Evidence of sperm removal behaviour in an externally fertilising species and compensatory behaviour for the risk of self-sperm removal

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Abstract

The removal of rival sperm from a female's sperm storage organ acts as a strong sperm competition avoidance mechanism, which has been reported only in internally fertilising species and not at all in externally fertilising species. This study demonstrated for the first time that nest-holding males of *Bathygobius fuscus*, an externally fertilising marine fish, remove the sperm of rival sneaker males from the spawning nest by exhibiting tail-fanning behaviour within the nest. Males showed tail-fanning behaviour when semen was artificially injected into the nest but not when seawater was injected, and in open nests this behaviour resulted in higher paternity rates for the focal male. The sperm removal behaviour entails the risk of removing their own sperm, therefore additional sperm release behaviour is likely necessary to benefit from the sperm removal effect. Consistent with this, males increased post-fanning sperm release behaviour more in the semen than in the seawater injection treatment. Moreover, males who had removed sperm for a longer time spent more time releasing sperm after the removal, suggesting that the additional sperm release behaviour compensated for the loss of their own sperm. These results suggest that sperm removal behaviour is not restricted to internally fertilizing organisms, and deserves further investigation in this and other species.

Keywords: post-copulatory sexual selection; sperm competition; sperm displacement; sperm removal

1. Introduction

Sperm competition over fertilisation among males is a major component of postcopulatory sexual selection [1-3] and can be a strong evolutionary pressure that shapes the evolution of male reproductive traits [2-4]. On the occasion of fertilisation involving sperm competition, the fertilisation success of each male depends on the relative number of their own sperm among those of rival males [2]. Therefore, the most common response by an individual or species under the presence of sperm competition is to increase sperm expenditure at mating [5-7]. However, since sperm production is costly, male ejaculate expenditure is predicted to increase to a maximum with two competitors [risk model; 8-12] but decrease when the number of competitors at a given spawning increases above two [intensity model; 13-17]. On the other hand, tactics that decrease the number of rival sperm and reduce rival's mating opportunities provide advantages for fertilisation [1, 3, 18].

Male sperm removal and displacement behaviour eliminates rival sperm from female reproductive organs before fertilisation avoiding or reducing sperm competition and enhancing male fertilization success [3]. For example, males of the damselfly *Calopteryx maculata* scrape out rival sperm previously deposited in the female's sperm storage organ before copulation with their penis that has a highly specialised morphology [19]. Last-male sperm precedence in fertilisation success is common in species where females accept several matings [9, 18, 20]. One of the reason for this is that the sperm of the last male deposited in the female's sperm storage organ is positionally most likely to be used for fertilisation, but it is possible that some of the rival sperm will be removed by the last male [21]. Similarly, the evolution of male mate-guarding behaviour after copulation may be affected by the presence of rival's sperm removal. Thus, sperm removal and displacement behaviour have an influence not only on the fertilisation success but also on the evolution of various traits associated with sperm competition. Unawareness of the existence of sperm removal behaviour would mislead our understanding of sperm competition dynamics.

Almost all sperm removal and displacement behaviours have been reported in insects [3] and, to the best of our knowledge, other than insects, only in birds [22], cuttlefish [23, 24], crayfish [25], nudibranches [26], and crabs [27]. These are all internally fertilising species [3] and externally fertilising species that perform sperm removal have not been reported to date. Considering the fertilisation mechanism and process, sperm removal is unlikely to occur in externally fertilising species. This may be because in the internally fertilising species, there generally is a certain amount of time between copulation and fertilisation and, during that period, males can remove any

sperm that is present in the female's sperm storage organ that was placed there by rival males. Males of externally fertilising species have no such time interval because ejaculation and fertilisation occur at approximately the same time. In addition, the released sperm of externally fertilising species are easily diffused and mixed, especially in water, making it difficult to remove the sperm of a particular male.

However, we found that males of a small marine fish, the dusky frillgoby *Bathygobius fuscus*, which is an externally fertilising species, exhibited sperm removal-like behaviour. Relatively large males of this species occupy rock holes as spawning nests, court females, and spawn in pairs in the nests (i.e., nest-holding tactics), while relatively small males intrude into the spawning nest and quickly ejaculate in the nest (i.e., sneaking tactics) [28-30]. Nest-holding males aggressively chase sneaker males out of the nests, but also exhibit tail-fanning behaviour towards the nest opening from inside the nest just after the sneaker males have left the nest (Y. Kanatani & A. Nakanishi, personal observation; see the electronic supplementary material for a video): this is clearly different from egg-fanning behaviour in the timing and intensity and from courtship-fanning behaviour in the place where it is performed. This tail-fanning behaviour may have the function of discharging the sperm of the sneaker male to the outside of the nest.

As mentioned above, to remove rival sperm, males need a certain amount of time between ejaculation and fertilisation. *B. fuscus* females intermittently deposit eggs over several hours (ca. 3–4 h; [29]) and sneaker males have long-lived sperm (mean survival rate at 3h after activation= 48.2%, range = 33.3-57.8%; [31]); therefore, sneaker male sperm can fertilise the deposited eggs even after their intrusion (i.e., ejaculation). Thus, nest-holding males have time to remove sneaker male sperm before fertilisation occurs. In addition, sperm released in the nest may be scarcely dispersed in the outside water owing to the closed structure of the nest. Based on this we hypothesise that tail-fanning behaviour of *B. fuscus* nest-holding males has a function of removing rival sperm from the nest. To test this hypothesis, we examined the sperm removal function of the tail-fanning behaviour and its effect on defending the paternity of nest-holding males by manipulating the water exchange in the nest.

