

# Female Pygmy Squid Cryptically Favour Small Males and Fast Copulation as Observed by Removal of Spermatangia

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

females cryptically favoured small males and fast copulation.

**Keywords** Cryptic female choice · Post-copulatory sexual selection · Mate choice · Spermatangia removal · Cephalopod · *Idiosepius paradoxus*

## Introduction

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

Cryptic female choice can arise when females might be unable to reject males or prevent them from mating, or when it could be very costly to do so. For instance, many crustacean species forcefully mate and females receive sperm from multiple males (Sainte-Marie et al. 1999; Thiel and Hinojosa 2003), and sexual harassment by males can halve the foraging efficiency of female mosquitofish, *Gambusia holbrooki* (Pilastro et al. 2003). In these cases,

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females may accept mating and then choose the sperm to use for fertilisation by CFC. Even with post-copulatory mate choice, males can choose their preferences based on general sexual traits (e.g., body size and colour), which males can assess before copulation. In insects, however, males show various behaviours during and after copulation (e.g., spiders using their legs to drum or rub on the female's abdomen), which is called copulatory courtship (Eberhard 1991, 1994). Some studies report that females increase the chances of paternity based on the intensity of the copulatory courtship behaviour (Edvardsson and Arnqvist 2000; Peretti and Eberhard 2010). Therefore, even if males do not perform any pre- or post-copulatory displays, females may express a preference using copulation itself (e.g., copulation duration and copulation movement) as a basis for the selection criteria and choose a male based on CFC.

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

Spermatangia or spermatophore removal is an ideal mechanism for studying CFC because we can directly observe sperm selection processes. In some field crickets (Orthoptera: Gryllidae), females remove the externally attached spermatophores during and after copulation (Sakaluk 1984; Simmons 1986). Sperm are transferred from the spermatophore to the spermathecae (sperm storage organs) on the females, and the longer the spermatophore remains attached, the higher are the chances of paternity (Sakaluk 1984; Simmons 1986; Bussière et al. 2006). *Teleogryllus commodus* females remove spermatophores before sperm transfer is complete when their mates are unattractive (Bussière et al. 2006; Hall et al. 2010). These studies suggest that these females cryptically chose males via spermatophore removal.

Coastal squid males also transfer spermatophores to females (Hanlon and Messenger 1996). During spermatophore transfer, a spermatophoric reaction occurs such that a spermatangium is extruded from the spermatophore and attaches to the female's body using a type of cement (Marian 2012; Sato et al. 2013b). Sperm are transferred from the spermatangia to the seminal receptacle located around the buccal mass (mouth) and stored until spawning (Sato et al. 2010). The seminal receptacle is a simple sac

that does not connect to the ovary in the mantle. It is thought that fertilisation occurs outside the body. Spermatangia are also able to act as a sperm storage tank, and the sperm contained therein directly contribute to fertilisation in the cuttlefish *Sepia apama* (Naud et al. 2005). Therefore, spermatangia removal by female pygmy squid would influence fertilisation success.

Female pygmy squid have been observed elongating their buccal mass and picking up the spermatangia within five minutes after copulation (Sato et al. 2013a, b). The length of time taken to elongate the buccal mass is a criterion for measuring effective spermatangia removal and, therefore, female preference. If the female changes the duration of buccal mass elongation and thereby the number of spermatangia removed in response to a male's morphological and behavioural traits, one might suggest that sexual selection via CFC is occurring in these pygmy squid. Male pygmy squid produce large spermatangia, which are attached to the base of the female's arms. The number of spermatangia removed and the number remaining can be counted, the number ejaculated can be evaluated, and the final storage can be assessed. In this study, we observed pre- and post-copulatory behaviour using video data from a previous experiment (Sato et al. 2013a) and analysed which variables associated with copulation resulted in spermatangia removal.

