

**Female pygmy squid cryptically favour small males and fast copulation as observed by removal of spermatangia**

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1 **Abstract**

2 Females can express mate (or fertilisation) preferences after copulation. In the  
3 Japanese pygmy squid, *Idiosepius paradoxus*, in which males do not show any  
4 conspicuous pre-copulatory displays, the females remove the spermatangia  
5 attached to their bodies after copulation. In this study, we observed pre- and  
6 post-copulatory behaviours and analysed which variables associated with  
7 copulation were correlated with spermatangia removal. When females mated with  
8 larger males or copulation lasted longer female squid elongated their buccal mass  
9 after copulation and removed more spermatangia. We also investigated the effects  
10 of spermatangia removal on the retained spermatangia to predict whether cryptic  
11 female choice (CFC) influenced fertilisation success. Spermatangia removal by  
12 females had a stronger effect on the number of spermatangia retained than did the  
13 number of spermatangia ejaculated by males. These results suggest that  
14 spermatangia removal after copulation by buccal mass elongation works as a CFC  
15 in Japanese pygmy squid, and females cryptically favoured small males and fast  
16 copulation.

17 **Keywords:** cryptic female choice, post-copulatory sexual selection, mate choice,  
18 spermatangia removal, cephalopod, *Idiosepius paradoxus*

**19 Introduction**

20 Most studies of sexual selection are of animals that show courtship or  
21 antagonistic displays before mating (Anderson 1994). Sexual selection is not  
22 thought to arise in animals that do not show pre-copulatory displays. However,  
23 over the past 30 years, research has explored the development of post-copulatory  
24 sexual selection, such as sperm competition (Parker 1970; Birkhead and Møller  
25 1998) and cryptic female choice (CFC) (Thornhill 1983; Eberhard 1996).  
26 Group-spawning males that do not show pre-copulatory displays invest their  
27 energy at the testis via sperm competition, suggesting that post-copulatory sexual  
28 selection takes place in these animals (Byrne et al. 2002; Ota and Kohda 2006).  
29 Few studies have examined CFC in these animals because it is thought that  
30 females choose males based on their pre-copulatory traits. Furthermore, no study  
31 has examined post-copulatory mate choice in animals that do not expressed  
32 conspicuous pre-copulatory traits.

33 Cryptic female choice can arise when females might be unable to reject  
34 males or prevent them from mating, or when it could be very costly to do so. For  
35 instance, many crustacean species forcefully mate and females receive sperm  
36 from multiple males (Sainte-Marie et al. 1999; Thiel and Hinojosa 2003), and  
37 sexual harassment by males can halve the foraging efficiency of female  
38 mosquitofish, *Gambusia holbrooki* (Pilastro et al. 2003). In these cases, females  
39 may accept mating and then choose the sperm to use for fertilisation by CFC.  
40 Even with post-copulatory mate choice, males can choose their preferences based  
41 on general sexual traits (e.g., body size and colour), which males can assess  
42 before copulation. In insects, however, males show various behaviours during and  
43 after copulation (e.g., spiders using their legs to drum or rub on the female's

44 abdomen), which is called copulatory courtship (Eberhard 1991, 1994). Some  
45 studies report that females increase the chances of paternity based on the intensity  
46 of the copulatory courtship behaviour (Edvardsson and Arnqvist 2000; Peretti and  
47 Eberhard 2010). Therefore, even if males do not perform any pre- or  
48 post-copulatory displays, females may express a preference using copulation itself  
49 (e.g., copulation duration and copulation movement) as a basis for the selection  
50 criteria and choose a male based on CFC.

51           The Japanese pygmy squid, *Idiosepius paradoxus*, shows neither  
52 antagonistic behaviour nor courtship displays, and males usually copulate with  
53 females freely (Kasugai 2000). Pygmy squid mate in a head-to-head position; the  
54 male darts toward the female, grasps hold of her, and attaches spermatangia,  
55 capsules containing spermatozoa, to the base of her arms. The squid do not engage  
56 in group spawning or any copulatory courtship behaviours. Recently, we observed  
57 that females frequently elongated their buccal mass, picked up the spermatangia,  
58 and removed them after copulation by eating them or by blowing water from the  
59 funnel (Sato et al. 2013). This behaviour may be a type of CFC.