If tail-fanning behaviour of nest-holding males has the function of removing sperm from the nest, they must remove some of their own sperm together with that of the rival sperm. This same risk has been shown in other species where copulation (ejaculation) and sperm removal occur at the same time (e.g. [32]). For example, the nudibranch *Chromodoris reticulata* removes rival sperm by withdrawing their penis with many backward-pointing spines after ejaculation, with some of their own sperm

also being removed [33]. Although it is expected that males attempt to reduce or compensate for this risk, there have been few studies on such compensatory behaviour. One example of the former is the adjustment of sperm removal duration in the kisslip cuttlefish *Sepia lycidas*, where males spend less time on sperm removal when they mate with the same female in succession [24]. In the present study, we expected that *B. fuscus* nest-holding males would increase their ejaculation behaviour after sperm removal to compensate for the risk of removing their own sperm as they repeatedly ejaculate during the lengthy female spawning.

2. Materials and methods

(a) Study species

The dusky frillgoby, *Bathygobius fuscus*, is a small marine fish, which mainly inhabits intertidal rocky shores in the Indo-Pacific Ocean including the coastal waters of southern Japan [34]. Relatively large males occupy small rock holes and crevices as spawning nests during the breeding season and court females (i.e., nest-holding tactic) [28, 29]. Spawning occurs between the nest-holding male and a female in the nest in synchronisation with semilunar periods [28, 35], which lasts for an average of 3–4 h [29]. Nest-holding males in some gobiid fishes including *B. fuscus* attach sperm-containing mucus onto the surface of the nest from before to during spawning and the eggs that are laid later are fertilised by the sperm released from the mucus [30, 36-38]. This pre-spawning sperm release behaviour is considered a counter tactic against the sneaking tactic [30, 37, 38] that enables nest-holding males to fertilise eggs before sneaker males [37]. It also allows nest-holding males to invest more time in nest guarding against sneaker males away from the egg-laying female because they do not need to always stay close to the female for ejaculation during the lengthy egg-laying period [36, 39, 40]. Nest-holding males usually accept several clutches of eggs from different females in a single tide [28, 41]. The eggs deposited on the inner surface of the nest are guarded and aerated by the nest-holding males until they hatch (4–5 days), whereas the females exhibit no parental care.

On the other hand, relatively small males do not have nests but intrude into nests where spawning is occurring to achieve parasitic fertilisation (i.e., sneaking tactic, [28, 29]). These sneaker males attach sperm-containing mucus onto the nest surface in the same way as that of the nest-holding males [30]. Sneaker males have relatively larger testes than those of nest-holding males [29] (also see [42]). Furthermore, the sperm of sneaker males are present at a higher concentration in the testes, are longer lived, and decrease in velocity more gradually than the sperm of nest-holding males [31]. Sneaker

males usually change their reproductive tactics into nest-holding tactics when nests and females are available [35], and nest-holding males sometimes adopt sneaking tactics [28].

Specimens used in this study were collected with a hand net in rocky intertidal pools on the Miezaki coast, Nagasaki, Japan (32°48' N; 129°44' E) during the breeding season (early June to late August) of this species. They were sexed by the shape of their genital papillae [43], and males > 73.3 mm total length (TL) were considered as nest-holding males and males < 48.5 mm TL were sneaker males, according to a previous study conducted at the same study site [29]. The males were stocked separately in glass tanks (60 × 30 × 30 cm) that were supplied with aerated seawater (salinity: 30–34‰; water temperature: 25–28°C; 14 h light and 10 h dark photoperiod) and contained 15 cm of water and sand covering the base (2 cm depth). Fish were offered frozen brine shrimp once per day to satiation until the beginning of the experiments.

(b) Experimental setup: artificial sneaking experiment

In this study, sperm removal function of tail-fanning behaviour and its effects on the paternity defence and sperm release behaviour were examined using artificial sneaking method: an injection of semen diluted with seawater into the spawning nest in the absence of sneaker males. Focal tail-fanning behaviour by nest-holding males was observed in the preliminary experiments using the artificial sneaking experiment. This method allowed us to control the timing and volume of ejaculation of the sneaker males.

The experimental glass tanks (45 × 30 × 30 cm) were set up the same as the stock tanks. A halved clay flower pot (upper diameter: 8.5 cm, lower diameter: 5.5 cm, height: 8.5 cm, volume: 166 cm^3) on a brick was attached to the side glass of the tank as the spawning nest (figure S1 in the electronic supplementary material). The nest opening area (7.9 cm^2) was created close to the same size as the average opening area of natural nests (7.4 cm^2; [35]). A silicone tube (inner diameter: 2 mm) was fixed through a hole drilled on the nest ceiling (figure S1) for the injection experiments. To collect the eggs after the experiment for subsequent paternity analysis, a plastic sheet was inserted into the inside wall of the nest. To observe spawning and tail-fanning behaviours, the activity inside the nest was recorded throughout the experiment using a digital video camera with night vision mode (HC-W850, Panasonic).

First, one large male was introduced into the experimental tank as a nest-holding male. After the male occupied the nest, one female was introduced. If spawning occurred, one sneaker male was sacrificed with a lethal dose of quinaldine (1250 ppm) and the testis was immediately isolated. The testis was placed in a laboratory dish and

cut with anatomical scissors. A small piece of testis was dropped into a microtube and the sperm were activated by adding 7 mL of artificial seawater (35‰, 26°C, pH 8.0). This initial sample water was made within 1 min of testis extraction. After 30 min from the beginning of pair spawning, 5 mL of the sample water was gently injected into the nest by a syringe via the silicone tube (ca 0.2 mL/s). To prevent any sperm from remaining in the tube, about 1.5 mL of additional seawater was injected into the tube. Because it was difficult to measure the volume of a single ejaculation by the sneaker males, the sperm concentration in the injection seawater (73–158 × 10^5 cells/mL) was adjusted according to our preliminary injection experiment that was performed in the same way as this study (n = 10). This sperm concentration led to a 3.4%–19.3% paternity of sneaker males, which was similar to the paternity rate obtained by a single real sneaker male intrusion observed in the tank (1.0%–29.2%; Y. Kanatani & T. Takegaki, unpublished data).

