

The effect of predation risk on post-copulatory sexual selection in the Japanese pygmy squid

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Running head: Relationship between predation risk and post-copulatory traits

1 **ABSTRACT**

2 Conspicuous male sexual traits (e.g. weapons for male–male competition and
3 displays for courting females) may attract predators. Under conditions of high predation
4 risk females typically become less choosy with respect to mates to reduce the time spent
5 on mate selection. However, post-copulatory sexual traits, such as sperm ejaculation for
6 sperm competition and sperm removal for cryptic female choice (CFC), may increase
7 with predation risk because they are more inconspicuous to predators. To examine this
8 hypothesis, we observed the reproductive behaviour in the Japanese pygmy squid,
9 *Idiosepius paradoxus*, in which the male attaches ejaculated spermatangia to the
10 female’s body and the female removes the spermatangia after copulation. Squid from
11 two populations (Ohmura and Oki), with low and high predation levels respectively,
12 were copulated in tanks under controlled presence/absence of predator conditions.
13 Among the Ohmura individuals spermatangia removal was suppressed in the presence
14 of a predator. Females may not be able to remove spermatangia effectively when facing
15 a predator because they feel threatened by the predator; as a result, more spermatangia
16 were retained during trials in which they were exposed to predators. In contrast for
17 squid from the Oki (high predation) population, which is exposed to a higher predation
18 risk, were not strongly affected by the predator presence. While the males ejaculated
19 more spermatangia, the females removed more of them. The effect of sexual conflict
20 may be greater than that of the predation risk in the pygmy squid. This suggests
21 adaptive differences in post-copulatory sexual selection traits linked to predation.

22 **Significance statement**

23 In general, the strength of pre-copulatory sexual selection decreases with
24 increasing predation risk because the sexual traits attract predators. However, post-
25 copulatory sexual traits which are often inconspicuous may not be influenced by
26 predation risk Post-copulatory behaviour of Japanese pygmy squid collected from two
27 populations experiencing different predation levels were investigated under
28 experimental predator presence/absence conditions. Among low predations individuals
29 sperm rejection by females (a post-copulatory trait) was suppressed in the presence of a
30 predator. In contrast individuals from the high predation population reported no change
31 in sperm rejection. As with pre-copulatory sexual selection, post-copulatory female
32 choice was suppressed by predator presence among individuals from a low-predation.
33 However, post-copulatory female choice was not affected by predator presence among
34 individuals from the high-predation population. This may indicate predation driven
35 adaptive differences and plastic responses in post-copulatory traits.

36 **Keywords**

37 Cryptic female choice, sperm competition, predation risk, sexual conflict,
38 cephalopod, *Idiosepius paradoxus*

39

40 INTRODUCTION

41 Across many species males often exhibit courtship behaviours involving
42 conspicuous sexual traits, such as dances, songs, and the display of body colours, which
43 allow them to obtain more mates than males with more inconspicuous traits (Andersson
44 1994). However, such courtship and copulatory behaviours are conspicuous not only to
45 females but also to predators meaning that predation risk may also influence female
46 mate choice (Lima and Dill, 1990; Magnhagen, 1991). In many cases, females are less
47 choosy when they face a higher predation risk (Forsgren 1992; Godin and Briggs 1996;
48 Karino et al. 2000; Bierbach et al. 2011) as exhibited through less extensive searching
49 for a mate (Hedrick and Dill 1993; Willis et al. 2012) and avoidance of males showing
50 conspicuous signals (Johnson and Basolo, 2003). Although fiddler crabs, *Uca beebei*,
51 are an exception in which female selectivity increases (Kim et al 2009) with increasing
52 predation risk, the strength of sexual selection generally decreases, male sexual traits
53 become less conspicuous, and females select their mates less carefully (Kelly and Godin
54 2001).

55 Predation risk may exert a different influence on post-copulatory sexual traits.
56 With the exception of for mate-guarding after copulation, most post-copulatory sexual
57 traits are probably inconspicuous to predators. For example, males invest in sperm traits,
58 such as ejaculation volume and sperm morphology, during sperm competition (Stockley
59 et al. 1997; Birkhead and Møller, 1998) but these traits do not attract predators. Females
60 are also thought to select sperm ejaculated by a preferred male; this is referred to as
61 cryptic female choice (CFC) (Thornhill 1983; Eberhard 1996). This selection process is
62 inconspicuous because females can choose mates after copulation without the presence
63 of males. By extension it may be predicted that for a given system (species/population)

64 post-copulatory sexual selection may be stronger under high predation conditions
65 compared to low predation conditions.