60           Spermatangia or spermatophore removal is an ideal mechanism for  
61 studying CFC because we can directly observe sperm selection processes. In some  
62 field crickets (Orthoptera: Gryllidae), females remove the externally attached  
63 spermatophores during and after copulation (Sakaluk 1984; Simmons 1986).  
64 Sperm are transferred from the spermatophore to the spermathecae (sperm storage  
65 organs) on the females, and the longer the spermatophore remains attached, the  
66 higher are the chances of paternity (Sakaluk 1984; Simmons 1986; Bussière et al.  
67 2006). *Teleogryllus commodus* females remove spermatophores before sperm  
68 transfer is complete when their mates are unattractive (Bussière et al. 2006; Hall

69 et al. 2010). These studies suggest that these females cryptically chose males via  
70 spermatophore removal.

71 Coastal squid males also transfer spermatophores to females (Hanlon and  
72 Messenger 1996). During spermatophore transfer, a spermatophoric reaction  
73 occurs such that a spermatangium is extruded from the spermatophore and  
74 attaches to the female's body using a type of cement (Marian 2012; Sato et al.  
75 2013). Sperm are transferred from the spermatangia to the seminal receptacle  
76 located around the buccal mass (mouth) and stored until spawning (Sato et al.  
77 2010). The seminal receptacle is a simple sac that does not connect to the ovary in  
78 the mantle. It is thought that fertilisation occurs outside the body. Spermatangia  
79 are also able to act as a sperm storage tank, and the sperm contained therein  
80 directly contribute to fertilisation in the cuttlefish *Sepia apama* (Naud et al. 2005).  
81 Therefore, spermatangia removal by female pygmy squid would influence  
82 fertilisation success.

83 Female pygmy squid have been observed elongating their buccal mass  
84 and picking up the spermatangia within five minutes after copulation (Sato et al.  
85 2013). The length of time taken to elongate the buccal mass is a criterion for  
86 measuring effective spermatangia removal and, therefore, female preference. If  
87 the female changes the duration of buccal mass elongation and thereby the  
88 number of spermatangia removed in response to a male's morphological and  
89 behavioural traits, one might suggest that sexual selection via CFC is occurring in  
90 these pygmy squid. Male pygmy squid produce large spermatangia, which are  
91 attached to the base of the female's arms. The number of spermatangia removed  
92 and the number remaining can be counted, the number ejaculated can be evaluated,  
93 and the final storage can be assessed. In this study, we observed pre- and

94 post-copulatory behaviour using video data from a previous experiment (Sato et al.  
95 2013) and analysed which variables associated with copulation resulted in  
96 spermatangia removal.

97 To test whether CFC influences fertilisation success, we investigated the  
98 effects of male traits and spermatangia removal behaviour on the final number of  
99 spermatangia retained. Finally, we examined the number of spermatangia retained  
100 and investigated the effects of buccal mass elongation, copulation time, and the  
101 numbers of spermatangia passed to the females to ascertain what effects these  
102 variables had on the numbers retained by the females.

### 103 **Material and Methods**

#### 104 Analysis of copulatory and post-copulatory behaviours

105 We collected and reared pygmy squid as previously described (Sato et al.  
106 2013) from the near-shore waters of the Chita Peninsula, central Honshu, Japan  
107 (34°43'N, 136°58'E), using a small dragnet on 12 and 29 April 2009. Live  
108 specimens were transported to the Usujiri Fisheries Station, Field Science Centre  
109 for Northern Biosphere, Hokkaido University, Japan (41°56'N, 140°56'E). Before  
110 being introduced into the experimental aquarium, all squid were separated by sex  
111 and maintained in four aquaria (60 × 45 × 45 cm) with closed circulation systems.

112 One male and one female were introduced into an experimental aquarium  
113 (30 × 40 × 20 cm). A plastic bar (1 × 15 cm) was placed on the sand in the bottom  
114 as a substrate for the squid to adhere to. The water temperature in this aquarium  
115 was the same as in the stock aquaria and maintained at 22°C. Since the squid  
116 needed several hours to become accustomed to the aquarium conditions, we split  
117 the aquarium into two areas using a partition and assigned each sex to an area (30

118 × 20 × 20 cm) 3 h before the experiment began. All trials were conducted between  
119 1000 and 1900 hr.

