Spermatangium formation and sperm discharge in the Japanese pygmy squid *Idiosepius paradoxus*

Noriyosi Sato[a,b,*], Takashi Kasugai[c], Hiroyuki Munehara[d]

[a] *Division of Biosphere Science, Graduate School of Environmental Science, Hokkaido University, Hokkaido 0600810, Japan*

[b] *Graduate School of Fisheries Science and Environmental Studies, Nagasaki University, Nagasaki 8528521, Japan*

[c] *Port of Nagoya Public Aquarium, Aichi 4550033, Japan*

[d] *Usujiri Fisheries Station, Field Science Centre for Northern Biosphere, Hokkaido University, Hokkaido 0411613, Japan*

[*] Corresponding author. Tel: +81 95 819 2819.

E-mail address: norico3000@gmail.com (N. Sato).

20 pages of text

9 figures

1 table

1 movie (supplementary component)

ABSTRACT

In cephalopods, sperm discharge is an important event not only for sperm transfer but also influencing sperm storage capacity of attached spermatangia (everted spermatophores). To investigate sperm discharge from spermatangia and the condition of naturally attached spermatangia in Japanese pygmy squid (*Idiosepius paradoxus*) we (i) investigated the morphology of spermatophores and spermatangia, and the process of spermatophore evagination and sperm discharge from spermatangia obtained in vitro; (ii) observed spermatangia that were naturally attached to female squids at 6, 12, 18, 24 and 48 h after copulation to investigate alterations in naturally attached spermatangia with time. The spermatophore of *I. paradoxus* is slender and cylindrical and consists of a sperm mass, a cement body and an ejaculatory apparatus, which is similar to those of loliginid squids. The spermatangium is fishhook-shaped, its distal end being open and narrow. After the spermatangium is formed, the sperm mass gradually moves to the open end of the spermatangium, from where sperm are released. Sperm discharge is a rapid process immediately after the beginning of sperm release, but within 5 min changes to an intermittent release of sperm. Although the volume of residual spermatozoa differed among spermatangia that were naturally attached to a single individual, the probability that spermatangia would be empty increased with time. Most naturally attached spermatangia discharged almost all of their spermatozoa within 24 h after copulation, and no spermatangia were attached to females 48 h after copulation. These results suggest that sperm transfer from the spermatangium to the seminal receptacle must occur within 24 h, and that the spermatangium functions as a transient sperm storage organ in pygmy squids.

Keywords: Cephalopoda; *Idiosepius paradoxus*; Sperm storage; Sperm transfer; Spermatophoric reaction

1. Introduction

Sperm transfer is a complex process in cephalopods, with males transferring intricate spermatophores to females during copulation (Mangold, 1987; Hanlon and Messenger, 1996). Through the so-called “spermatophoric reaction”, the spermatophore everts itself, forming a spermatangium (Austin et al., 1964; Mann et al., 1966; Takahama et al., 1991) which is attached to the female body through distinct mechanisms, e.g., mechanical anchorage provided by the ejaculatory apparatus and chemical adhesion by the cement body (Marian, 2012a, b). Additionally, several female decapodiforms bear sperm storage organs (called “seminal receptacles”) on their buccal membrane, e.g., *Loligo forbesi* (Lum-Kong, 1992), *Loligo pealii* (Drew, 1911), *Loligo vulgaris* (van Oordt, 1938), *Sepia apama* (Naud et al., 2005), *Sepia officinalis* (Hanlon et al., 1999), *Todarodes pacificus* (Ikeda et al., 1993). Other decapodiforms have specialized receptacles for spermatophores, such as nuchal receptacles (e.g., *Lycoteuthis lorigera*, Hoving et al., 2007) or a posterior seminal sac (e.g., *Heteroteuthis dispar*, Hoving et al., 2008). In some oceanic and deep-sea squids, however, spermatangia are implanted externally and special receptacles are absent (Hoving and Laptikhovsky, 2007; Hoving et al., 2009). In cases where a sperm storage organ is present, it is not known how the spermatozoa reach the seminal receptacle from the attached spermatangia; the spermatangia are attached externally on the buccal membrane, but sperm are stored inside the seminal receptacle (Drew, 1911; van Oordt, 1938; Lum-Kong, 1992).