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

## Materials and Methods

### Analysis of Copulatory and Post-Copulatory Behaviours

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

One male and one female were introduced into an experimental aquarium (30 cm × 40 cm × 20 cm). A

plastic bar (1 cm × 15 cm) was placed on the sand in the bottom as a substrate for the squid to adhere to. The water temperature in this aquarium was the same as in the stock aquaria and maintained at 22 °C. Since the squid needed several hours to become accustomed to the aquarium conditions, we split the aquarium into two areas using a partition and assigned each sex to an area (30 cm × 20 cm × 20 cm) 3 h before the experiment began. All trials were conducted between 1,000 and 1,900 h.

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

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

### Statistical Analysis

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

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

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

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

We used a GLM with a negative binomial distribution and log link function to analyse whether male traits (copulation duration and male DML) influenced the numbers of spermatangia removed and retained until the end of the experiment. GLM was also used to assess which sex strongly influenced the number of spermatangia retained until the end of the experiment by evaluating the effects of copulation duration and buccal mass elongation on this number. The number of spermatangia retained until the end of the experiment was set as the response variable and was treated as count data that followed a Poisson distribution. The significance of the fixed effects on the dependent variable was assessed with a likelihood ratio test using the log likelihood model (including the fixed effect) and the null model (without the fixed effect). Finally, we investigated whether the numbers of attached spermatangia and removed spermatangia influenced the number of spermatangia retained until the end of the experiment. We evaluated the effects of the explanatory variables based on  $\Delta\text{AIC}$  by removing variables from the model. We used R version 2.15.2 for all analyses (R Development Core Team 2012).

### Results

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

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

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

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

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

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

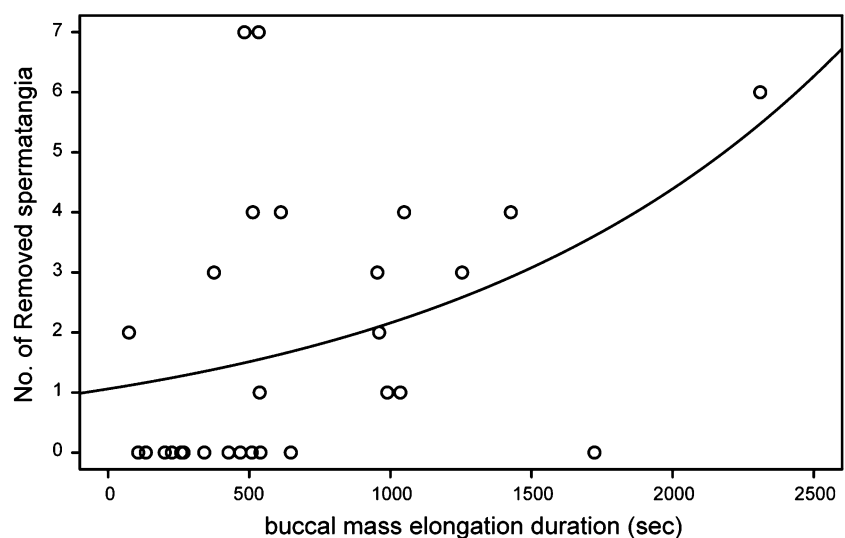
number of spermatangia showed that numbers of removed spermatangia and attached spermatangia had a large effect on the final number of spermatangia, with the former having a stronger effect than the latter ( $\Delta\text{AIC} = 41.65$  and  $22.10$ , respectively, Table 2). Copulation duration did not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