120           Thirty-two trials were conducted between 16 April and 12 May 2009. In  
121 total, 32 females (dorsal mantle length (DML);  $11.89 \pm 1.61$  mm) and 32 males  
122 (DML;  $8.65 \pm 0.95$  mm) were used, and each squid was used in only one trial. At  
123 the start of the experiment, the partition was removed, and copulation was  
124 observed. Males copulated with females immediately after the partition was  
125 removed. After one copulation was observed, the male was removed from the  
126 aquarium, and the post-copulatory behaviour of the female was recorded by video  
127 for 1 h. All trials were analysed from the video data, and copulation duration  
128 (defined as the time from when the male began to grasp the female to the time  
129 when he left the female) and elongating duration (defined as the time from when  
130 the female began to elongate the buccal mass to the time when she finished) were  
131 recorded. In five trials, the males were removed after two copulations because  
132 they copulated again immediately after the first copulation. We counted the  
133 number of spermatangia passed during copulation, the number of attached  
134 spermatangia (i.e. spermatangia not blown off by a water jet soon after  
135 copulation), the number of spermatangia removed by buccal mass elongation, and  
136 the number of spermatangia retained on the female's body until the end of the  
137 experiment.

138           All squid observed were anaesthetised with 1% ethanol and fixed in  
139 Bouin's solution after the experiment, and DML was measured to the nearest 0.01  
140 mm.

141 Statistical analysis

142 We used a generalised linear model (GLM) with a Poisson binomial  
143 distribution and log link function to analyse whether the duration of buccal mass  
144 elongation influenced the number of spermatangia removed. The significance of  
145 buccal mass elongation duration on the number of removed spermatangia was  
146 assessed using the likelihood ratio test.

147 A GLM was used to analyse the association between buccal mass  
148 elongation and other variables. The duration of buccal mass elongation (BE) for  
149 each trial was set as the response variable and the data were assumed to follow a  
150 gamma distribution. We incorporated potential factors that could affect buccal  
151 mass elongation duration and formed the initial model using the following log link  
152 function:

$$153 \quad \log(\text{BE}) = \alpha_0 + \alpha_1 MS + \alpha_2 FS + \alpha_3 SR + \alpha_4 AS + \alpha_5 CD,$$

154 where *MS* is the male DML, *FS* is the female DML, *SR* is the size ratio (male  
155 DML / female DML), *AS* is the number of attached spermatangia, *CD* is the  
156 copulation duration (for the five trials in which the males copulated twice, we  
157 used the average duration of the two copulations), and  $\alpha_0 - \alpha_5$  are the estimated  
158 parameters of interest. To identify factors affecting elongation duration, we  
159 performed model selection based on Akaike's information criteria (AIC). The  
160 model with the smallest AIC value was chosen as the best fit. We then evaluated  
161 the effects of the explanatory variables based on increments of the AIC ( $\Delta\text{AIC}$ ) by  
162 removing variables from the best-fit model one at a time.

163 We used a GLM with a negative binomial distribution and log link  
164 function to analyse whether male traits (copulation duration and male DML)  
165 influenced the numbers of spermatangia removed and retained until the end of the  
166 experiment. GLM was also used to assess which sex strongly influenced the



167 number of spermatangia retained until the end of the experiment by evaluating the  
168 effects of copulation duration and buccal mass elongation on this number. The  
169 number of spermatangia retained until the end of the experiment was set as the  
170 response variable and was treated as count data that followed a Poisson  
171 distribution. The significance of the fixed effects on the dependent variable was  
172 assessed with a likelihood ratio test using the log likelihood model (including the  
173 fixed effect) and the null model (without the fixed effect). Finally, we investigated  
174 whether the numbers of attached spermatangia and removed spermatangia  
175 influenced the number of spermatangia retained until the end of the experiment.  
176 We evaluated the effects of the explanatory variables based on  $\Delta$ AIC by removing  
177 variables from the model. We used R version 2.15.2 for all analyses (R  
178 Development Core Team 2012).