A few studies have attempted to explain this process through direct observation of spermatophores, spermatangia and the spermatophoric reaction, or by investigating the morphology of the seminal receptacle (e.g. Drew, 1919; van Oordt, 1938; Austin et al., 1964; Mann et al., 1966, 1970; Takahama et al., 1991; Lum-Kong, 1992; Sato et al., 2010; Marian, 2012a; Marian and Domaneschi, 2012). In *Loligo pealii*, Drew (1919) observed that the distal tip of the spermatangium is open after the spermatophoric reaction, with spermatozoa being immediately released from the opening and becoming active in contact with seawater. The same pro-

cess of immediate sperm discharge from spermatangia was reported for *Doryteuthis plei* (Marian 2012a). Histological evidence from spermatozoa stored in seminal receptacles suggests that sperm reach the seminal receptacle from the spermatangia by actively swimming (Drew, 1919; Sato et al., 2010). Therefore, understanding sperm discharge from spermatangia is an important step towards a thorough comprehension of sperm transfer mechanisms in squids.

Attached spermatangia may also function as a means of sperm storage; some studies reported that sperm from attached spermatangia contribute directly to fertilization (e.g., in *S. apama*; Naud et al., 2005). In *L. bleekeri*, males that attach spermatangia inside the female mantle have higher fertilization success (Iwata et al., 2005). While sperm discharge is related to fertilization success, it also influences sperm depletion in spermatangia, affecting how long spermatangia store sperm on the female body. Sperm discharge is thus an important event not only for sperm transfer but also for both the fertilization ability and sperm storage capacity of attached spermatangia. Nevertheless, knowledge of these processes remains deficient.

The Japanese pygmy squid *Idiosepius paradoxus* mates in the head-to-head position (Kasugai, 2000; Nabhitabhata and Suwanamala, 2008). During copulation, the male squid darts towards the female, grasping her body and attaching spermatangia at the arm base (Kasugai, 2000; Sato et al., 2013b). Females have a seminal receptacle in the ventral portion of the buccal membrane (Sato et al., 2010). The pygmy squid is an ideal species for studying sperm discharge because, apart from the ease of culturing and maintaining live animals, the location of spermatophore placement is distinct from the site of sperm storage, which implies that sperm discharge plays an important role in sperm transfer. In the present study, we describe the gross morphology of spermatophores and spermatangia and the spermatophoric reaction of the Japanese pygmy squid, and we investigate sperm discharge from spermatangia obtained in vivo. Additionally, we investigate the condition of naturally attached spermatangia and sperm discharge in vivo.

2. Materials and methods

2.1. Sample collection

Mature pygmy squids were collected with a small drag net (1 m × 2 m, mesh size: 1.5 mm) near small stocks of the seagrass *Zostera marina* in nearshore waters of the Chita Peninsula, central Honshu, Japan (34°71'N, 136°97'E), on 29 April 2009 and 14 March 2013. Living specimens collected in 2009 were transported by parcel delivery service to the Usujiri Fisheries Station, Field Science Center for Northern Biosphere, Hokkaido University (41°94'N, 140°95'E) for in vitro observation of spermatophores, the spermatophoric reaction, spermatangia and sperm discharge. Living specimens collected in 2013 were transported to Nagasaki University, Japan (32°79'N, 129°86'E) for in vivo observation of naturally attached spermatangia.

2.2. In vitro observation of spermatophores, the spermatophoric reaction, spermatangia and sperm discharge

All pygmy squids were maintained in an aquarium (60 cm × 45 cm × 45 cm) with a closed circulation system until the start of the experiment. Before dissection, pygmy squids were anaesthetized with 1% ethanol (Sato et al., 2013a). Spermatophoric sacs containing spermatophores were removed from 31 male squids (dorsal mantle length (DML): 8.69 mm ± 1.00 SD). The number of spermatophores contained in a spermatophoric sac ranged from 5 to 50. We used 60 spermatophores from 12 males for in vitro observation. Spermatophoric sacs were either opened immediately after dissection or left unopened overnight at 4 °C in small dishes containing seawater and opened the next day. To observe the morphology of spermatophores and spermatangia, the spermatophoric reaction and sperm discharge, spermatophores were transferred from a freshly opened sac into a Petri dish filled with sea water at 20 °C. The spermatophoric reaction was induced by physical stimulation with a paper string (created by

