



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

Noriyosi Sato^{1,2} · Yu Uchida³ · Takeshi Takegaki¹

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Abstract

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

Significance statement

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

Keywords Cryptic female choice · Sperm competition · Predation risk · Sexual conflict · Cephalopod · *Idiosepius paradoxus*

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✉ Noriyosi Sato
norico3000@gmail.com

² Present address: Oki Marine Biological Station, Shimane University, Oki, Japan

³ Faculty of Fisheries, Nagasaki University, Nagasaki, Japan

¹ Graduate School of Fisheries and Environmental Sciences, Nagasaki University, Nagasaki, Japan

Introduction

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

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

Male Japanese pygmy squid, *Idiosepius paradoxus*, do not use agonistic or elaborate courtship behaviour to acquire mates (Kasugai 2000). Rather, they grab females for copulation without any display and pass spermatophores via the hectocotylus, which is a specialised arm used for copulation (Sato et al. 2013a). Spermatangia are discharged from spermatophores when they are pulled from the funnel, which is a muscular structure like the siphon used for water jetting and respiration, and attached to the base of the female arm by males (Sato et al. 2014a). Spermatozoa are then released from the tip of the spermatangium and are activated on contact with seawater, transferred to the seminal receptacle by swimming actively, and stored there (Sato et al. 2010, 2014a). Paternity analysis of egg masses collected in the field revealed each studied female to have copulated with about 10 males, indicating a highly promiscuous mating system (Sato 2017). The sperm ejaculated by each male form a mixture in the seminal

receptacle, and the relative sperm volume remaining in the female's body is correlated with fertilisation success (Sato et al. 2017). Females elongate their mouth (buccal mass) and choose successful mates cryptically post-copulation by picking up and removing spermatangia before completing sperm transfer (Sato et al. 2013b, 2014b). The volume of removed spermatangia is dependent on not only buccal mass elongation but also copulation duration and body size, and females prefer smaller males, but their preferences for long or short copulation duration vary from year to year (Sato et al. 2014b and 2017).

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

Methods

Census of fish fauna and evaluation of predation risk

The fish fauna was investigated using line censuses of Ohmura Bay and Oki Island to evaluate the degree of predation risk for two populations of pygmy squid (Supplemental Material 1). In Ohmura Bay, fewer fish were observed than at Oki Island, and 90% of the fish were a small goby (*Sagamia geneionema*), which feed mainly on gammaridean amphipods (Sano 1998). At Oki Island, the presence of potential predators on Japanese pygmy squid, such as rockfish, sculpin and greenling, was noted. Two rockfishes (*Sebastes flavidus* and *S. alutus*) living in the north-eastern Pacific Ocean have been reported to feed on cephalopods (Brodeur and Pearcy 1984), and we have occasionally observed predatory attacks by rockfish (unidentified *Sebastes*) on pygmy squid (N. Sato, personal observation). *Pseudoblennius percooides* (sculpin) is a major predator of juvenile and young fish in seagrass beds (Horinouchi and Sano 2000), and *Hexagrammos otakii*

(greenling) feeds on fish, crustaceans and polychaetes (Kwak et al. 2005). Accordingly, the predation risk to pygmy squid is potentially higher in the Oki population than in the Ohmura population.