The sperm in the remaining 2 mL sample water was stained with rose bengal (0.2%, 28 µL), and fixed with 1.4 mL of 10% formalin. This solution was filtered under vacuum through a membrane filter (MF-Millipore, 0.22 µm pore size × 25 mm diameter, Merck Millipore). The filtered membrane was dried at 40°C for 48 h and then mounted on a slide and cleared with immersion oil [44]. The slide was covered with a coverslip and examined under a phase-contrast microscope (× 400, ECLIPSE Ci-S, Nikon). The sperm were counted in three 25 × 25 µm grids in a field of view and this was performed at four different fields of view. The total number of sperm on each membrane was estimated as the average value of these measurements. The sperm concentration and the total number of injected sperm was calculated from the estimated total number of sperm and the volume of sample water.

(c) Sperm removal function of tail-fanning behaviour

To demonstrate the effect of tail-fanning behaviour on sperm removal, we should have compared between the males exhibiting tail-fanning behaviour and males not exhibiting tail-fanning behaviour. However, in our preliminary experiments (A. Nakanishi & T. Takegaki, unpublished data), only one male did not exhibit tail-fanning behaviour when diluted semen was injected into the nests (n=12). Therefore, in this study, we compared the males exhibiting tail-fanning behaviour in the nests with and without a nest entrance cover (i.e., closed and open treatments, respectively; figure S1). For the closed treatment, the nest entrance was covered with a transparent acrylic board from just before the injection until 150 s after the injection (figure S1); most tail-fanning behaviour was performed within 150 s after injection (91% in the present study). To

minimise water exchange through the gap between the nest entrance and the cover, soft silicone tubing was placed along the rim of the nest entrance. The nest entrance cover treatment controlled not only the tail-fanning behavioural effect but also the diffusion effect; however, the diffusion effect might be much smaller than the fanning effect.

To confirm the sperm removal function of tail-fanning behaviour, the difference in sperm concentration in the nest before and after tail-fanning behaviour was compared between closed (n = 5) and open treatments (n = 6) in the semen injection experiment (table S1 in the electronic supplementary material). Just after the completion of the semen injection, nest water (12.0-21.0 mL) was sampled with a syringe via the tube as the before-removal sample. Then, 150 s after the first sampling, the second sample of nest water (12.2-20.0 mL) was obtained as the after-removal sample. The sperm in the sample water was stained with rose bengal (0.2%, 60-80 μL), and fixed with 10% formalin (2.4-4.0 mL). The sperm concentration of the sample water was measured by the above mentioned method. The sperm concentration of the nest water at the time of the second sampling was estimated by taking the influence of the reduced number of sperm by the first sampling. The experiments were conducted on different males for each treatment.

(d) Tail-fanning behaviour in response to rival sperm

To confirm whether nest-holding males performed tail-fanning behaviour in response to the presence of semen in the nests, a seawater injection experiment was conducted as the control treatment (n = 7; 5 closed and 2 open treatments; table S1). As with the semen injection experiment, after 30 min from the start of pair spawning, 5 mL of seawater was injected into the nests. The tail-fanning behaviour by the nest-holding males was recorded for 10 min before and after the seawater injection, and the time spent fanning and sperm release behaviour was compared with that in the semen injection experiments.

Nest-holding males sometimes perform tail-fanning behaviour to remove sands, seaweeds and silt deposited in the nests. To exclude the possibility of removing sperm as such foreign matters, a silt injection experiment was conducted. The silt was made by homogenizing sand particles with a mortar and pestle and by sieving (grain size = 5-75 μm). They were dried 48h at 80°C and then mixed with seawater (silt concentration: 0.2%). The silt containing seawater was whitish, which was closely resembling the semen containing seawater used in this study. The silt injection was performed at 30 min after 7 of the 11 semen injection experiment (table S1), all of which were done without a nest entrance cover. The occurrence of tail-fanning behaviour was observed

for 10 min after injections. Moreover, to ensure the male response to the sperm, 30 min after silt injection, semen injection experiment was conducted again in 3 of the 7 cases that the spawning had continued until that time (table S1).

(e) Effect of tail-fanning behaviour on paternity defence

To examine the effect of tail-fanning behaviour by the nest-holding males on their paternity defence, semen injection experiments were conducted for closed (n = 6) and open (n = 6) treatments (table S1). To control for the effects of the length of pair spawning on the fertilisation success of each male, spawning behaviour was terminated by lifting the nest at 60 min after the semen injection (figure S1). After the experiment, the eggs deposited on the plastic sheet (2207–8791 eggs, n = 12) were collected from the nests and incubated in another tank until hatching (3–4 days). The newly hatched larvae were anesthetised with quinaldine (600 ppm) and fixed with 99% ethanol for paternity analysis. There was no size difference in nest-holding males between open (mean TL ± SD = 73.68 ± 4.79 mm, range = 67.10–79.75 mm) and closed (75.61 ± 4.73 mm, 68.70–82.20 mm) treatments (two-sample t-test, $t = 0.70$, $p = 0.50$).

(f) Sperm release after sperm removal

Sperm removal by *B. fuscus* nest-holding males entails a risk of removal of their own sperm. We expected that nest-holding males would increase sperm release behaviour after sperm removal to compensate for this risk. To test this hypothesis, time spent on sperm release behaviour 10 min before and after tail-fanning behaviour was observed in the semen injection experiments for paternity defence (n = 12; 6 with cover and 6 without cover) and seawater injection experiments (n = 7; 5 with cover and 2 without cover; table S1). Sperm release behaviour is the behaviour of rubbing its genital papilla onto the nest substrate with a wriggling body movement, which is clearly differentiated from sperm removal and egg-fanning behaviours.