twisting a Kimwipe into a string; Kimberly-Clark Corp., Irving, TX, USA) at the oral region of the spermatophore, which was placed on a glass slide with seawater for observation (Fig. 1A). Observation was conducted using a microscope and photographs were taken with a digital camera (VB-7010; Keyence Corp., Osaka, Japan). Nomenclature follows Marian (2012a) and Marian and Domaneschi (2012).

2.3. In vivo observation of spermatangia

Based on the presence of white testes in males, and ripe eggs, nidamental glands and a larger body size in females, all pygmy squids were separated by sex and maintained in two aquaria (60 cm × 45 cm × 45 cm) with closed circulation systems under a 14/8 h light/dark photoperiod; the aquaria were exposed to outdoor air temperatures, which ranged from 12 to 14 °C. Pygmy squids were fed live mysid shrimp (*Neomysis intermedia*) twice daily. Five plastic plates (1 cm × 30 cm) were placed on the sand bottom in each aquarium as an adhering substrate for pygmy squids.

To conduct the experiments, two males and one female were introduced into an experimental aquarium (60 cm × 45 cm × 45 cm). Before female introduction we confirmed that the female did not have any spermatangia attached to its body. We split the aquarium into two areas with a partition and confined each sex to one area for more than 3 h before the experiment began to acclimate the animals to aquarium conditions. A plastic plate (1 cm × 20 cm) was placed on the sand bottom in each area as an adhering substrate. All trials were conducted between 0900 and 2100 hours.

At the beginning of the experiment, we removed the partition and allowed the squids to approach each other for 30 min. After copulation and confirmation that spermatangia had been transferred to the female, we segregated the female from the males again using the partition. After 6, 12, 18, 24 and 48 h, 5, 10, 7, 14 and 11 females were anaesthetized with 1% ethanol, respectively, the attached spermatangia were examined using a microscope and photographs

were taken with a digital camera (EC3; Leica, Wetzlar, Germany). We used a generalized linear mixed model (GLMM) with a binomial distribution and logit link function to determine if the rate of empty spermatangia was influenced by time. The presence or absence of sperm in remaining spermatangia in four experimental treatments (6, 12, 18 and 24 h) was used as the response variable (1 = empty, 0 = sperm present). The order of the trials was a random effect. The significance of the effect of time was assessed using a Wald test. We used R version 2.15.2 (R Development Core Team, 2012) for all analyses.

3. Results

3.1. Spermatophores, spermatophoric reaction and spermatangia

Spermatophores are slender, cylindrical, and ~2.5 mm in length (Fig. 1A). The aboral region of the spermatophore is filled by the sperm mass (Fig. 1B). The sperm mass is ~1350 μm long and connected to the cement body at the middle of the spermatophore by a thin connecting cylinder (Fig. 1C). The cement body and the ejaculatory apparatus are ~850 and ~300 μm long, respectively. Except for its aboral end, the cement body is covered by the outer membrane, the middle membrane and the inner tunic, the inner tunic bearing several folds (Fig. 1C and D). The cement body is wider at the aboral and oral ends, the intermediate region being thinner and much longer than either end (Fig. 1A, C and D). The ejaculatory apparatus is composed of the inner tunic and the outer, middle and inner membranes (Fig. 1D). The cap bears a long cap thread at the oral end of the spermatophore (Fig. 1D).

At the beginning of the spermatophoric reaction, the ejaculatory apparatus tube is extruded and everted from the oral end of the spermatophore (Fig. 2A; see also movie 1 in the supplementary online Appendix). During the elongation of the everting ejaculatory apparatus tube, the cement body and the sperm mass move to the everting tube (Fig. 2B). The cement body is compressed when it reaches the oral end of the tube (Fig. 2C), and the sperm mass is briefly stored within the tube that is formed by the everting outer membrane and inner tunic (Fig. 2C

and D). This causes swelling of the everting tube, with its diameter almost doubling. The forming spermatangium is curved and filled with the sperm mass (Fig 2C and D). Finally, the outer membrane and the inner tunic separate from the remaining empty case (middle membrane and outer and middle tunics) and the spermatophoric reaction is completed (Fig. 3A–C). The duration of the spermatophoric reaction (from extruding the ejaculatory apparatus to completing spermatangium formation) was 20.75 ± 4.03 s (mean ± SD, n = 4).