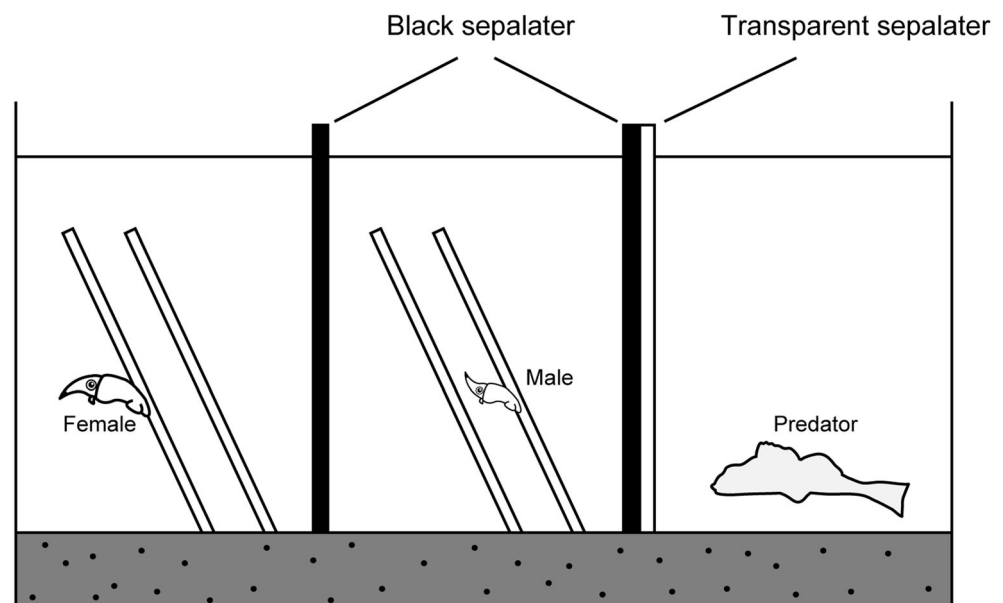
Aquarium experiment

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

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

The aquaria (45 × 20 × 30 cm) used for the experiments were partitioned, by black and transparent plastic boards, into three areas (male, female and predator areas), each measuring 15 × 20 × 30 cm, and a female, male, and sculpin (or no sculpin) was introduced into each area (Fig. 1). Three plastic plates (30 × 0.6 cm) were placed on the sandy bottom of each of the two areas for use by squid as perches. To acclimatise to the aquarium conditions, the animals were introduced 30 min before the trials began. We then gently removed the black partitions between the male and female areas and between the female and predator areas. This allowed the male to access the female while exposed to a threat of predation without any risk of predator attack. Behaviour was recorded using a digital video camera (HDR-XR 520V; Sony, Tokyo, Japan). The male was removed from the aquarium soon after copulation and we counted the spermatangia and observed the sperm-removing behaviour of the female. Video recording was continued for 30 min after copulation by which time a female has usually ceased engaging in sperm-removing behaviour (Sato et al. 2013b). If no copulation occurred within 30 min, the trial was stopped. A copulatory trial was conducted twice for each individual to see whether the behaviour changed with the presence of a predator. Thirty-seven pairs (22 from Ohmura and 15 from Oki) were allocated for the experiment with a predator in the first trial and 51 pairs (36 from Ohmura and 15

Fig. 1 The aquarium used for the experiments. Three plastic plates were placed in each squid-containing area to serve as perches. During a trial, a predator was or was not placed in the predator compartment. All trials started after removing the black separators



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

We noted any display behaviour by the squid to assess whether predation risk influenced reproductive behaviour. These included attempted copulation, female resistance (i.e. whether females showed resistance to grabbing during copulation), copulation duration (defined as the time from when the male began to grasp the female to when he left the female) and buccal mass elongation duration (indicative of female effort expended on sperm removal) (defined as the time from when the female began to elongate the buccal mass to when she finished elongation). We also counted the numbers of spermatangia that the male attached to the female (ejaculated spermatangia), that the female removed (removed spermatangia) and that the female kept on her body (remaining spermatangia). The method of counting spermatangia followed Sato et al. (2014b, 2017).