(g) Primer development and paternity analysis

We developed three DNA markers and then genotyped 12 data sets consisted of 12 nest-holding males, 12 sneaker males, 12 females, and 1087 embryos (85–93 embryos each) (table S2 in the electronic supplementary material). Paternity was inferred using the exclusion methods described in detail previously [45].

(g) Statistical analysis

The presence of tail-fanning behaviour by the nest-holding males was compared between semen and seawater injection experiments by Fisher's exact test. The proportion of sperm remaining in the nest after semen injection was compared between open and closed treatment by two-sample t-test.

The time spent on tail-fanning behaviour by nest-holding males was compared between open and closed treatments by a two-sample t-test. The effect of the amount of rival sperm on tail-fanning behaviour was analysed by a generalised linear model (GLM). Since the response variable (time spent on tail-fanning behaviour) is a continuous variable that does not include zero value, a gamma distribution (log-link function) was used in this analysis. The effect of the number of injected sperm was treated as an explanatory variable. Male body size (TL) was included in this model as an explanatory variable because body size may affect the water exchange effect in the nest [46]. To examine the effect of tail-fanning behaviour on paternity defence of the nest-holding males, a GLM with a binomial distribution and logit link function was performed in the semen injecting experiments. The paternity rate was treated as a response variable and the nest entrance treatment (open or closed), the duration of sperm release behaviour 10 min before and after injection, and the number of injected sperm were treated as explanatory variables.

To examine the effects of semen injection and tail-fanning behaviour on the subsequent sperm release behaviour, GLMs with Gaussian distribution and log-link function were used. However, the dataset was highly unbalanced because tail-fanning behaviour was not observed in the seawater injection treatment except one male (details in Results). Therefore, in this study, the effect of tail-fanning behaviour was analysed using only the data of semen injection treatment. In the first analysis, the difference in time spent for sperm release behaviour between the before and after injection experiment was treated as a response variable, and the injection treatment (semen or seawater) and nest entrance treatment (open or closed) were treated as explanatory variables, and in the second analysis, the time spent for tail-fanning behaviour and the nest entrance treatment were treated as explanatory variables. In these GLM analyses, the significance of the fixed effects was assessed with a likelihood ratio test using chi-square approximation. All statistical analyses were performed with R version 3. 5. 1 [47].

3. Results

(a) Occurrence of tail-fanning behaviour and its effect on discharging sperm

In the semen injection experiments, tail-fanning behaviour by the nest-holding males was observed after injection in all 26 experiments irrespective of entrance cover treatment (mean time spent for fanning ± SD = 31.1 ± 26.9 s/10 min, range = 3–112 s/10 min, n = 23, not including 2nd semen injection experiments), whereas one male also fanned before the injection (3 s). In the seawater injection experiment (n = 7), there was no male exhibiting tail-fanning behaviour, except for one male (3 s after injection). The tail-fanning behaviour occurred at a significantly higher rate in the semen injection experiment than in the seawater injection experiments (Fisher's exact test, $p < 0.0001$, n = 29; one male which fanned before the semen injection was removed from the analysis). All females continued egg laying during and after the semen injection without going out of the nest. No females exhibited tail-fanning behaviour.

The remaining proportion of sperm at 150 s after semen injection was significantly higher in the closed treatment (mean ± SD = 65.9 ± 14.4 %, range = 47.9-80.5 %, n = 5) than in the open treatment (12.4 ± 8.8 %, 1.9-23.7 %, n = 6; two-sample t-test, $t = 7.59$, $p < 0.0001$). Silt injection did not induce tail-fanning behaviour of the nest-holding males (n = 7) except one male (4 s): the occurrence rate of tail-fanning behaviour was significantly different from that of the semen injecting experiment (Fisher's exact test, $p < 0.0001$, n =29). The 2nd sperm injection subsequent to the silt injection experiments induced tail-fanning behaviour in all cases (n = 3).

(b) Effect of sperm removal on paternity defence

In the semen injection experiment, the time spent on tail-fanning behaviour by nest-holding males did not differ between open (27.8 ± 15.8 s/10 min, range = 7–43 s/10 min, n = 6) and closed (44.0 ± 40.1 s/10 min, range = 6–112 s/10 min, n = 6) treatments (two-sample t-test, $t = 0.92$, $p = 0.38$). Neither the number of injected sperm of sneaker males nor the body size of the nest-holding males affected the time spent on tail-fanning behaviour (table 1). No females exhibited tail-fanning behaviour.

Nest-holding males showed significantly higher paternity rates in the open treatment than that in the closed treatment (table 2). The paternity rate decreased with the number of injected sperm of sneaker males but was not affected by the time spent for sperm release behaviour before and after sperm injection (table 2).

(c) Sperm release after sperm removal

In the semen injection experiments, 9 out of the 12 nest-holding males increased their time spent on sperm release behaviour after the injection. The difference in time spent

on sperm release behaviour before and after semen injection was significantly larger than that in the seawater injection experiments (figure 1 and table 3). In the semen injection experiments, males that had spent more time on tail-fanning behaviour had a higher sperm release behaviour after injection (figure 2 and table 4).