66 Male Japanese pygmy squid, *Idiosepius paradoxus*, do not use agonistic or
67 elaborate courtship behaviour to acquire mates (Kasugai 2000). Rather, they grab
68 females for copulation without any display and pass spermatophores via the
69 hectocotylus, which is a specialised arm used for copulation (Sato et al. 2013a).
70 Spermatangia are discharged from spermatophores when they are pulled from the funnel,
71 which is a muscular structure like the siphon used for water jetting and respiration, and
72 attached to the base of the female arm by males (Sato et al. 2014a). Spermatozoa are
73 then released from the tip of the spermatangium and are activated on contact with
74 seawater, transferred to the seminal receptacle by swimming actively and stored there
75 (Sato et al. 2010, 2014a). Paternity analysis of egg masses collected in the field revealed
76 each studied female to have copulated with about 10 males, indicating a highly
77 promiscuous mating system (Sato 2017). The sperm ejaculated by each male form a
78 mixture in the seminal receptacle and the relative sperm volume remaining in the
79 female's body is correlated with fertilisation success (Sato et al. 2017). Females
80 elongate their mouth (buccal mass) and choose successful mates cryptically post-
81 copulation by picking up and removing spermatangia before completing sperm transfer
82 (Sato et al. 2013b, 2014b). The volume of removed spermatangia is dependent on not
83 only buccal mass elongation but also copulation duration and body size, and females
84 prefer smaller males, but their preferences for long or short copulation duration vary
85 from year to year (Sato et al. 2014b and 2017).

86 Given the importance of post-copulatory sexual selection in Japanese pygmy
87 squid they represent an excellent model to study the relationship between such sexual

88 selection dynamics (i.e. sperm competition and CFC) and predation risk. Individuals
89 may invest more towards post-copulatory sexual traits when they are exposed to high
90 predation risk because post-copulatory sexual traits are more inconspicuous to predators
91 than pre-copulatory sexual traits. Accordingly we tested if ejaculated sperm volume
92 would increase when male pygmy squid were exposed to increased predation risk. We
93 also tested if female mate choice after copulation would be reinforced by predation risk,
94 and that they would be choosier when selecting mates. To examine this hypothesis, we
95 observed the reproductive behaviour of the Japanese pygmy squid, with and without
96 exposure to a predator. To assess the roles of behavioural plasticity and/or adaptation
97 underpinning the relationships between predation pressure and post-copulatory sexual
98 selection the experiments were performed for individuals sourced from two natural
99 populations subject to high and low predations pressures.

100 **METHODS**

101 *Census of fish fauna and evaluation of predation risk*

102 The fish fauna was investigated using line censuses of Ohmura Bay and Oki
103 Island to evaluate the degree of predation risk for two populations of pygmy squid
104 (Supplemental Material 1). In Ohmura Bay, fewer fish were observed than at Oki Island,
105 and 90% of the fish were a small goby (*Sagamia geneionema*), which feed mainly on
106 gammaridean amphipods (Sano 1998). At Oki Island, the presence of potential predators
107 on Japanese pygmy squid, such as rockfish, sculpin, and greenling, was noted. Two
108 rockfishes (*Sebastes flavidus* and *S. alutus*) living in the north-eastern Pacific Ocean
109 have been reported to feed on cephalopods (Brodeur and Pearcy 1984), and we have
110 occasionally observed predatory attacks by rockfish (unidentified *Sebastes*) on pygmy

111 squid (N. Sato, personal observation). *Pseudoblennius percoides* (sculpin) is a major
112 predator of juvenile and young fish in seagrass beds (Horinouchi and Sano 2000), and
113 *Hexagrammos otakii* (greenling) feeds on fish, crustaceans, and polychaetes (Kwak et
114 al. 2005). Accordingly, the predation risk to pygmy squid is potentially higher in the Oki
115 population than in the Ohmura population.

116 *Aquarium experiment*

117 Japanese pygmy squids were collected from seagrass beds in nearshore waters at
118 two locations, Ohmura Bay (32°52'N 129°58'E) and Oki Island (36°10'N 133°16'E),
119 Japan, between April and May 2014 using a small drag net or a hand net and SCUBA
120 diving. The specimens were collected in the reproductive season and all squid were
121 mature. The squid were distinguished by sex based on the presence of a hectocotylus
122 and maintained separately in two closed-circulation aquaria (60 × 30 × 35 cm). Ten
123 plastic plates (25 × 0.6 cm) were placed on the sandy bottom of the aquaria as adhering
124 substrates, because the squid have an adhesive organ in the dorsal mantle and attach to
125 the substrates, most of the time (Sasaki 1923). The squid were fed live mysid shrimps
126 (*Neomysis intermedia*) or prawns (*Palaemon* sp.) *ad libitum*, twice daily. Predatory fish
127 were collected from seagrass beds in nearshore waters at two locations, Sado Island
128 (38°04'N 138°14'E) and Oki Island, between April and May 2014 using a hand net and
129 SCUBA diving. As predators, *Pseudoblennius cottoides* (total body length (TL) ±
130 standard deviation (SD) = 84.65 ± 17.12 mm, n = 8) and *P. percoides* (72.41 ± 6.80
131 mm, n = 11) were used because these species prey on Japanese pygmy squid in the field
132 (N. Sato, personal observation). For the experiments using the Ohmura population, six
133 *P. cottoides* collected around Sado Island were used because few were found in Ohmura

134 Bay. All *P. percoides* were used only for the Oki population. These two species were not
135 classified before the experiments because these two species are very similar in
136 behaviour and morphology. Therefore, we judged that their likely effects on the squid
137 are expected to be similar, and used these predators as a single category. These sculpins
138 were maintained in closed-circulation aquaria (40 × 25 × 30 cm) at an ambient
139 temperature of 20–25°C under a 12-h light/12-h dark cycle. The sculpins were fed live
140 prawns (*Palaemon* sp.) *ad libitum* daily.