## 179 **Results**

180 The number of spermatangia passed by males to females during  
181 copulation (mean  $\pm$  SD =  $3.64 \pm 1.72$ ,  $n = 37$ ) was positively correlated with  
182 copulation duration ( $4.57 \pm 2.59$  s,  $n = 37$ ; Spearman's rank correlation:  $r_s = 0.41$ ,  
183  $P = 0.013$ ). Neither male DML ( $8.65 \pm 0.95$  mm,  $n = 32$ ) nor female DML ( $11.89$   
184  $\pm 1.61$  mm,  $n = 32$ ) was significantly related to copulation duration (male DML:  
185  $r_s = -0.03$ ,  $n = 37$ ,  $P = 0.87$ ; female DML:  $r_s = -0.13$ ,  $n = 37$ ,  $P = 0.44$ ) or the  
186 number of spermatangia (male DML:  $r_s = 0.18$ ,  $n = 37$ ,  $P = 0.28$ ; female DML:  $r_s$   
187  $= -0.15$ ,  $n = 37$ ,  $P = 0.38$ ).

188 All the females blew water from the funnel immediately after copulation.  
189 An average of  $1.67 \pm 0.87$  spermatangia were removed in nine of 32 trials, and all  
190 the spermatangia were removed by water jetting in two trials. The number of

191 spermatangia that were successfully attached was not significantly related to both  
192 male DML ( $r_s = -0.15$ ,  $n = 37$ ,  $P = 0.36$ ) and copulation duration ( $r_s = 0.28$ ,  $n =$   
193  $37$ ,  $P = 0.09$ ).

194 In 28 trials, the females elongated the buccal mass within 5 min after  
195 copulation. Fifteen females picked up  $3.47 \pm 2.00$  spermatangia and removed  
196 these by either eating them or jetting. In eight females, all the spermatangia were  
197 removed. The remaining 13 females had not picked up any spermatangia by the  
198 end of the observation period. In 28 trials, 20 females stopped elongation even if  
199 some spermatangia remained on the body. Elongation duration had a significant  
200 effect on the number of spermatangia removed (GLM with likelihood ratio test:  $\chi^2$   
201  $= 9.609$ ,  $P < 0.01$ , see Fig. 1). Four females did not elongate their buccal mass by  
202 the end of the observation period in 32 trials; in one of these, all of the  
203 spermatangia were removed by a water jet.

204 For buccal mass elongation duration, the optimal model selected by AIC  
205 was as follows:  $\log(\text{BE}) = \alpha_0 + \alpha_1 MS + \alpha_3 SR + \alpha_4 AS + \alpha_5 CD$ . The estimated  
206 parameters in the GLM are shown in Table 1. The best-fit models did not include  
207 female DML. Male DML and copulation duration had large effects in the models,  
208 and the effect of buccal mass elongation duration increased with male size and  
209 copulation duration. Both male DML and copulation duration were significantly  
210 related to removed spermatangia (GLM with a likelihood ratio test: male DML  $\chi^2$   
211  $= 6.388$ ,  $P < 0.05$ , see Fig. 2a; copulation duration  $\chi^2 = 4.544$ ,  $P < 0.05$ , Fig. 2b).

212 Neither copulation duration nor male DML had a significant effect on the  
213 number of spermatangia kept until the end of the experiment (GLM with a  
214 likelihood ratio test: copulation duration  $\chi^2 = 0.332$ ,  $P = 0.56$ ; male DML  $\chi^2 =$   
215  $0.003$ ,  $P = 0.96$ ). The GLM model for the final number of spermatangia showed

216 that numbers of removed spermatangia and attached spermatangia had a large  
217 effect on the final number of spermatangia, with the former having a stronger  
218 effect than the latter ( $\Delta\text{AIC} = 41.65$  and  $22.10$ , respectively, Table 2). Copulation  
219 duration did not have a significant effect on the final number of spermatangia  
220 (GLM with a likelihood ratio test:  $\chi^2 = 0.013$ ,  $P = 0.91$ ), but elongation duration  
221 did have a significant effect (GLM with a likelihood ratio test:  $\chi^2 = 6.867$ ,  $P <$   
222  $0.01$ , Fig. 3).

## 223 **Discussion**

224 Female pygmy squid cryptically favoured small males and short  
225 copulation duration as ascertained by the numbers of removed spermatangia.  
226 Eberhard suggested that female bias during or after copulation could be associated  
227 with particular male characteristics, indicating CFC (Eberhard 1996). Our study  
228 suggested that spermatangia removal by buccal mass elongation following  
229 copulation is a mechanism of CFC in Japanese pygmy squid. The number of  
230 spermatangia removed increased with the duration of buccal mass elongation,  
231 which suggested that females attempted to decrease the number of retained  
232 spermatangia by buccal mass elongation. Duration of buccal mass elongation  
233 models showed that male traits (male DML and copulation duration) had large  
234 effects. There was a significant relationship between male traits and the numbers  
235 of spermatangia removed.