4. Discussion

(a) Evidence of sperm removal function in externally fertilising species

This study strongly suggests that the tail-fanning behaviour of nest-holding males of *Bathygobius fuscus* just after sneaker male intrusion has a function of removing rival sperm to outside the nest and contribute to defend their paternity. Firstly, all nest-holding males exhibited tail-fanning behaviour directed towards the nest opening when injecting sperm of sneaker males into the nest, whereas no tail-fanning behaviour was observed in the seawater and silt injection experiments, except for one male. Secondly, tail-fanning had an effect of decreasing sperm number in the nest by exchanging nest water with outside water, though the water diffusion might have partially affected: the number of sperm decreased more in the open nest-entrance treatment than in the closed treatment. Thirdly, the sperm removal behaviour contributed to defend the paternity of nest-holding males: lower paternity rate was observed in the closed treatment. To the best of our knowledge, this is the first study that shows sperm removal behaviour for externally fertilising species.

No previous studies have reported on sperm removal behaviour in externally fertilising species. One of the obvious reasons is the lack of time for the removal of sperm because ejaculation and fertilisation occur at approximately the same time in most species. However, in internally fertilising species, there is a time interval between copulation and fertilisation, during which time the males can remove the sperm present in the spermatheca that was placed there by rival males. This difference in the fertilisation mode makes it difficult to evolve sperm removal behaviour in externally fertilising species. Although *B. fuscus* is an externally fertilising species, the nest-holding males do have time to remove sperm between ejaculation and fertilisation because of the long-lasting intermittent female egg deposition (3–4 h) and extremely long-lived sperm (mean survival rate at 3h after activation= 48.2%; [31]). This species also utilise a spatially closed nest for spawning, such as a rock hole or crevice. In general, released sperm of externally fertilising species are easily diffused, especially in water [48]; however, the sperm of *B. fuscus* may be retained in the nest for a long period. Since similar reproductive characteristics including sperm release behaviour are

observed in several gobies, such as *G. niger, Zosterisessor ophiocephalus, Knipowitschia panizzae, Pomatoschistus minutus* [36-39], they may have a potential for removal of sperm. Moreover, many anuran species seem to have enough time to remove rival sperm. For example, in a Leptodactylid frog *Leptodactylus chaquensis,* when the spawning starts, not only paired male but also sneaker males churn the foam nest by kicking with their legs [49]. This churning behaviour is considered to promote fertilization of their own sperm, but it also suggests that males may have time to remove rival sperm and that this behaviour may have a function of removing sperm.

The sperm removal behaviour of nest-holding males was induced in the semen injection experiments, implying that the presence of sneaker males and their nest intrusion are not essential stimuli for the occurrence of sperm removal behaviour. In addition, nest-holding males showed almost no reaction before semen injection and after seawater and silt injection, even though their own sperm was present in the nest. These results suggest that nest-holding males can recognise sperm and/or semen of other males probably by chemical stimulation. For example, territorial males of the black goby *Gobius niger* increase aggressive behaviour in response to the sperm of other territorial males through the stimulation by the steroid produced by the mesorchial gland that acts as a sexual pheromone [50]. On the other hand, nest-holding males of *B. fuscus* may not be capable of recognising the amount of injected sperm of rival males. This is because they did not remove the sperm for a longer period when a higher concentration (i.e., amount) of rival sperm was injected, and thus the concentration of the injected sperm had a strong negative effect on the paternity rate of nest-holding males.

Sperm removal directly affects on the male reproductive success and could be a strong selection pressure that shapes the related reproductive traits. Actually, in the present study, the fertilisation success of sneaker males was decreased to one-third, on average, by sperm removal and the subsequent sperm release by nest-holding males. Therefore, if the existence of sperm removal behaviour is overlooked, the evolution of relevant traits could be misunderstood. For example, the enlarged testes of sneaker males of *B. fuscus* is considered to be evolved to increase the ejaculate volume under a high risk of sperm competition with nest-holding males; however, testes size is not related to the fertilisation success of sneaker males (Kanatani Y & Takegaki T, unpublished data). The effects of testes and ejaculate size might be masked by the sperm removal effect. Moreover, fanning behaviour in fish is known as a typical behaviour for egg care to provide oxygen but also act as a courtship display [51]. It is generally considered that females choose males providing high quality care of eggs on the basis of fanning behaviour [51], but sperm removal fanning that prevents sneaker

males from fertilization might be associated with the evolution of male fanning behaviour as a sexual ornament, because sneaker participation in spawning is expected to provide benefits and costs in various aspects for females [52]. Thus, our findings suggest that the framework for post-copulatory sexual selection in externally fertilising species needs to be extended in future studies.

(b) Compensation for the risk of removal of own sperm

Generally, in species exhibiting male sperm removal behaviour, males remove rival sperm before copulation and ejaculation, and therefore the removal of their own sperm does not occur. However, for example, males of the beetle *Tenebrio molitor* [32] and nudibranch *Chromodoris reticulata* [33] remove their own sperm together with rival sperm because copulation and sperm removal occur at the same time. Nest-holding males of *B. fuscus* also entail the risk of removing their own sperm from the nest because their sperm are always present in the nest during and even before spawning. Sperm concentration in the nest decreased to 13% in average at about 180 s after injection (open treatment) suggesting a high effect of sperm removal behaviour and high risk of the removal of own sperm. Assuming that the sperm of sneaker males and nest-holding males are removed from the nest at the same rate, nest-holding males can obtain the effect of sperm removal on paternity defence only by increasing more own sperm after sperm removal. Additional sperm of the nest-holding males are released from the mucus attached on the inside wall of the nest before and after sperm removal [30]. Because sneaker males also attach sperm-containing mucus at the sneaker male intrusion [30], there is a potential for an increase in sneaker sperm after removal, even though sneaker sperm did not increase after sperm removal in the present study because the sperm were artificially injected into the nest water. The sperm attached by sneaker males may not be retained on the nest wall for longer than those attached by nest-holding males because sneaker males have much smaller sperm duct glands, the reproductive accessory organs near the testes that produce mucus, than that of nest-holding males. In our previous tank observation, sperm attached by a sneaker male was large enough in volume to be visible; however, the sperm mass disappeared into the water in seconds ([30]; http://www.momo-p.com/index.php?movieid=momo161222bf01b&embed=on). Thus, the sperm of sneaker males may increase temporarily just after the sneaker male intrusion, yet, their sperm dissolved out from the mucus after sperm removal would be much less than those of nest-holding males.