The spermatangium is ~2 mm long and ~80 μm in diameter and is fishhook-shaped (Fig. 4A). The base of the spermatangium is composed of a burst cement body (Fig. 4A) and its distal end is open and sharp (Fig. 4B). The remaining empty case of the spermatophore is composed of the outer tunic case and part of the evaginated ejaculatory apparatus tube (Fig. 5A and B). The spiral filament is conspicuous in the remaining empty case (Fig. 5C).

3.2. Sperm discharge

The sperm mass that is packed within the outer membrane moves gradually to the open end of the spermatangium (see also Fig. 3) and then spermatozoa are released from it. In some spermatophores, the sperm mass was not completely packed within the spermatangium before the end of the spermatophoric reaction and spermatozoa were also released directly from the remaining empty case (Fig. 6; see also movie 1 in the online Appendix). Discharged spermatozoa actively swam when in contact with seawater.

In 50 spermatangia, the velocity of sperm discharge was fast soon after the formation of the spermatangium (Fig. 6; movie 1 in the online Appendix). During the first 5 min of sperm release, the discharge velocity became gradually slower and the pattern changed to intermittent sperm release. During intermittent release, groups of residual spermatozoa located near the open end became activated, and then actively swam out of the spermatangium intermittently.

In 10 spermatangia, spermatozoa discharge occurred slowly even shortly after spermatangium formation, the following steps proceeding as above.

All spermatangia that were observed on glass slides stopped discharging after about 1 h. We transferred 10 spermatangia to Petri dishes soon after the spermatophoric reaction. Although sperm discharge from these spermatangia followed the same pattern as the glass slide observations and discharge stopped after about 1 h, two spermatangia continued to release spermatozoa for more than 6 h. However, spermatozoa release had stopped when we observed these spermatangia after 10 h, even though several spermatozoa remained in the spermatangium. We could not determine how long a spermatangium can store sperm by in vitro observation.

3.3. In vivo sperm discharge

The volume of remaining sperm in the naturally attached spermatangia differed (Fig 7A), with some spermatangia having a larger amount of sperm while others were empty (Fig 7B) after the same period of time after copulation. Table 1 lists the probabilities of females having empty spermatangia for the four experimental periods. There was only one empty spermatangium in the 6 h experimental treatment group, and the probability of empty spermatangia increased with time (GLMM with binomial error distribution, logarithm link: Wald's $Z = 3.606$, $P < 0.001$; Fig. 8). We observed several broken spermatangia (i.e., they only consisted of a cement body) among intact spermatangia in the 12-, 18-, and 24-h treatment groups (Fig 9). However, there were no intact spermatangia in the 48-h experimental treatment group, and except for 2 females that only had broken spermatangia, the remaining 9 females did not have any spermatangia attached to their bodies after 48 h.

4. Discussion

4.1. Morphology of the spermatophore and spermatangium

Recent studies showed that the ejaculatory apparatus of squids may contain numerous minute stellate particles within the spiral filament and attached to the inner membrane at the level of the oral region of the cement body (e.g., Marian, 2012a; Marian and Domaneschi, 2012). It

has been suggested that during the spermatophoric reaction stellate particles provide anchorage for attaching spermatangia before the adhesion provided by the cement body (Marian, 2011, 2012a, 2012b; Marian et al., 2012). The spiral filament is present in several species (see review by Marian 2012a, b, 2014). Stellate particles were also detected in major coleoid taxa (e.g., Austin et al., 1964; Takahama et al., 1991; Hoving et al., 2009; Marian, 2012a, b, 2014). Marian (2012b) suggested that the spiral filament and stellate particles play a major role during spermatangia attachment in decapodiforms. The morphology of the spermatophore in the pygmy squid is similar to that of loliginids (Hess, 1987), but we did not observe a conspicuous spiral filament structure in intact spermatophores of the Japanese pygmy squid. The spiral filament became conspicuous only in its everted state, after the spermatophoric reaction, in remaining empty cases (Fig. 5C). The everted spiral filament was very short, in accordance with the length of the intact ejaculatory apparatus, which in this species is shorter than the cement body.