## Discussion

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

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

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



**Table 1** The coefficient estimates in the optimal model for the elongation duration of buccal mass

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*

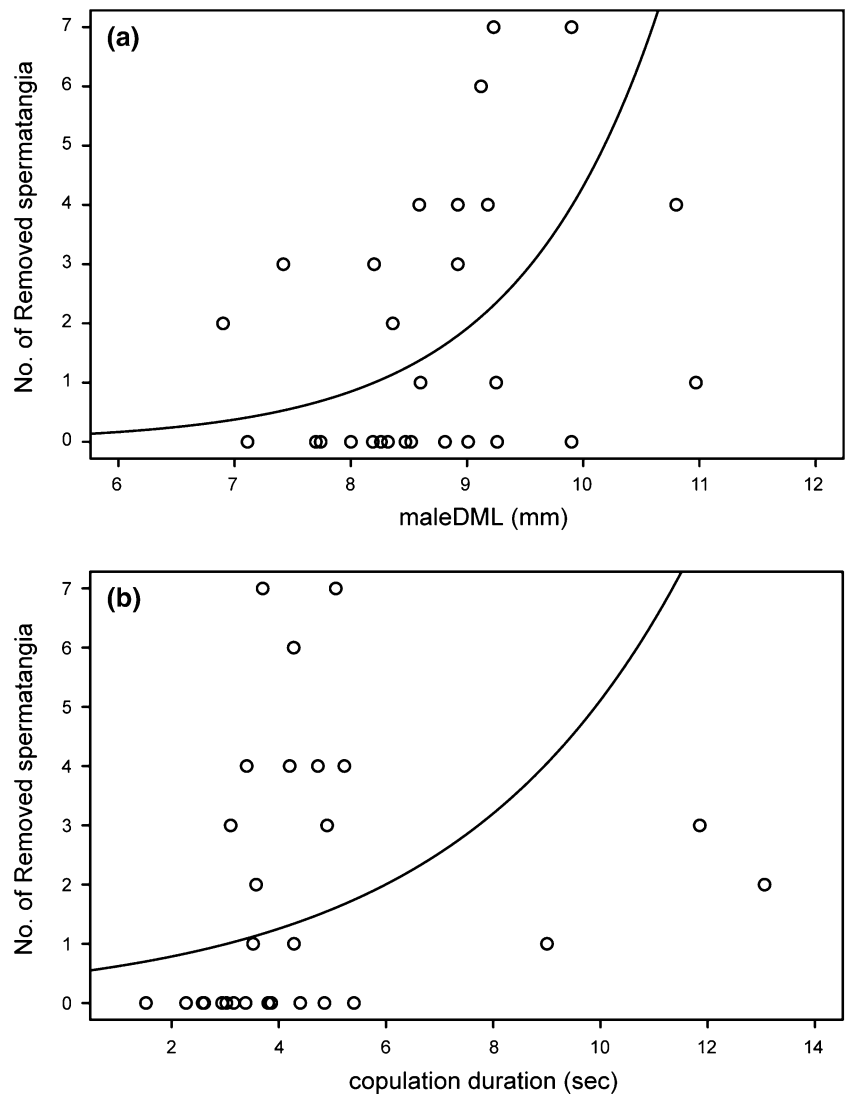
$\Delta AIC$  indicates the increment of Akaike information criteria if the explanatory variable is removed from the best-fit model. *P* indicates *P* value based on Wald statistics

\* significant at *P* = 0.01 level

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

Female pygmy squid also preferred short copulation duration. This preference may relate to predation risk. Predation risk is higher during copulation because of the

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





increased conspicuousness of a mating pair (Magnhagen 1991; Magurran and Nowak 1991; Siemers et al. 2012). Fast copulation would be less conspicuous, which may increase survival rates. Predation risk may be high for pygmy squid because many fish live in the seagrass beds where pygmy squid mate (Nakamura et al. 2003; Horinouchi and Sano 2000), and the pygmy squid is so small that most fish may predate on them. Additionally, they do not show pre-copulatory displays, which may support this hypothesis.