During the lengthy female egg deposition period, nest-holding males intermittently repeat sperm release behaviour even in the absence of sneaker male intrusion. In the present study, the nest-holding males increased their time spent on sperm release behaviour after semen injection compared to the seawater injection treatment. A probable reason for the increase in sperm release behaviour is a response to the increased risk of sperm competition due to the presence of rival sperm. For example, nest-holding males of the sand goby *P. minutus* attach sperm-containing mucus more frequently in the presence of sneaker males [38]. More noteworthy is that *B. fuscus* nest-holding males who had removed sperm for a longer time spent more time to release sperm after semen injection. As is the case with the sperm competition risk, it is possible that nest-holding males removed and released sperm more by recognizing intensity of sperm competition from the amount of injected sperm. However, it cannot reasonably be assumed that they can recognize the amount of sperm in the nest, because sperm removal duration was not affected by the number of injected sperm (table 2) and nest entrance manipulation did not affect sperm release duration (table 4). Thus, the change in sperm release duration with the sperm removal duration may not be as a result of response to the intensity of sperm competition.

Another possibility is that the sperm release behaviour just after sperm removal is compensating for the loss of their own sperm that was removed by their sperm removal behaviour. Although all nest-holding males performed sperm removal behaviour when injecting rival sperm, they all lost a part of their paternity and their paternity rate was strongly influenced by the amount of injected sperm, despite the sperm removal and additional sperm release behaviours. These imply that sperm removal was not complete and the subsequent sperm release behaviour might not be enough to overwhelm the remaining sneaker male sperm. The presence of extremely short removal behaviour suggests that the imperfect removal may be mainly caused by the removal risk of own sperm rather than energy cost of removal behaviour: the removal risk entails the cost of adding sperm after the removal. Furthermore, as mentioned above, there is a high possibility that nest-holding males can not recognize the amount of sperm in the nest. If they can not recognize how much sperm they removed and remaining in the nest, adjusting the sperm release duration based on the duration of just preceding removal behaviour might be one of the effective strategy to maintain the amount and proportion of own sperm. The behaviour to reduce the removal risk of self sperm has been reported in the cuttlefish [24], but there has been no study showing compensatory behaviour for the risk of self-sperm removal.

Risk-taking behaviour entails a trade-off between cost and benefit, and the decision-making process has been mainly considered in the optimisation model.

However, an extended model should be developed to understand the sperm removal behaviour of *B. fuscus* nest-holding males. In their sperm removal, there seems to be a risk of removal of their own sperm and a benefit of removing rival sperm, and the cost arising form the risk and the benefit would be closely tied to each other. However, the proportion of both males' sperm within the nest after removal is constant if non-selective removal occurs; therefore, both the cost and benefit may not occur from removal behaviour itself. The benefit (i.e., evolution) of sperm removal behaviour must be considered together with the subsequent additional sperm release behaviour, similar to the sperm removal and subsequent copulation in many sperm-removing species. The amount of sperm removed by nest-holding males may be affected by how much additional sperm they can produce because the energy cost of sperm release behaviour might be much larger than that of removal behaviour due to the extra costs associated with sperm production. To achieve a comprehensive understanding of sperm removal and subsequent sperm-releasing behaviours, first of all, the dynamics of the amount of sperm released by males of different tactics must be investigated.

Ethics. These experiments and observations were approved by the Animal Care and Use Committee of the Faculty of Fisheries, Nagasaki University (permission no. NF-0008 and 0022), in accordance with the Guidelines for Animal Experimentation of Faculty of Fisheries (fish, amphibians, and invertebrates), Nagasaki University.

Data accessibility. DNA sequences: DDBJ accessions DRA008291. Data available from the Mendeley Data: http://dx.doi.org/10.17632/6sx3nbkb7w.3 [53].

Authors' contributions. Conceptualisation, A.N., S.K., and T.T.; Methodology, A.N., Y.K., S.K., M.Y., N.S., and T. T.; Investigation, A.N., Y.K., and T.T.; Writing – Original Draft, A.N. and T.T.; Writing – Review & Editing, T.T.; Project Administration and Funding Acquisition, A.N. and T.T.

Competing interests. We declare no competing interests.

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Table 1. Effects of the number of injected sperm and body size (TL) of nest-holding (NH) males on time spent on tail-fanning behaviour.

	Estimate	SE	LRT $\chi 2$	*P*
Intercept	0.022	0.120	–	–
No. injected sperm	0.000003	0.000006	0.223	0.637
NH body size	−0.0001	0.0016	0.007	0.932

Table 2. Effects of the number of injected sperm, the presence of entrance cover (open or closed) and sperm release duration on paternity rate. The estimates for the effects of entrance cover use the closed treatment as the reference factor level.

	Estimate	SE	LRT χ 2	*P*
Intercept	3.214	0.563	–	–
Entrance cover (open)	1.406	0.248	38.030	6.97E−10
Sperm release duration	0.002	0.002	0.624	0.430
No. injected sperm	−0.0003	0.0001	11.106	0.00086

Table 3. Effects of the presence of entrance cover (open or closed) and the injection treatment (semen or seawater) on the difference in time spent for sperm release behaviour between before and after the injection. The estimates for the effects of entrance cover and injection treatment use the closed treatment and seawater treatment as the reference factor level, respectively.