141 The aquaria (45 × 20 × 30 cm) used for the experiments were partitioned, by
142 black and transparent plastic boards, into three areas (male, female, and predator areas),
143 each measuring 15 × 20 × 30 cm, and a female, male, and sculpin (or no sculpin) was
144 introduced into each area (Fig.1). Three plastic plates (30 × 0.6 cm) were placed on the
145 sandy bottom of each of the two areas for use by squid as perches. To acclimatise to the
146 aquarium conditions, the animals were introduced 30 min before the trials began. We
147 then gently removed the black partitions between the male and female areas, and
148 between the female and predator areas. This allowed the male to access the female
149 while exposed to a threat of predation without any risk of predator attack. Behaviour
150 was recorded using a digital video camera (HDR-XR 520V; Sony, Tokyo, Japan). The
151 male was removed from the aquarium soon after copulation and we counted the
152 spermatangia and observed the sperm-removing behaviour of the female. Video
153 recording was continued for 30 min after copulation by which time a female has usually
154 ceased engaging in sperm-removing behaviour (Sato et al. 2013b). If no copulation
155 occurred within 30 min the trial was stopped. A copulatory trial was conducted twice
156 for each individual to see whether the behaviour changed with the presence of a
157 predator. Thirty-seven pairs (22 from Ohmura and 15 from Oki) were allocated for the

158 experiment with a predator in the first trial and 51 pairs (36 from Ohmura and 15 from
159 Oki) were first allocated for the experiment without a predator (Fig 2). We conducted
160 113 (58 without a predator) and 60 (30 without a predator) experiments on the Ohmura
161 and Oki populations, respectively. Small numbers of the pygmy squid were collected
162 from Oki, so the sample size was smaller. It was impossible to evaluate trials blindly as
163 the presence of a predator was visible in the video. The same sculpins were used
164 repeatedly. All squid were anaesthetised with 1% ethanol and fixed in 10%
165 formaldehyde seawater after the experiment. The dorsal mantle length (DML; body
166 size) of the fixed squid samples was measured to 0.01 mm. Body weight and testis
167 weight were also measured to calculate the gonad somatic index (GSI; [testis weight /
168 body weight] × 100) to 0.0001 g. Sculpins collected from Sado Island were
169 anaesthetised by immersion in 600 ppm MS-222 and fixed in 10% formaldehyde
170 seawater after measuring the TL for another study, while sculpins collected from Oki
171 Island were released at the collection site after measuring their TL.

172 We noted any display behaviour by the squid to assess whether predation risk
173 influenced reproductive behaviour. These included attempted copulation, female
174 resistance (*i.e.* whether females showed resistance to grabbing during copulation),
175 copulation duration (defined as the time from when the male began to grasp the female
176 to when he left the female), and buccal mass elongation duration (indicative of female
177 effort expended on sperm removal) (defined as the time from when the female began to
178 elongate the buccal mass to when she finished elongation). We also counted the
179 numbers of spermatangia that the male attached to the female (ejaculated spermatangia),
180 that the female removed (removed spermatangia), and that the female kept on her body

181 (remaining spermatangia). The method of counting spermatangia followed Sato et al.
182 (2014b, 2017).

183 *Statistical analysis*

184 We analysed the factors that affected squid behaviours (inking, copulation,
185 female resistance, copulation duration, and buccal-mass-elongation duration), as well as
186 the numbers of ejaculated, removed, and remaining spermatangia, using generalised
187 linear mixed models (GLMMs) in each population (Ohmura or Oki) (Crawley 2005;
188 Bolker et al. 2009). In all analyses, a full model was constructed to test the main effects
189 of the following explanatory variables: male body size, female body size, copulation
190 duration (except for the inking, copulation, and copulation duration models), predator
191 presence, and trial order (first or second trial). Interactions among copulation duration
192 and predator presence, male body size and predator presence, and female body size and
193 predator presence were also investigated, because when females express a preference
194 for any male traits, those preferences may change when there is a predation risk. We
195 used squid identification as a random factor because each of the males and females was
196 used twice in this study. A binomial error distribution and logit link function were
197 applied in the inking, copulation, and female-resistance models. A gamma distribution
198 and log link function were applied in the models of copulation duration and buccal mass
199 elongation duration. A Poisson error distribution and log link function were applied in
200 the models of spermatangia ejaculated, removed, and retained. We subsequently
201 compared the Akaike information criterion (AIC) among all models. To verify the
202 variables selected by the AIC the significance of each explanatory variable was assessed

203 using the likelihood ratio test (LRT). We used R software (ver. 3.4.2; R Development
204 Core Team 2017) for the analyses.