236 When females copulated with larger males or when the copulation  
237 duration was longer, female squid elongated the buccal mass for a longer period of  
238 time after copulation, and more spermatangia were removed. This suggests that  
239 small males with short copulation duration were favoured. Generally, larger males

240 are favoured because a larger body is better for male–male rivalry or male–female  
241 interactions (Cote and Hunte 1989; Cooper Jr. and Vitt 1993; Shine and Mason  
242 2005; Charlton et al. 2007). In flying animals, small males are preferred, as they  
243 have lower flight costs and increased agility. For example, in the polygynous bat  
244 *Saccopteryx bilineata*, small males are better at defending the harem than are  
245 larger ones (Voigt et al. 2005), and in the kestrel *Falco tinnunculus*, small males  
246 are better providers in courtship and at feeding offspring (Hakkarainen et al. 1996).  
247 Although we do not know whether small pygmy squid are highly agile, agility  
248 would not be of a great benefit because they do not form harems or provide  
249 parental care.

250           Female pygmy squid also preferred short copulation duration. This  
251 preference may relate to predation risk. Predation risk is higher during copulation  
252 because of the increased conspicuousness of a mating pair (Magnhagen 1991;  
253 Magurran and Nowak 1991; Siemers et al. 2012). Fast copulation would be less  
254 conspicuous, which may increase survival rates. Predation risk may be high for  
255 pygmy squid because many fish live in the seagrass beds where pygmy squid  
256 mate (Nakamura et al. 2003; Horinouchi and Sano 2000), and the pygmy squid is  
257 so small that most fish may predate on them. Additionally, they do not show  
258 pre-copulatory displays, which may support this hypothesis.

259           Having a small body might also be an advantage in avoiding predation.  
260 The pygmy squid can adhere to the substrata, such as seagrass, using an adhesive  
261 organ on the dorsal mantle, where they can hide to avoid predation (Sasaki 1923).  
262 Their small body size would make them less conspicuous and thus increase their  
263 survival rates. This could be why females prefer small males; however, further  
264 studies are needed to investigate the relationships among predation risk,

265 copulation duration, and body size.

266           Critic female choice might be an important mechanism in the  
267 determination of fertilisation success in the pygmy squid. The most important  
268 variable in the final spermatangia model was not ejaculation but the number of  
269 spermatangia removed. Moreover, only buccal mass elongation duration, which  
270 indicated effort directed toward spermatangia removal, was significantly related to  
271 the number of spermatangia that remained. Male traits did not influence the  
272 number of spermatangia that remained. In these cephalopods, spermatangia  
273 attached to the female body release sperm from the oral end after completion of  
274 the spermatophoric reaction (Drew 1919; Marian 2012). A previous study on  
275 pygmy squid suggested that sperm from released spermatangia actively swam to  
276 the seminal receptacle (Sato et al. 2010). This would suggest that the volume of  
277 sperm stored would increase with the number of attached spermatangia.  
278 Unfortunately, it is not known whether sperm volume stored in the seminal  
279 receptacle is related to fertilisation success in cephalopods. In a mating pair of  
280 loliginid squid, a male can pass spermatangia at the opening of the egg duct,  
281 which ensures the highest paternity (Iwata et al. 2005); however, this does not  
282 appear to be a priority in cuttlefish, which pass spermatangia around the buccal  
283 mass (Naud et al. 2005). The sperm storage system in the pygmy squid, which  
284 would be similar to that in cuttlefish, relied on the seminal receptacle. Histological  
285 studies in cephalopods have found that spermatozoa are not layered in the seminal  
286 receptacle (e.g., Drew, 1911; Naud et al. 2005; Sato et al. 2010). The sperm  
287 volume in the seminal receptacle might be directly related to fertilisation success.  
288 However, many studies of sperm competition have shown that sperm precedence  
289 influences fertilisation success (Birkhead and Møller 1998; Urbani et al. 1998;

290 Price et al. 1999). To confirm this hypothesis, it would be necessary to conduct  
291 paternity analysis.

292         The longer male pygmy squid copulate, the more spermatangia they pass  
293 to the females. However, females dislike longer copulation and remove more  
294 spermatangia when the copulation duration is longer. These results suggest  
295 possible conflict between the sexes over copulation duration (Arnqvist and Rowe  
296 2005). Pygmy squid transfer spermatangia two to three times during one  
297 copulation (Sato et al. 2013). Males change the location of spermatangia  
298 attachment during each transfer, which may be a defence against spermatangia  
299 removal by females.