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

Cryptic female choice might be an important mechanism in the determination of fertilisation success in the pygmy squid. The most important variable in the final spermatangia model was not ejaculation but the number of spermatangia removed. Moreover, only buccal mass

elongation duration, which indicated effort directed toward spermatangia removal, was significantly related to the number of spermatangia that remained. Male traits did not influence the number of spermatangia that remained. In these cephalopods, spermatangia attached to the female body release sperm from the oral end after completion of the spermatophoric reaction (Drew 1919; Marian 2012). A previous study on pygmy squid suggested that sperm from released spermatangia actively swam to the seminal receptacle (Sato et al. 2010). This would suggest that the volume of sperm stored would increase with the number of attached spermatangia. Unfortunately, it is not known whether sperm volume stored in the seminal receptacle is related to fertilisation success in cephalopods. In a mating pair of loliginid squid, a male can pass spermatangia at the opening of the egg duct, which ensures the highest paternity (Iwata et al. 2005); however, this does not appear to be a priority in cuttlefish, which pass spermatangia around the buccal mass (Naud et al. 2005). The sperm storage system in the pygmy squid, which would be similar to that in cuttlefish, relied on the seminal receptacle. Histological studies in cephalopods have found that spermatozoa are not layered in the seminal receptacle (e.g., Drew 1911; Naud et al. 2005; Sato et al. 2010). The sperm volume in the seminal receptacle might be directly related to fertilisation success. However, many studies of sperm competition have shown that sperm precedence influences fertilisation success (Birkhead and Møller 1998; Urbani et al. 1998; Price et al. 1999). To confirm this hypothesis, it would be necessary to conduct paternity analysis.

The longer male pygmy squid copulate, the more spermatangia they pass to the females. However, females dislike longer copulation and remove more spermatangia when the copulation duration is longer. These results suggest possible conflict between the sexes over copulation

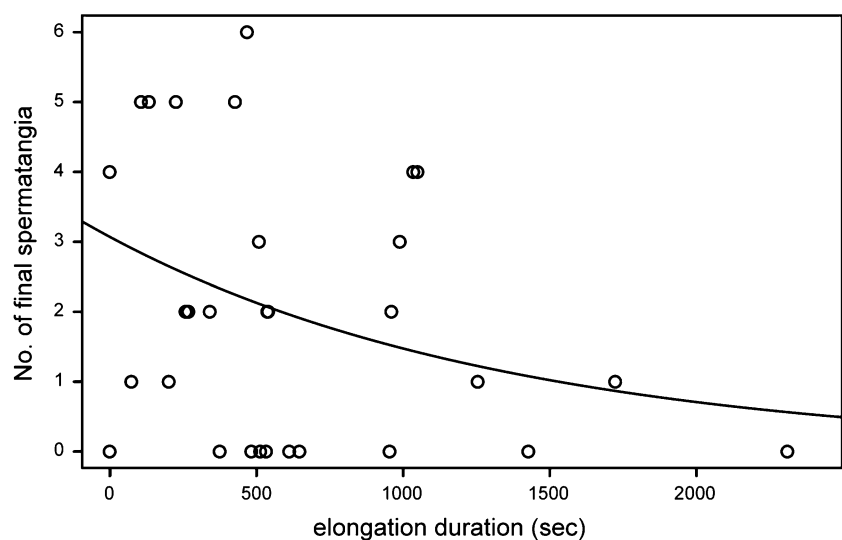
**Table 2** The coefficient estimates in the optimal model for the number of finally retained spermatangia

Explanatory variable	Estimate (SE)	<i>P</i>
Removed spermatangia	$\Delta\text{AIC} = 41.65$ −0.560 (0.113)	<0.001*
Passed spermatangia number	$\Delta\text{AIC} = 22.10$ 0.374 (0.079)	<0.001*

*P* indicates *P* value based on Wald statistics

\* significant at *P* = 0.01 level

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



duration (Arnqvist and Rowe 2005). Pygmy squid transfer spermatangia two to three times during one copulation (Sato et al. 2013a, b). Males change the location of spermatangia attachment during each transfer, which may be a defence against spermatangia removal by females.

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

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

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