205 **RESULTS**

206 The body sizes of both males and females in the Ohmura Bay population ($n =$
207 64, mean \pm SD DML = 8.44 ± 1.05 for males; $n = 58$, 11.55 ± 1.48 for females) were
208 significantly smaller than those of the Oki Island population ($n = 30$, 10.67 ± 1.10 for
209 males; $n = 30$, 15.10 ± 1.61 for females; ANOVA: $F = 3.94$, d.f. = 1, $p = 0.001$ for
210 males; $F = 3.95$, d.f. = 1, $p = 0.001$ for females). Of 174 (86 without a predator)
211 experiments, four experiments were removed from the analysis because the female
212 attacked the male during the observation. Ultimately, 109 (56 without a predator) and 60
213 (30 without a predator) experiments were analysed for the Ohmura and Oki populations,
214 respectively (Table 1). The GSI of the Ohmura Bay population ($n = 60$, 6.20 ± 1.11) was
215 significantly greater than that of the Oki Island population ($n = 30$, 5.04 ± 0.46 ;
216 ANOVA: $F = 20.88$, d.f. = 1, $p = 0.0001$).

217 Inking behaviour was observed in 40 trials: by males in 27 trials and by females
218 in 18 trials (both sexes in five trials). Predator was included in the best fitted models for
219 Ohmura (Table 2) and the factor was a significant variable in the best model in the LRT
220 (Table 3), which suggested that squid released ink more frequently when a predator was
221 present. In the Oki population, predator, male size, and the interaction between male
222 size and predator (Table 2) were significant according to the LRT (Table 3). Squid
223 released ink more frequently when a predator was present and when the male was larger,
224 but the effect of male size decreased in the presence of predators.

225 Males grabbed females for copulation in 79 of 169 trials. Female size was
226 included in the three minimum AIC models for both populations (Table 2). In the best
227 model, this factor was significant only for the Ohmura population in the LRT (Table 3),
228 and copulation was observed more frequently when the female was smaller. Across the
229 79 trials, eight males failed to copulate, or the females escaped before the males had
230 completed sperm transfer.

231 Of the 71 copulation successes, copulation resistance was shown by females in
232 26 cases. When female resistance occurred during copulation, female body size and
233 copulation duration were included in the three and four minimum AIC models for
234 Ohmura and Oki, respectively (Table 2), but copulation duration did not have a
235 significant effect on squid behaviour in the LRT (Table 3). Females showed greater
236 resistance to copulation as body size decreased in the Ohmura population.

237 Female body size, male body size, predator, trial order, and the interactions
238 between female size and predator and between male size and predator were included in
239 the best model of copulation duration for the Ohmura population; the AIC of this model
240 was over 2 points less than that of the next-best model (Table 2). All variables were
241 significant in the LRT (Table 3). On the other hand, predator and trial order were
242 included in the best model for the Oki population (Table 2). The effects of both factors
243 were significant in the LRT, whereby copulation duration increased with predator
244 presence, but female and male body size and their interactions weakened the effect of
245 predation in the Ohmura population (Table 3).

246 The buccal mass elongation duration was not related to any variable because no
247 variables were included in the lowest AIC model, and the variables of all five minimum
248 AIC models differed (Table 2).

249 In the best model of the number of ejaculated spermatophores, male body size,
250 copulation duration, predator, and the interactions between male size and predator and
251 between copulation duration and predator were included in the two minimum AIC
252 models for the Ohmura population (Table 2). Except for copulation duration, these
253 variables were significant in the LRT (Table 3), which means that more spermatangia
254 were ejaculated when the male was smaller and predators were absent, but the effect of
255 predation decreased with male size and copulation duration. Therefore, the average
256 number estimated by the best model was similar between predator absence and presence
257 (Fig. 2). Regarding the Oki population, only trial order was included in the lowest AIC
258 model (Tables 2 and 3), but different or no variables were included in each of the five
259 minimum AIC models.

260 Male size and predator were included in the four minimum AIC models of
261 removed spermatangia for the Ohmura population (Table 2). In the best model, both
262 variables were statistically significant according to the LRT (Table 3). This suggests
263 that females removed more spermatangia when they copulated with smaller males and a
264 predator was present for the Ohmura population (Fig. 2). Although predator was
265 included in the four minimum AIC models for the Oki population, this variable was not
266 significant in the LRT ($p = 0.056$) (Tables 2 and 3).

267 In the model selection for the number of remaining spermatangia, only predator
268 presence was included in the five lowest models in Ohmura population (Table 2), and
269 this variable was significant in the LRT (Table 3). The females retained more
270 spermatangia after copulation when a predator was present in the Ohmura population
271 (Table 3, Fig. 2).