Statistical analysis

We analysed the factors that affected squid behaviours (inking, copulation, female resistance, copulation duration

and buccal-mass-elongation duration), as well as the numbers of ejaculated, removed, and remaining spermatangia, using generalised linear mixed models (GLMMs) in each population (Ohmura or Oki) (Crawley 2005; Bolker et al. 2009). In all analyses, a full model was constructed to test the main effects of the following explanatory variables: male body size, female body size, copulation duration (except for the inking, copulation and copulation duration models), predator presence and trial order (first or second trial). Interactions among copulation duration and predator presence, male body size and predator presence and female body size and predator presence were also investigated, because when females express a preference for any male traits, those preferences may change when there is a predation risk. We used squid identification as a random factor because each of the males and females was used twice in this study. A binomial error distribution and logit link function were applied in the inking, copulation and female-resistance models. A gamma distribution and log link function were applied in the models of copulation duration and buccal mass elongation duration. A Poisson error distribution and log link function were applied in the models of spermatangia ejaculated, removed and retained. We subsequently compared the Akaike information criterion (AIC) among all models. To verify the variables selected by the AIC, the significance of each explanatory variable was assessed using the likelihood ratio test (LRT). We used R software (ver. 3.4.2; R Development Core Team 2017) for the analyses.

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

Results

The body sizes of both males and females in the Ohmura Bay population ($n = 64$, mean \pm SD DML = 8.44 ± 1.05 for males; $n = 58$, 11.55 ± 1.48 for females) were significantly smaller

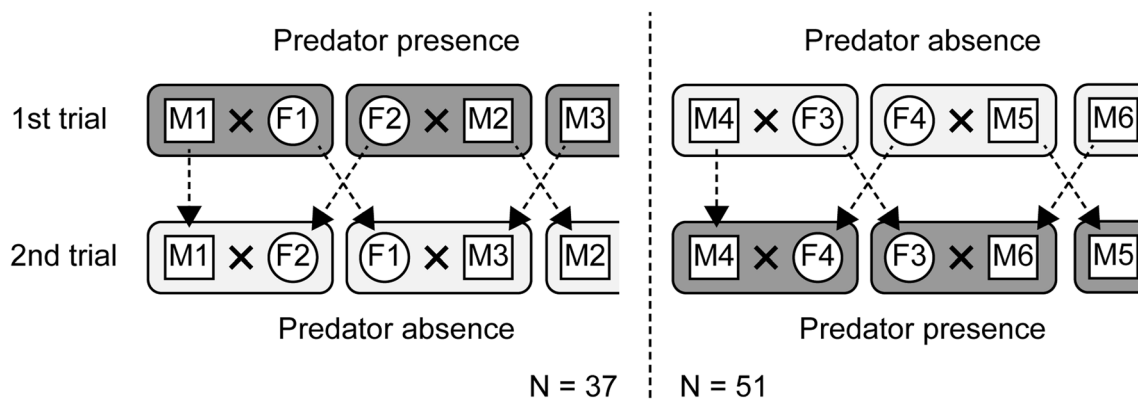


Fig. 2 Diagram showing experimental design. Grey and white boxes indicate each trial. Left and right sides indicate the initial presence and absence of a predator, respectively

than those of the Oki Island population ($n = 30$, 10.67 ± 1.10 for males; $n = 30$, 15.10 ± 1.61 for females; ANOVA: $F = 3.94$, $df = 1$, $p = 0.001$ for males; $F = 3.95$, $df = 1$, $p = 0.001$ for females). Of 174 (86 without a predator) experiments, four experiments were removed from the analysis because the female attacked the male during the observation. Ultimately, 109 (56 without a predator) and 60 (30 without a predator) experiments were analysed for the Ohmura and Oki populations, respectively (Table 1). The GSI of the Ohmura Bay population ($n = 60$, 6.20 ± 1.11) was significantly greater than that of the Oki Island population ($n = 30$, 5.04 ± 0.46 ; ANOVA: $F = 20.88$, $df = 1$, $p = 0.0001$).

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

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

Of the 71 copulation successes, copulation resistance was shown by females in 26 cases. When female resistance occurred during copulation, female body size and copulation duration were included in the three and four minimum AIC

models for Ohmura and Oki, respectively (Table 2), but copulation duration did not have a significant effect on squid behaviour in the LRT (Table 3). Females showed greater resistance to copulation as body size decreased in the Ohmura population.