	Estimate	SE	LRT $\chi 2$	*P*
Intercept	−8.548	8.109	–	–
Entrance cover (open)	2.419	9.595	0.075	0.78
Injection treatment (semen)	21.005	9.821	4.778	0.029

Table 4. Effects of the presence of entrance cover (open or closed) and fanning duration on the difference in time spent for sperm release behaviour between before and after the semen injection. The estimates for the effects of entrance cover use the closed treatment as the reference factor level.

	Estimate	SE	LRT χ 2	*P*
Intercept	4.422	8.400		–
Fanning duration	0.331	0.142	5.657	0.017
Entrance cover (open)	−5.310	8.247	0.541	0.46

Figure caption

Figure 1. Comparison of the difference in time spent on sperm release behaviour before and after injection between the semen (n = 12) and seawater (n = 6) injection treatments (details in table S1). The boxplots show medians, 25% and 75% quartiles, 10% and 90% percentiles (whiskers), and outliers (dots).

Figure 2. The relationship between the difference in time spent on sperm release behaviour before and after semen injection and time spent on sperm removal behaviour (n = 12; details in table S1).

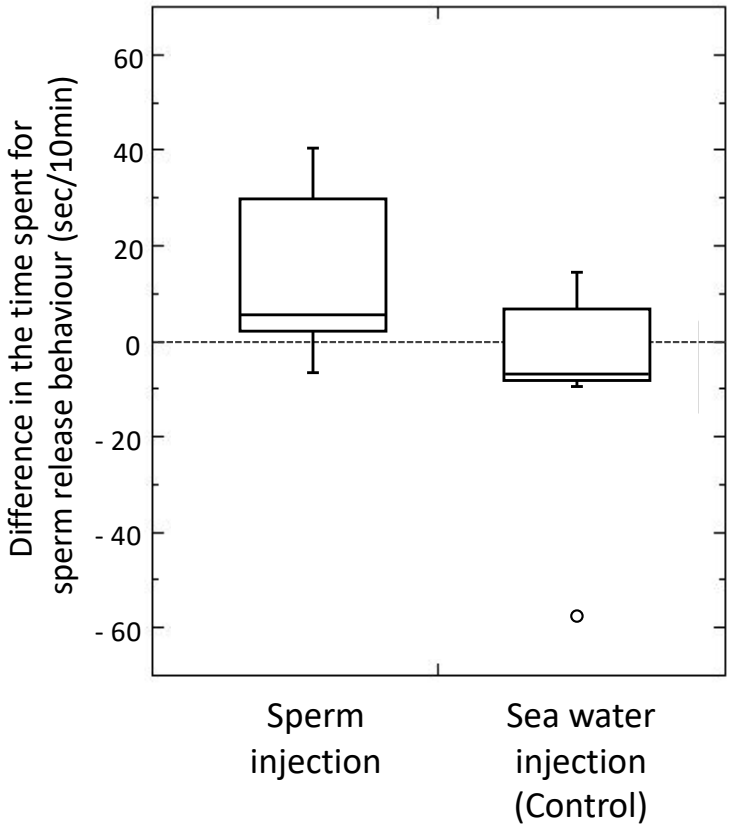
Difference in the time spent for sperm release behaviour (sec/10min)
60
40
20
0
- 20
- 40
- 60
Sperm injection
Sea water injection (Control)

figure 1

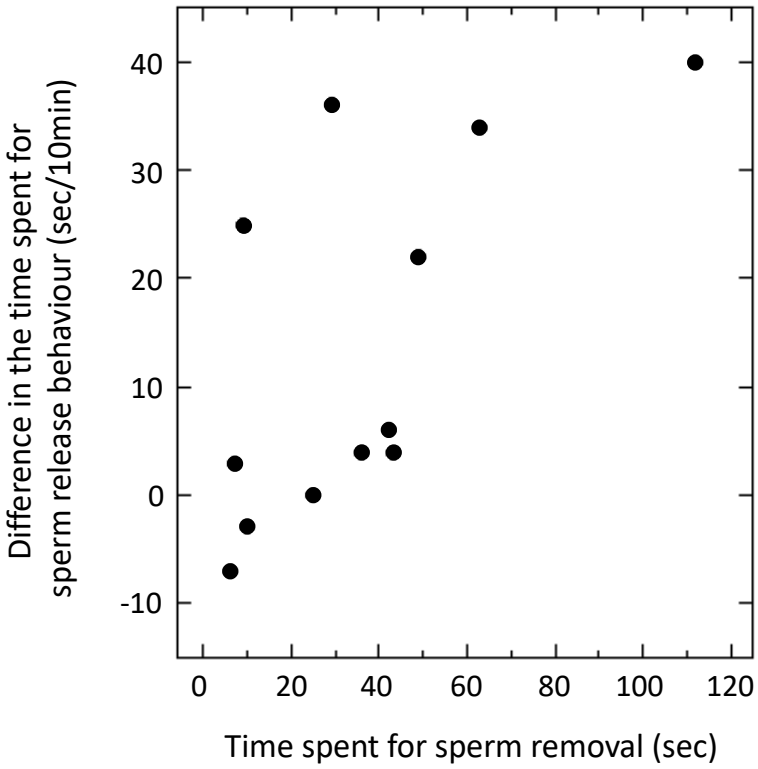
Difference in the time spent for sperm release behaviour (sec/10min)
40
30
20
10
0
-10
0
20
40
60
80
100
120
Time spent for sperm removal (sec)

figure 2

Table S1. Sample size for each injection experiment with different entrance treatment (open & closed). The kinds of experiments corresponding to each Result section are shown by grey bars. Note that silt injection experiments were conducted after a part of semen injection experiments (1st), and 2nd semen injection exeriments were conducted after a part of silt injection experiments (arrows show the flow of these experiments). See details in Materials and method section.