272 **DISCUSSION**

273 The squid they frequently discharged ink in the trials in which a predator was
274 present indicating a perception of threat. Predator presence did not influence most
275 copulatory behaviours, such as copulation occurrence, female resistance, and buccal
276 mass elongation duration. However, the number of spermatangia ejaculated decreased in
277 the presence of a predator in the best model of the Ohmura (high predation) population.
278 Although the interaction between predator presence and male traits did not influence the
279 number of spermatangia removed, female squid removed fewer spermatangia after
280 copulation when exposed to a predator and the spermatangia remaining also changed.
281 These results were opposite to our hypothesis that males ejaculate more sperm and
282 females become choosier and remove more sperm when they are exposed to a high
283 predation risk. In the eriophyoid mite, *Aculops allotrichus*, in which males deposit
284 spermatophores on a substrate while females pick up sperm regardless of the presence
285 of males, the males deposited fewer spermatophores when the predation risk was high
286 (Michalska 2016). Reported results for precopulatory mate choice are similar to our
287 results (*e.g.* Forsgren 1992; Johnson and Basolo, 2003; Willis et al. 2012). The pygmy
288 squid may suppress behaviours related to spermatangia ejaculation and removal.
289 Immobility enhances the crypsis of prey animals against visually hunting predators
290 (Nishiumi and Mori 2015). However, we did not find a significant relationship between
291 the presence of a predator and buccal mass elongation duration, which indicates that
292 males and females did not dedicate less time to spermatangia removal. Although
293 copulation duration increased with the presence of a predator, this effect was
294 counteracted by other factors such as squid body size and interactions. Consequently,
295 female squid might not be able to remove spermatangia as effectively as usual; as a

296 result, more spermatangia were retained during trials in which they were exposed to
297 predators.

298 The effect of predation risk was remarkable individuals from the Ohmura
299 population where the pygmy squid is exposed to a lower predation risk. In contrast,
300 individuals from the Oki population reported negligible influence by the presence of a
301 predator. This overall pattern could reflect a combination of the Ohmura individuals
302 being highly sensitive to predator presence on account of limited previous experience,
303 and/or the greater experience and potential of local adaptation of Oki individuals to
304 predator presence. Some studies have reported that predation risk influences the
305 intensity of sperm competition. For example, predation decreases sperm competition in
306 the agile frog, *Rana dalmatina* (Lodé et al. 2004). The male frog is selectively predated
307 by polecats and the sex ratio has less of a male bias in a high-predation-risk
308 environment. On the other hand, predation was responsible for a higher level of sperm
309 competition in the guppy, *Poecilia reticulata* (Neff et al. 2008; Elgee et al. 2010). Elgee
310 et al. (2010) suggested that female guppies become unresponsive to courtship in the
311 presence of predation, and males change their copulatory behaviour to a “sneaky” type
312 of copulation without courtship, which increases sperm competition. In our study,
313 although the Oki population, which ejaculated and removed more spermatangia than the
314 Ohmura population, may be adapted to a high-predation risk environment, its GSI was
315 lower. The many ejaculations by males may be due to their larger body size and females
316 may remove more spermatangia in response to the greater number of ejaculations.
317 Therefore, predation risk would not reinforce sperm competition in the pygmy squid.

318 Predation risk had no influence on pre and peri-copulatory behaviours such as
319 attempted copulation, female resistance to attempted copulation, the duration of

320 copulation, and buccal mass elongation. The Japanese pygmy squid is likely already
321 exposed to a high predation risk in its natural environment. This species has the smallest
322 body size among all cephalopods, but they are solitary and do not have any traits that
323 protect their bodies from predatory attacks, such as scales or a shell (Lu and Dunning,
324 1998). Therefore, their reproductive behaviour may already be adapted to predation and
325 they may not need to adjust their behaviour much, even if a predator is present. Their
326 copulation is quite simple in cephalopods, is completed quickly, and the females
327 perform CFC. Many coastal squid, such as loliginid squid and cuttlefish, perform
328 remarkable displays before copulation, while copulation continues for a long time in the
329 octopus (Hanlon and Messenger 1998). However, copulation duration is not affected by
330 the presence of predators in some animals, including squid (Maier et al. 2000; Taylor et
331 al. 2005; Franklin et al. 2014). Therefore, it is unlikely that predation influences the pre
332 and peri-copulatory behaviour of the pygmy squid.

333 The males ejaculated more spermatangia when they were smaller, but the
334 females removed more spermatangia ejaculated by smaller males. Moreover, while Oki
335 males transferred more spermatangia, Oki females removed more spermatangia. These
336 results show that there is sexual conflict between sperm transfer by males and sperm
337 acceptance by females. Arnqvist (2005) showed many examples of sexual conflict that
338 occurred after mating (e.g. males ejaculated sperm with seminal toxins and females
339 showed decreased sensitivity to the substances as a counter adaptation). In our results,
340 we do not know the cost for females or why females remove more spermatangia, but
341 females may avoid sperm storage bias for a certain male as genetic “bet-hedging”. The
342 egg batch of Japanese pygmy squid collected in the field was sired by many males and
343 the fertilisation success was not skewed (Sato 2017). Genetic bet-hedging is an

344 important factor to secure genetic diversity and offspring compatibility (Jennions and
345 Petrie 2000; Garcia-Gonzalez et al. 2015). Reproductive success in the pygmy squid
346 may be influenced by obtaining sperm from various males.