300         However, the possibility that spermatangia removal is not CFC remains  
301 because we did not know the exact sperm volumes of the spermatangia passed by  
302 males and those retained spermatangia in females, or fertilisation success. A large  
303 amount of sperm might not be needed for storage or fertilisation in pygmy squid.  
304 Surplus sperm passed by males might serve as a nuptial gift and provide  
305 nutritional resources. For example, female land snails can digest stored sperm  
306 (Rigby 1963). The behaviour might also be performed simply to keep clean. To  
307 resolve this issue, we should clarify the mechanism of sperm transfer and storage  
308 in cephalopods and determine how many sperm are stored in the seminal  
309 receptacle and used for fertilisation.

310         In conclusion, although pygmy squid do not show any pre-copulatory  
311 behaviour, females cryptically favour small males and fast copulation, as  
312 demonstrated by post-copulatory spermatangia removal. Even without copulatory  
313 displays, sexual selection may arise via CFC because females can keep or reject  
314 sperm by establishing mate preference during copulation.

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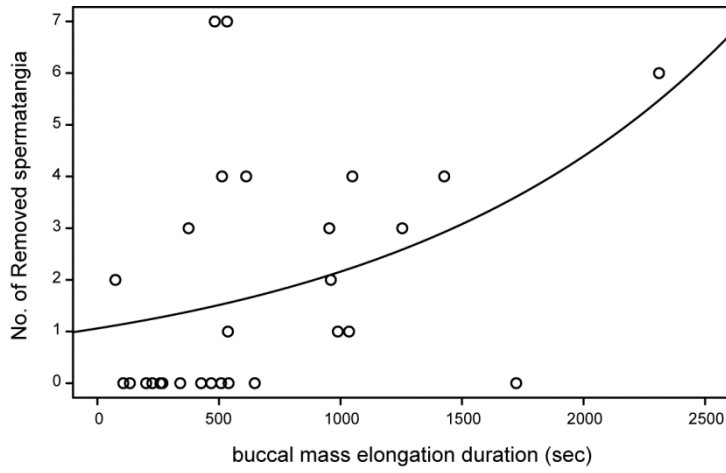
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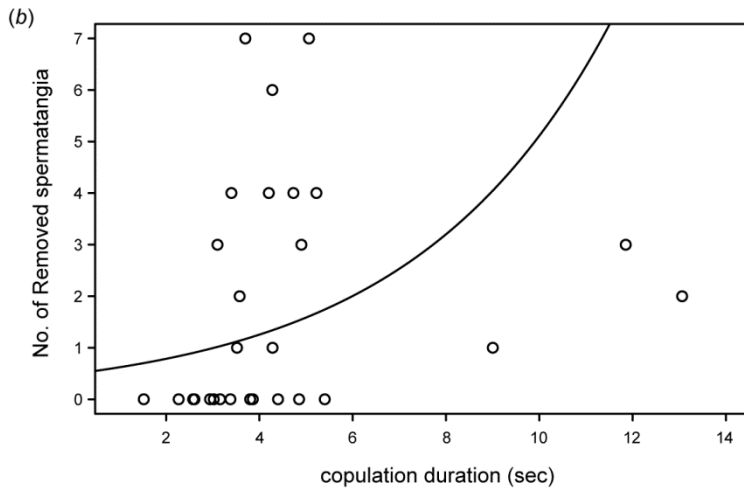
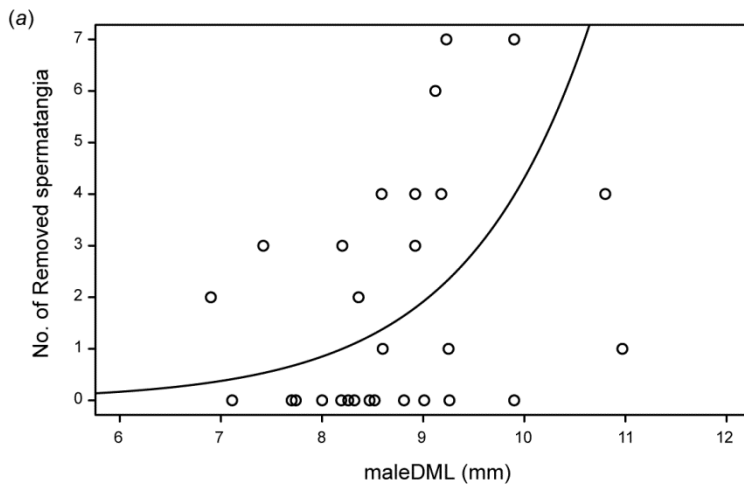
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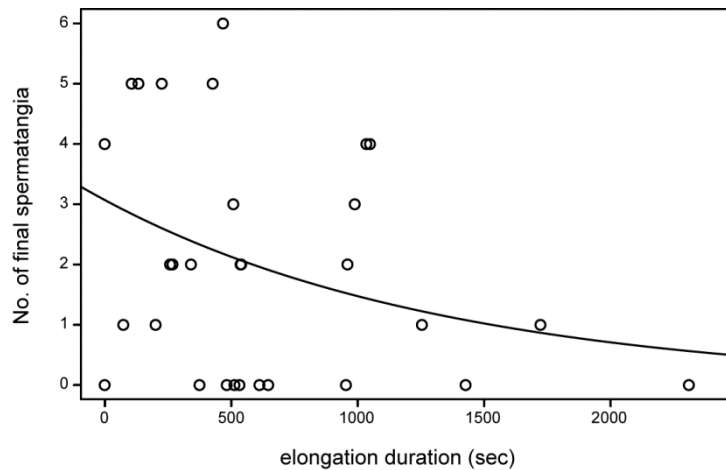