Result section		(a) Occurrence of tail-fanning behaviour and its effect on discharging sperm			(b) Effect of sperm removal on paternity defence	(c) Sperm release after sperm removal
Injection experiment		Semen (1st)	Silt	Semen (2nd)	Seawater	Semen
Entrance treatment	Open	5 →	7 →	3	2	6
	Closed	6 ↗	–	–	5	6

Table S2. Characteristics of the microsatellite loci of *Bathygobius fuscus* used for parentage analysis

Locus	Repeat motif	Primer sequence (5'–3')	Size range	No. alleles	H_O	H_E	Null allele frequency	GenBank Accession No.	Exclusion probability
Kumohaze2*	$(TCAT)_{16}$	F:GGCTTTGCAGTGAACCAGTG R:GTGGCACCTTTTCTACTTAAAAGT	73–137	15	0.657	0.903	0.150	LC461190	0.778
Kumohaze13	$(ACT)_{21}$	F:ATGCCTGTCAACCCCACAAT R: ACCGTCAAAGACCTGATAGCTC	120–238	26	0.911	0.953	0.017	LC461191	0.883
Kumohaze15	$(TCT)_{21}$	F: ACATGGACGGCTGGTATAAGTC R: TCCTGGTTATGACACAAAAGTGA	114–290	31	0.864	0.960	0.049	LC461192	0.896
								Combined EP	0.997

H_O: observed heterozygosity; H_E: expected heterozygosity; *: indicates significant deviation from Hardy–Weinberg equilibrium ($P < 0.01$); EP: Exclusion probability

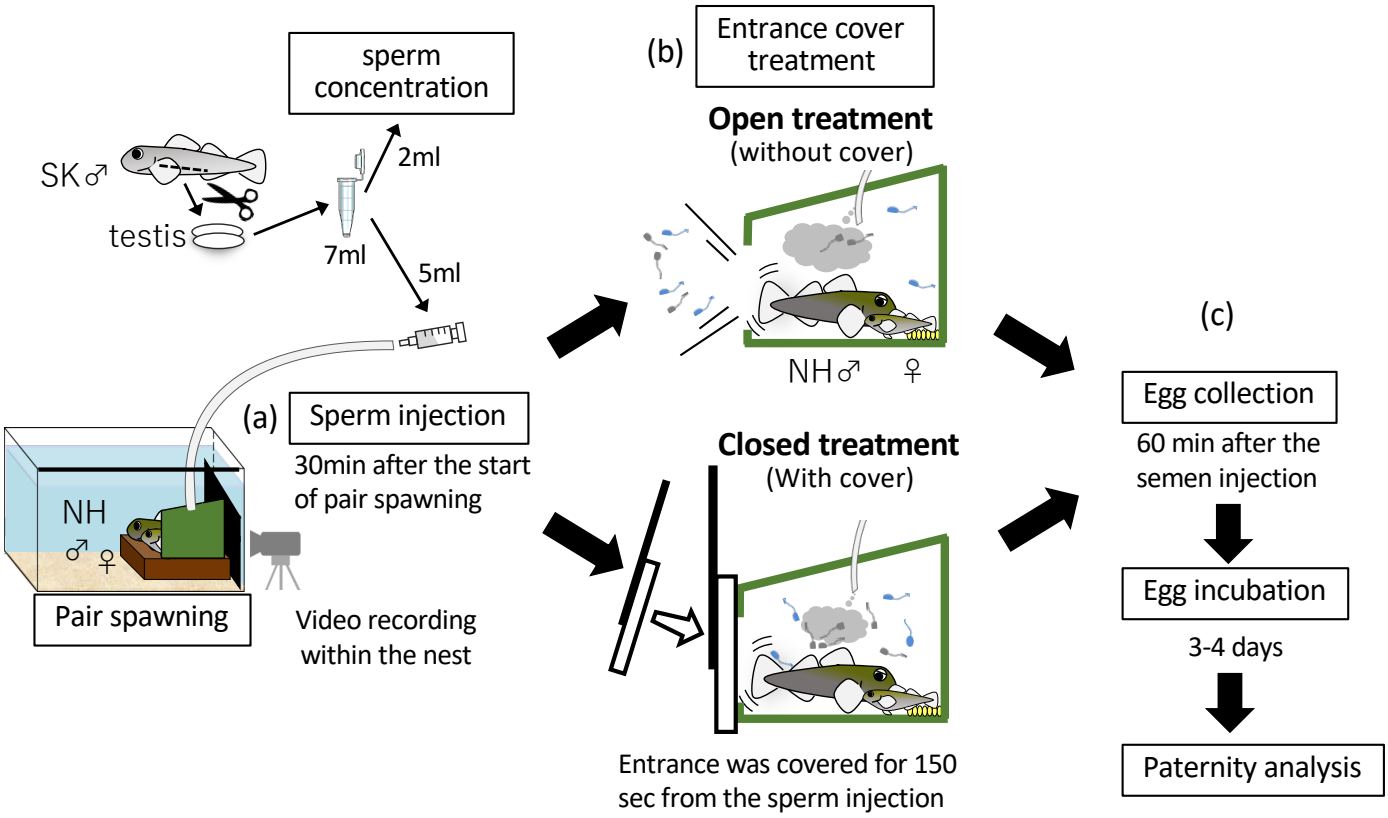

Figure S1. Illustration of the artificial sneaking experiment. (a) After 30 min of the start of pair spawning, 5 mL of the sample water (semen or seawater) was injected into the nest through a tube. (b) A nest entrance cover was used (or not) to control the effect of tail-fanning behaviour on sperm removal from the nest (open and closed treatment). For the closed treatment, the nest entrance was covered from just before the injection until 150 s after the injection. (c) Spawning behaviour was terminated at 60 min after the semen injection to control for the effects of the length of spawning on fertilisation success. The eggs were incubated until hatching (3–4 days) and the newly hatched larvae were used for paternity analysis.