347 In conclusion, individuals from a high predation population did not change
348 ejaculation and removal behaviours with predator presence indicating potential
349 adaptation to high-predation conditions. Alternatively, for individuals from low
350 predation copulation, post-copulatory traits of ejaculation and removal of spermatangia
351 were suppressed by predation risk indicating a plastic (non-genetic) response. Overall
352 the results indicate that predation does effect post-copulatory sexual selection but such
353 effects showed by a balance between local adaptation and plasticity. Including
354 copulatory behaviour, the post-copulatory traits of the Japanese pygmy squid may not
355 be influenced greatly by predation risk. Does this phenomenon occur in other animals?
356 Predation might not have a large impact on reproductive behaviour in promiscuous
357 species that do not perform copulatory displays. Our results may be typical for
358 promiscuous species. However, many studies have reported a trade-off between traits
359 for pre- versus post-copulatory sexual selection (Simmons and Emlen 2006; Pitcher et
360 al. 2009; Yamane et al. 2010). In the dung beetle *Onthophagus sagittarius*, hornless
361 males have larger testes than horned males. In salmon (*Oncorhynchus kisutch*), sperm
362 swimming velocities are lower in males that invest more in secondary sexual coloration
363 (Pitcher et al. 2009). Males exposed to predation risk would likely invest in traits related
364 to post-copulatory sexual selection and suppress the expression of traits for
365 precopulatory sexual selection. It is necessary to study the impact of predation on post-
366 copulatory sexual selection in animals with different mating systems. However,
367 spermatangia removal was strongly influenced by ejaculation volume. In the evolution

368 of post-copulatory sexual traits, the effect of sexual conflict would be greater than that
369 of the predation risk.

370 Data availability

371 The dataset supporting this manuscript is available as electronic supplementary material.

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380

381 Ethical approval

382 This study was approved by the Animal Care and Use Committee of Faculty of
383 Fisheries, Nagasaki University (permission no. NF-0014), in accordance with the
384 Guidelines for Animal Experimentation of Faculty of Fisheries (fish, amphibians, and
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386 University.

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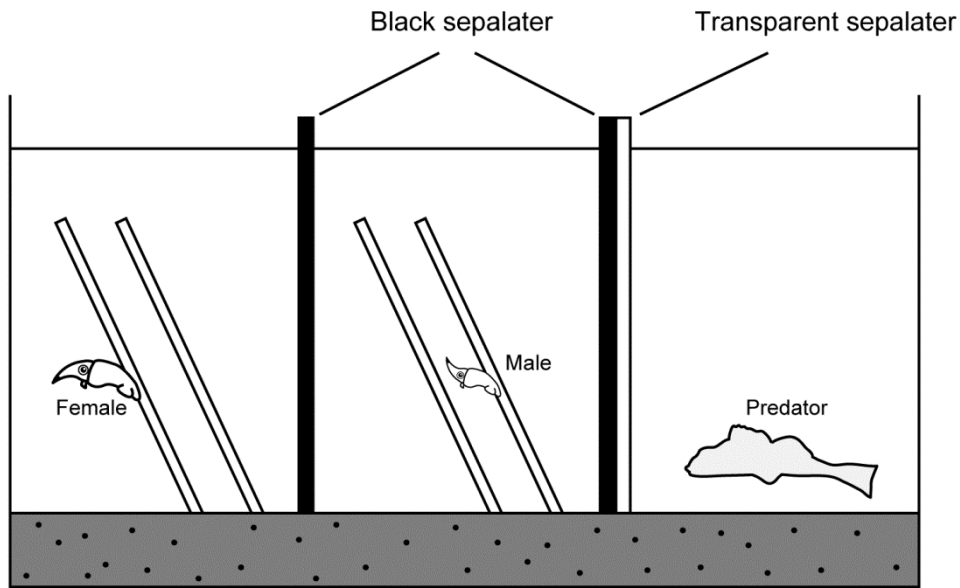
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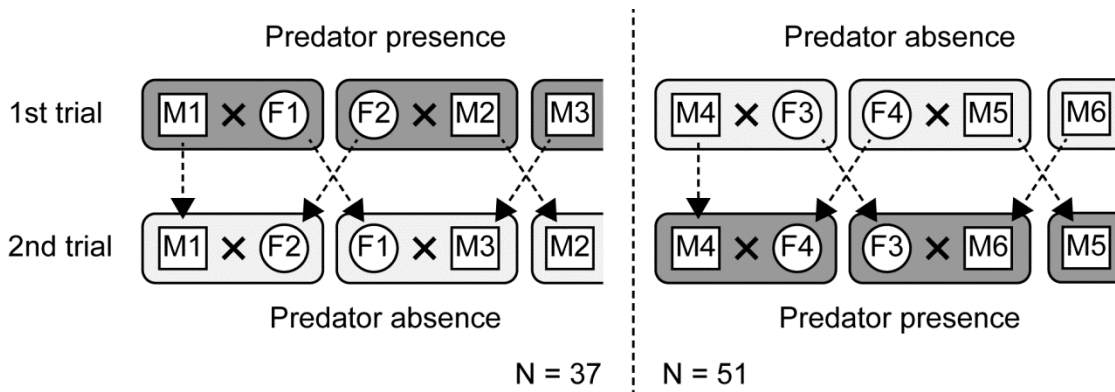
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492 **Fig. 1**