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438 **Fig. 1**, The relationship between the number of spermatangia removed and the  
 439 duration of buccal mass elongation by females after copulation ( $n = 28$ ). The solid  
 440 line represents a Poisson regression ( $y = e^{(0.0591x + 0.0007)}$ ,  $P < 0.001$ ).



441 **Fig. 2**, The relationship between the number of spermatangia removed by females  
 442 after copulation and (a) the size of the mating partner ( $n = 28$ ) and (b) copulation  
 443 duration ( $n = 28$ ). The solid lines represent a negative binomial regression ((a):  $y$   
 444  $= e^{(0.8120x - 7.7308)}$ ,  $P < 0.01$ ; (b):  $y = e^{(0.2341x - 7.7308)}$ ,  $P < 0.05$ ).



445 **Fig. 3**, The relationship between the final number of spermatangia kept until the  
 446 end of the experiment and the duration of buccal mass elongation by females after  
 447 copulation ( $n = 32$ ). The solid line represents a Poisson regression ( $y = e^{(-0.0007x +$   
 448  $1.1533)}$ ,  $P < 0.05$ ).

**Table 1.**

The coefficient estimates in the optimal model for the elongation duration of buccal mass.  $\Delta$ AIC indicates the increment of Akaike information criteria if the explanatory variable is removed from the best-fit model.  $P$  indicates  $P$  value based on Wald statistics.

explanatory variable	estimate (SE)	$P$	
maleDML	$\Delta$ AIC = 11.67		
	0.851 (0.208)	< 0.001	*
femaleDML	—	—	
size ratio	$\Delta$ AIC = 0.34		
	-2.380 (1.495)	0.125	
attached spermatangia number	$\Delta$ AIC = 0.36		
	-0.121 (0.080)	0.144	
copulation duration	$\Delta$ AIC = 7.88		
	0.183 (0.055)	0.003	*
* Significant at $P = 0.01$ level; -explanatory variables removed through model selection.			

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**Table 2.**

The coefficient estimates in the optimal model for the number of finally retained spermatangia.  $P$  indicates  $P$  value based on Wald statistics.

explanatory variable	estimate (SE)	$P$	
removed spermatangia	$\Delta$ AIC = 41.65		
	-0.560 (0.113)	< 0.001	*
passed spermatangia number	$\Delta$ AIC = 22.10		
	0.374 (0.079)	< 0.001	*
* Significant at $P = 0.01$ level			

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