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

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

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

Table 1 Summary of the experiment data

| | Ohmura | | Old | |
|-------------------|-------------------|--------------------|--------------------|--------------------|
| | Absence | Presence | Absence | Presence |
| 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

Table 2 The best five A IC models

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

Italics indicate AIC models within 2 at ΔAIC from the best AIC model

BM buccal mass, *D* duration, *Num* number, *Sp* spermatangia, *Fsize* female body size, *M size* male body size, *Cop D* copulation duration, *Pop* population, *Pre* predation, *Ord* order

Table 3 Estimates of the best model based on the AIC in each CI_M_M analysis

| Population | Response variable | (Intercept) | Fsize | M size | Cop D | Pre (presence) | Ord | Fsize × Pre | M size × Pre | Cop D × Pre |
|------------------|-------------------|---------------|------------------|---------------|--------------|----------------|---------------|---------------|---------------|---------------|
| Ohmura | Inking | - 2.19(0.99) | - | - | - | 3.13(0.80)*** | - 0.87(0.55) | - | - | - |
| | Copulation | 4.95(2.09) | - 0.44(0.18)** | - | 0.77(0.50) | - | - | - | - | - 393(1.63)** |
| | Female Resist | 96.68(38.58) | - 10.01(3.93)*** | - | - | 33.12(14.52)* | - | - | - | - |
| | 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) | 4.63(2.12)** | - | - | 0.65(0.23)* | 0.16(0.05)** |
| | Rejected Sp Num | 5.78(1.79) | - | 0.61(0.21)** | - | 4.68(0.31)* | - | - | - | - |
| Oki | Remaining Sp Num | - 1.46(0.56) | - | - | 0.06(0.03) | 0.67(0.31)* | 0.57(0.31) | - | - | - |
| | 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 | 291(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. Italics indicate a factor listed in AIC models within 2 at AIC. Asterisks indicate a significant difference by the LRT (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

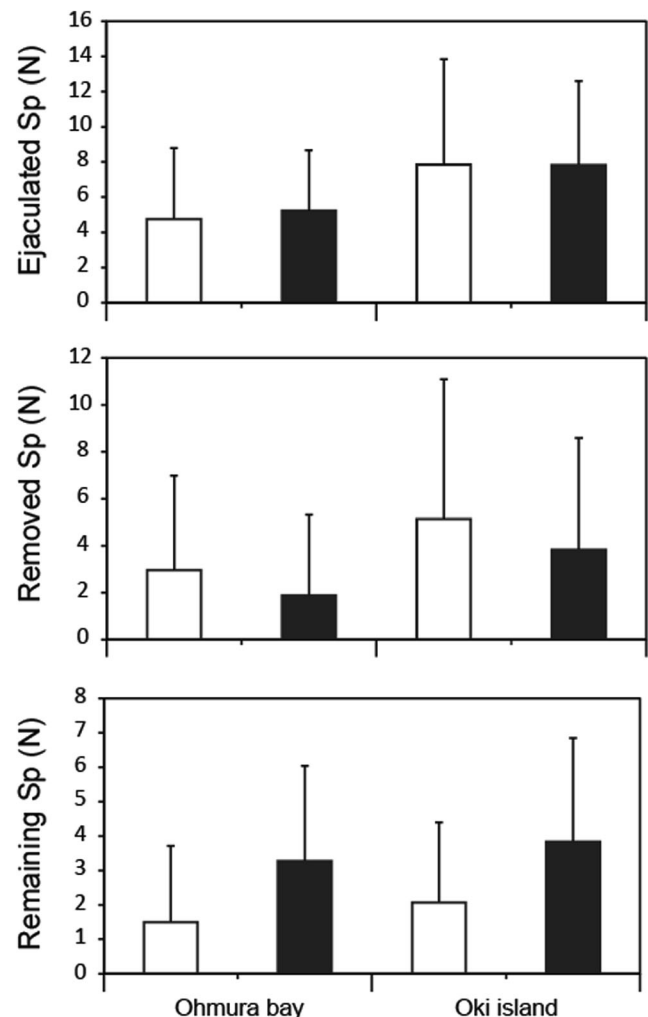


Fig. 3 The average numbers of ejaculated, removed and remaining spermatangia between presence (solid graph) and absence (open graph) of a predator in the two populations. The vertical bars denote the standard deviation. Sp: spermatangia