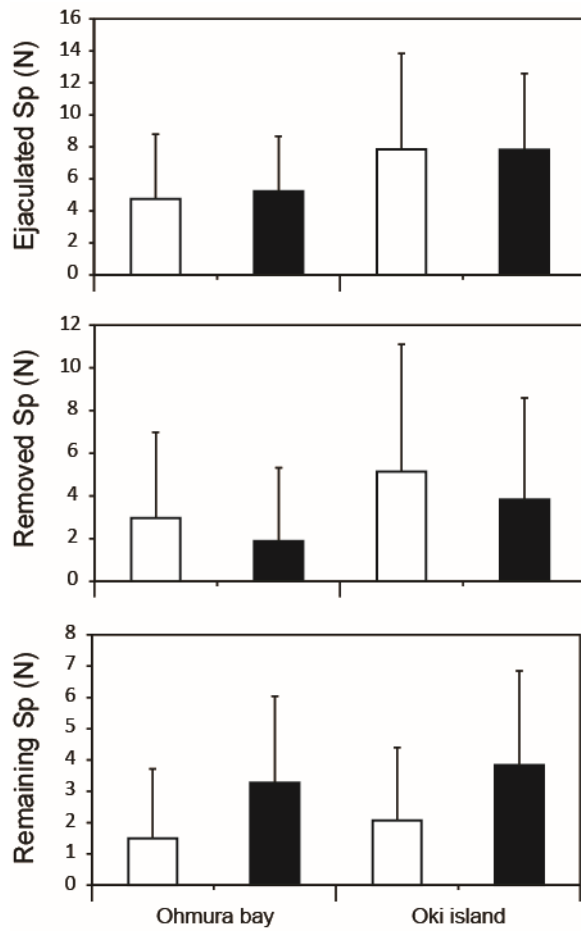
493 The aquarium used for the experiments. Three plastic plates were placed in each squid-
 494 containing area to serve as perches. During a trial, a predator was or was not placed in
 495 the predator compartment. All trials started after removing the black separators.



496

497 **Fig. 2**

498 Diagram showing experimental design. Grey and white boxes indicate each trial. Left
 499 and right sides indicate the initial presence and absence of a predator, respectively.



500 **Fig. 3**

501 The average numbers of ejaculated, removed, and remaining spermatangia between
 502 presence (solid graph) and absence (open graph) of a predator in the two populations.

503 The vertical bars denote the standard deviation. Sp: spermatangia

504

Table 1. Summary of the experiment data				
	Ohmura		Oki	
	absense	presense	absense	presense
No of experiments	56	53	30	30
Inking	2	21	5	12
Copulation	27	25	15	12
Female Resist	11	9	2	4
Copulation D	8.02 ± 6.7	6.70 ± 3.8	12.37 ± 10.3	11.77 ± 8.7
Ejaculate Sp Num	4.64 ± 4.0	4.85 ± 4.0	7.86 ± 6.0	7.83 ± 4.8
BM Elongation D	620.0 ± 285.6	530.35 ± 542.6	1012.0 ± 382.0	946.50 ± 557.0
Rejected Sp Num	2.96 ± 2.9	1.8 ± 2.4	5.50 ± 4.2	3.83 ± 3.3
Remaining Sp Num	1.44 ± 2.2	2.95 ± 2.8	2.07 ± 2.3	3.83 ± 3.0
BM, buccal mass; D, Duration; Num, number; Sp, spermatangia				

