuitive, suggesting that individuals that chose to express costly agonistic
279 behaviours were in poorer condition than those that refrained from fighting injuriously.
280 However, pre-fight lysozyme levels of those that fought non-injurious are likely to reflect a
281 higher infection load such that these individuals were in a worse condition to begin with.
282 This explains why these individuals then chose not to engage in agonistic behaviours that
283 would lead to self-inflicted damage during the contest. If they are already fighting infections
284 through elevated lysozyme activity then they would have a reduced capacity to cope with
285 the additional costs of damaged acrorhagi.

286 Although pre-fight immune response did not predict fight outcome, fight outcome
287 itself had a significant effect on post-fight immune response. Individuals of all three fight
288 outcomes (loser, draw and winner) exhibited a significant reduction in lysozyme activity

289 within one hour of the fight, with individuals involved in fights with clear outcomes
290 (win/lose) exhibiting the highest degree of reduction. However, over the next 24 hours the
291 patterns of winners and losers began to diverge with the lysozyme activity of losers
292 continuing to decrease while the lysozyme activity of winners increased, albeit not to pre-
293 fight levels. This suggests that while both winners and losers suffer a similar reduction in
294 immunity immediately after a fight, winners are able to recover from this loss quicker. These
295 results also suggest that being involved in a fight with a clear outcome has a greater effect
296 on post-fight immune response compared to being in a fight that ends in a draw, possibly
297 indicating that 'clear cut' fights are more costly.

298 Post-fight change in immune response was also affected by injury state. Individuals
299 that were involved in non-injurious fights as well as those that received attacks exhibited a
300 severe reduction in lysozyme activity one hour after the fight, a pattern similar to that seen
301 in winners and losers. This reduction continued over the next 24 hours for individuals that
302 had received attacks but appeared to level off for those involved in non-injurious fights.
303 Individuals that used their weapons (acrorhagi) to inflict attacks on their opponents on the
304 other hand did not exhibit any significant change in lysozyme activity after the fight,
305 maintaining their pre-fight lysozyme level at both post-fight time points. This result suggests
306 that while individuals that inflicted attacks had lower lysozyme activity before the contest,
307 they were better able to maintain this stable immune state following a fight. Differences in
308 post-fight immune response between individuals that inflicted and individuals that received
309 attacks might be expected due to differences in injury accrual. In *A. equina*, individuals are
310 unable to inflict attacks on their opponent without also injuring themselves (self-inflicted
311 damage – Lane and Briffa, 2017), ripping holes in their acrorhagi in order to leave behind
312 necrotising peels. However, while both attackers and recipients incur injuries in *A. equina*,
313 the type of injury accrued is not the same. Attack recipients are damaged by the application
314 of nematocyst-filled 'peels' which cause localised necrosis while attackers are left with open
315 wounds in their acrorhagi. Arguably we might therefore expect the risk of infection to be
316 higher for attackers. For this reason, the ability to sustain pre-fight levels of immune
317 response after a fight may be beneficial to attackers. However, even if the injuries received
318 by attackers do not result in an increased susceptibility to infection, the necrosis caused by
319 peels will still require a heightened immune response to heal. Thus, the continued reduction

320 in post-fight immunity seen in recipients may result in an extended healing time for the
321 wounds incurred. However, it should be noted that we have measured only one aspect of
322 immune response here, while the immunity of *A. equina* as with all individuals is multi-
323 faceted (Stabili et al., 2015) and thus the whole picture is likely more complex.

324 Taken together, our findings indicate that immunity can influence strategic decisions
325 in a fight (to attack or not) and that fighting can significantly affect subsequent immunity in
326 *A. equina*. Furthermore, the severity of this effect depends both on fight outcome and on
327 the agonistic behaviours displayed during the fight. Fighting experience is known to have
328 substantial effects on an individual's subsequent fighting success. For example, individuals
329 that win a fight often go on to win subsequent fights, while those that lose keep on losing
330 (winner effect and loser effect, respectively – Hsu et al., 2006; Rutte et al., 2006). Although
331 the mechanism behind these experience effects has been debated, one suggestion was that
332 they derive from physiological shifts or constraints induced by the first fighting experience
333 (Hsu et al., 2006; Rutte et al., 2006). Our data show how differences in post-fight immunity,
334 and moreover in the ability to maintain/recover pre-fight immunity levels, could go on to
335 affect individuals' subsequent fights. If such immunological shifts are common, they may
336 provide a mechanistic explanation for both strategic decisions and the effects of fighting
337 experience on subsequent contests. Thus, immune status can be added to the factors
338 already known to influence the decision to fight, including resource value (Arnott and
339 Elwood, 2008) and morphological correlates of RHP (Lailvaux and Irschick, 2006).