Discussion

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

The effect of predation risk was remarkable individuals from the Ohmura population where the pygmy squid is exposed to a lower predation risk. In contrast, individuals from the Oki population reported negligible influence by the presence of a predator. This overall pattern could reflect a combination of the Ohmura individuals being highly sensitive to predator presence on account of limited previous experience, and/or the greater experience and potential of local adaptation of Oki individuals to predator presence. Some studies have reported that predation risk influences the intensity of sperm competition. For example, predation decreases sperm competition in the agile frog, *Rana dalmatina* (Lodé et al. 2004). The male frog is selectively preyed by polecats and the sex ratio has less of a male bias in a high-predation-risk environment.

On the other hand, predation was responsible for a higher level of sperm competition in the guppy, *Poecilia reticulata* (Neff et al. 2008; Elgee et al. 2010). Elgee et al. (2010) suggested that female guppies become unresponsive to courtship in the presence of predation, and males change their copulatory behaviour to a “sneaky” type of copulation without courtship, which increases sperm competition. In our study, although the Oki population, which ejaculated and removed more spermatangia than the Ohmura population, may be adapted to a high-predation risk environment, its GSI was lower. The many ejaculations by males may be due to their larger body size and females may remove more spermatangia in response to the greater number of ejaculations. Therefore, predation risk would not reinforce sperm competition in the pygmy squid.

Predation risk had no influence on pre- and peri-copulatory behaviours such as attempted copulation, female resistance to attempted copulation, the duration of copulation and buccal mass elongation. The Japanese pygmy squid is likely already exposed to a high predation risk in its natural environment. This species has the smallest body size among all cephalopods, but they are solitary and do not have any traits that protect their bodies from predatory attacks, such as scales or a shell (Lu and Dunning 1998). Therefore, their reproductive behaviour may already be adapted to predation and they may not need to adjust their behaviour much, even if a predator is present. Their copulation is quite simple in cephalopods, is completed quickly and the females perform CFC. Many costal squid, such as loliginid squid and cuttlefish, perform remarkable displays before copulation, while copulation continues for a long time in the octopus (Hanlon and Messenger 1998). However, copulation duration is not affected by the presence of predators in some animals, including squid (Maier et al. 2000; Taylor et al. 2005; Franklin et al. 2014). Therefore, it is unlikely that predation influences the pre- and peri-copulatory behaviour of the pygmy squid.

The males ejaculated more spermatangia when they were smaller, but the females removed more spermatangia ejaculated by smaller males. Moreover, while Oki males transferred more spermatangia, Oki females removed more spermatangia. These results show that there is sexual conflict between sperm transfer by males and sperm acceptance by females. Arnqvist and Rowe (2005) showed many examples of sexual conflict that occurred after mating (e.g. males ejaculated sperm with seminal toxins and females showed decreased sensitivity to the substances as a counter adaptation). In our results, we do not know the cost for females or why females remove more spermatangia, but females may avoid sperm storage bias for a certain male as genetic “bet-hedging”. The egg batch of Japanese pygmy squid collected in the field was sired by many males and the fertilisation success was not skewed (Sato 2017). Genetic bet-hedging is an important factor to secure genetic diversity and offspring compatibility (Jennions and Petrie 2000; Garcia-Gonzalez et al. 2015). Reproductive

success in the pygmy squid may be influenced by obtaining sperm from various males.

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

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Compliance with ethical standards

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

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