505

506

Table 2. The best five AIC models						
Response variable	Ohmura			Okii		
	Explanatory variables	AIC	ΔAIC	Explanatory variables	AIC	ΔAIC
Inking	Pre + Ord	95.9	—	Msize + Pre + Ord + Msize:Pre	68.9	—
	Pre	96.4	0.58	Msize + Pre + Msize:Pre	69.7	0.82
	Fsize + Pre + Ord	97.1	1.23	Fsize + Msize + Pre + Ord + Msize:Pre	70.6	1.67
	Msize + Pre + Ord	97.8	1.99	Fsize + Msize + Pre + Msize:Pre	71.1	2.23
	Fsize + Pre	98.1	2.25	Fsize + Msize + Pre + Ord + Fsize:Pre + Msize:Pre	72.1	3.17
Copulation	Fsize	150.0	—	Fsize	84.2	—
	Fsize + Msize	151.6	1.62	Fsize + Pre	85.3	1.07
	Fsize + Ord	151.8	1.80	Fsize + Ord	85.3	1.07
	Fsize + Pre	152.0	2.00	None	85.4	1.22
	Fsize + Pre + Fsize:Pre	153.1	3.08	Fsize + Pre + Fsize:Pre	85.7	1.47
Female Resist	Fsize + Pre + Fsize:Pre	53.0	—	Fsize + Msize + CopD	33.2	—
	Fsize	54.5	1.46	CopD	33.4	0.24
	Fsize + CopD + Pre + Fsize:Pre + CopD:Pre	54.5	1.52	CopD + Pre	33.7	0.49
	Fsize + Msize + CopD + Pre + Ord + Msize:Pre + CopD:Pre	55.4	2.41	Msize + CopD	33.7	0.53
	Fsize + Msize + CopD + Pre + Ord + Fsize:Pre + Msize:Pre + CopD:Pre	55.9	2.85	None	34.1	0.91
Copulation D	Fsize + Msize + Pre + Fsize:Pre + Msize:Pre	-582.7	—	Pre + Ord	-319.0	—
	Msize + Pre + Ord	-580.1	2.64	Fsize + Msize + Ord	-313.7	5.25
	Fsize + Msize + Pre	-579.0	3.73	Fsize + Ord	-302.3	16.65
	Pre	-565.4	17.31	Fsize + Msize + Pre + Ord + Fsize:Pre	-301.9	17.14
	Msize + Pre + Ord + Msize:Pre	-561.9	20.82	Fsize + Pre + Ord	-296.2	22.79
Ejaculate Sp Num	Msize + CopD + Pre + Msize:Pre + CopD:Pre	236.1	—	Ord	167.3	—
	Msize + CopD + Pre + Ord + Msize:Pre + CopD:Pre	236.3	0.25	Msize + Ord	168.0	0.66
	Msize + Ord	237.4	1.32	CopD + Ord	168.2	0.90
	Fsize + Msize + CopD + Pre + Msize:Pre + CopD:Pre	238.0	1.94	CopD	168.6	1.28
	Msize + CopD + Ord	238.3	2.19	Fsize + Ord	168.7	1.44
BM Elongation D	None	611.2	—	None	420.3	—
	CopD	612.9	1.70	Fsize	421.4	1.06
	Msize	613.0	1.80	Msize	421.6	1.29
	Pre	613.1	1.91	Ord	422.1	1.81
	Fsize	613.2	1.94	CopD	422.2	1.88
Rejected Sp Num	Msize + Pre	190.2	—	Pre	148.8	—
	Msize + Pre + Ord	191.6	1.41	CopD + Pre	149.8	1.04
	Msize + Pre + Msize:Pre	191.9	1.66	CopD + Pre + CopD:Pre	149.9	1.08
	Fsize + Msize + Pre	192.0	1.84	Pre + Ord	150.1	1.33
	Msize + CopD + Pre	192.2	2.00	Msize + Pre	150.4	1.63
Remaining Sp Num	CopD + Pre + Ord	178.2	—	Fsize + Pre + Ord	122.2	—
	Pre + Ord	178.7	0.49	Fsize + CopD + Pre + CopD:Pre	122.4	0.16
	Msize + CopD + Pre + Msize:Pre + CopD:Pre	179.6	1.40	Fsize + Ord	123.0	0.80
	CopD + Pre	179.7	1.47	Fsize + CopD + Pre + Ord	123.1	0.84
	Msize + CopD + Pre + Ord + Msize:Pre + CopD:Pre	179.7	1.50	CopD + Pre + CopD:Pre	123.1	0.85

Bold type indicates AIC models within 2 at ΔAIC from the best AIC model.

BM, buccal mass; D, Duration; Num, number; Sp, spermatangia; Fsize, Female body size; Msize, Male body size; Cop D, Copulation Duration; Pop, Population; Pre, Predation; Ord, Order

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Table 3. Estimates of the best model based on the AIC in each GLMM analysis										
Population	Response variable	(Intercept)	Fsize	Msize	Cop D	Pre (presence)	Ord	Fsize x Pre	Msize x Pre	Cop D x Pre
Ohmura	Inking	-2.19(0.98)	—	—		3.13(0.80)***	-0.87(0.55)	—	—	—
	Copulation	4.95(2.09)	-0.44(0.18)**	—		—	—	—	—	—
	Female Resist	96.68(38.58)	-10.01(3.93)**	—	0.77(0.50)	33.12(14.52)*	—	—	—	-3.93(1.63)**
	Copulation D	1.86(1.95)	-0.01(0.13)***	-0.02(0.16)***		3.28(2.10)***	—	-0.08(0.15)***	-0.26(0.17)***	—
	Ejaculate Sp Num	4.90(1.45)	—	-0.43(0.17)*	0.01(0.02)	-6.63(2.12)**	—	—	0.65(0.23)*	0.16(0.05)**
	Rejected Sp Num	5.78(1.79)	—	-0.61(0.21)**	—	-0.68(0.31)*	—	—	—	—
	Remaining Sp Num	-1.46(0.56)	—	—	0.06(0.03)	0.67(0.31)*	0.57(0.31)	—	—	—
Oki	Inking	-21.51(9.97)	—	1.63(0.86)*		25.58(10.48)**	1.13(0.69)	—	-2.21(0.93)**	—
	Copulation	-7.49(4.52)	0.48(0.29)	—		—	—	—	—	—
	Female Resist	-5.70(7.59)	-0.67(0.45)	1.22(0.77)	0.10(0.06)	—	—	—	—	—
	Copulation D	1.72(0.19)	—	—	—	0.13(0.14)***	0.31(0.08)***	—	—	—
	Ejaculate Sp Num	1.21(0.39)	—	—	—	—	0.45(0.21)*	—	—	—
	Rejected Sp Num	1.61(0.01)	—	—	—	-0.62(0.01)	—	—	—	—
	Remaining Sp Num	2.91(2.00)	-0.22(0.13)	—	—	0.59(0.34)	0.63(0.34)*	—	—	—

The values in parentheses show the standard error of each estimated coefficient of the explanatory variables. Bold indicate a factor listed in AIC models within 2 at AIC. Asterisks indicate a significant difference by the LRT (*p < 0.05, **p < 0.01, ***p < 0.001).

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