340

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344 **Competing interests**

345 No competing interests declared.

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348

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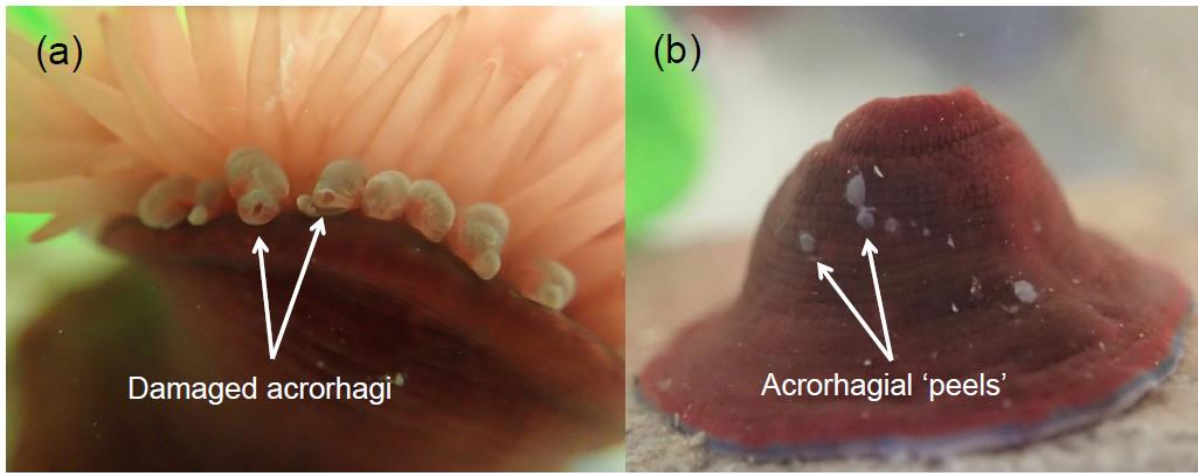
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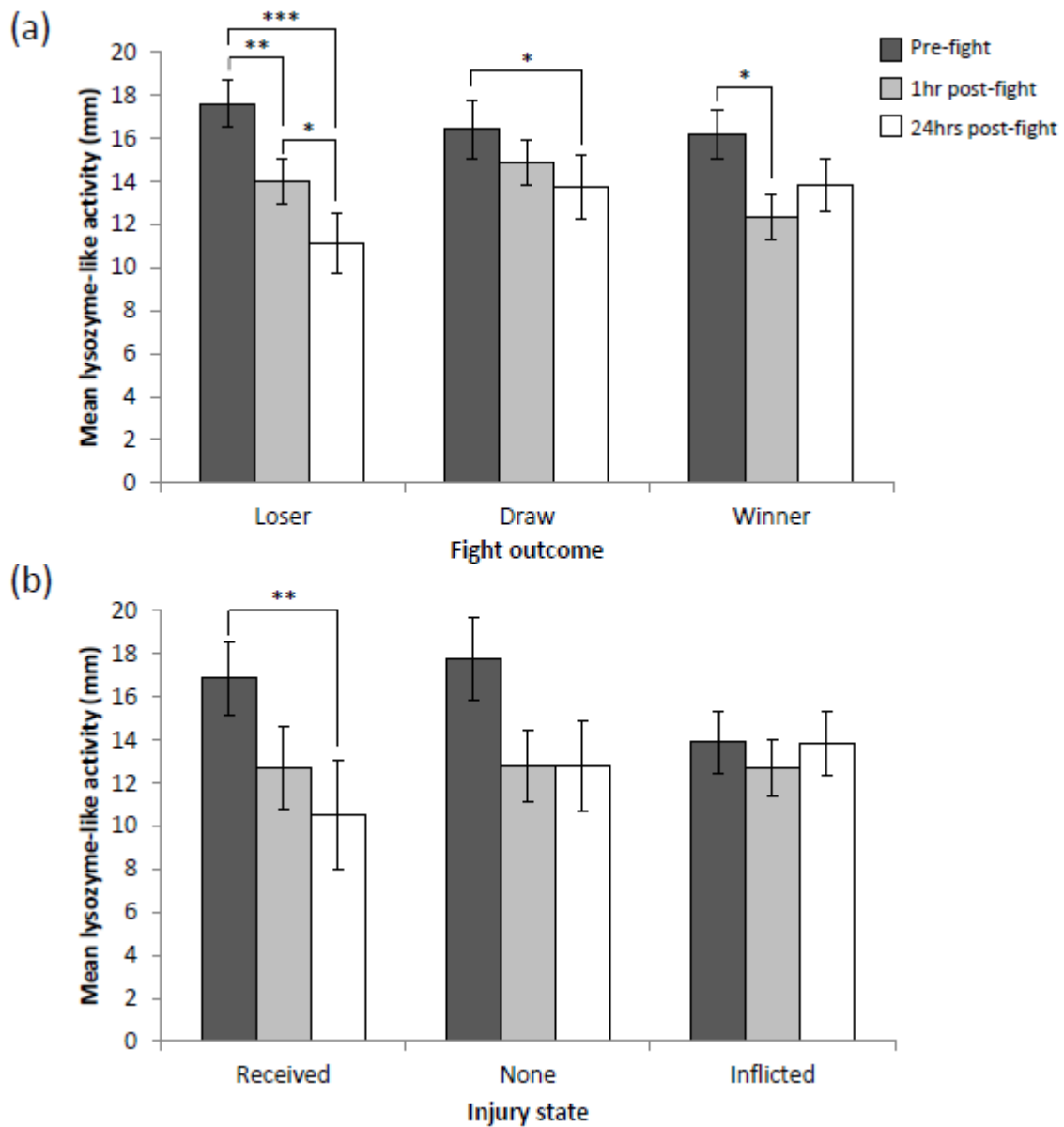
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471 **Figure 1** (a) Damage to the attacker – holes left in acrorhagi after attack and (b) damage to

472 the recipient – necrotising acrorhagial 'peels' left behind by attacker



473

474 **Figure 2** Average \pm SE lysozyme-like activity (mm) between (a) fight outcomes (n = loser - 25;
 475 draw - 22; winner - 26) and (b) injury states (n = received - 11; no peels - 50; inflicted - 12)
 476 across all three occasions. Asterisks indicate significant differences within groups across the
 477 three occasions as determined by least squares means ($*$ \leq 0.05; $**$ \leq 0.01; $***$ \leq 0.001).