48 h after copulation, most females did not bear spermatangia on their bodies, and in several cases we could not even observe the remnants of the cement body. Moreover, attached spermatangia were easily removed using tweezers (Sato, pers. obs.). These results suggest that spermatangia attachment in *I. paradoxus* is loose, which may be associated with the fact that they have a short ejaculatory apparatus and respective spiral filament, which contains stellate particles that are supposedly involved in spermatophore anchorage. However, stellate particles are difficult to detect (e.g., Marian 2012a, b; Marian and Domaneschi, 2012), especially in this case, where the spermatophore was less than 3 mm long. Future histological and scanning electron microscopy studies should confirm if stellate particles are present in the pygmy squid spermatophore.

Pygmy squids have dimorphic hectocotyli. A recent study showed that the right hectocotylus is used as a guide for spermatophore transfer by the left hectocotylus (Sato et al., 2013b).

229 Considering that spermatangia attachment appears to be loose in this species, the dimorphic
230 hectocotyli may assure successful spermatophore transfer.
231 The shape of cephalopod spermatangia is highly variable, appearing as a short pole in *S. of-*
232 *ficinalis* (Hanlon et al., 1999) and *S. esculenta* (Wada et al., 2005), a teardrop in *T. pacificus*
233 (Takahama et al., 1991) and irregularly coiled in *H. dispar* (Hoving et al., 2008). Iwata and
234 Sakurai (2007) reported that the spermatangia of *L. bleekeri* differ between dimorphic males;
235 large males have rope-like spermatangia, while small males have drop-like ones. Japanese
236 pygmy squids have fishhook-shaped spermatangia. The folded inner tunic in intact spermato-
237 phores possibly allows for expansion after eversion, permitting the formation of a spermatan-
238 gium with a larger volume. The open distal end of the spermatangium of the Japanese pygmy
239 squid is narrower than that in other decapodiforms (e.g., *S. esculenta*, Wada et al., 2005; *D.*
240 *plei*, Marian, 2012a). This characteristic morphology might be intimately related with sperm
241 discharge, a hypothesis that is discussed below.
242
243 *4.2. Sperm discharge from the spermatangium*
244 In almost all spermatangia of *I. paradoxus* observed in vitro, the flow of sperm discharge be-
245 came weak within 5 min after the beginning of sperm release, stopping within 1 h even if the
246 spermatangium contained a large number of spermatozoa. In previous studies that described
247 sperm discharge in squids, no early decrease in sperm discharge has been reported (Drew,
248 1919; Marian, 2012a). This difference in the sperm discharge pattern may be related to the
249 morphology of the distal end of the spermatangia. While the distal end of the spermatangia of
250 pygmy squids is narrow and sperm discharge decreases early, the distal end of loliginid squids
251 is generally wider. This narrow open end in pygmy squids may function as a discharge con-
252 troller. Slow discharge speeds would be advantageous in this case because the relatively small
253 sperm volume of the spermatangia would last longer. Short-term discharge carries a risk for
254 sperm transfer, because female squids continue to move actively after copulation, potentially

contributing to sperm dilution. Therefore, a narrow distal end might help reduce rapid sperm release and avoid the risk of complete sperm transfer failure.

Spermatangia in Petri dishes discharged spermatozoa for a longer period than those on glass slides. This difference might be related to sperm motility and to the activation of sperm during intermittent release. In fish, sperm motility is regulated by osmotic pressure (Takai and Morisawa, 1995). On glass slides, the osmotic pressure will easily change due of water evaporation. However, changes in osmotic pressure in Petri dishes should be slower than those on glass slides because they contain more seawater. It is not known whether osmotic pressure regulates sperm motility in cephalopods, but some studies have reported that motility is regulated by the surrounding environment (e.g., by CO_2, Hirohashi et al., 2013; or by sperm concentration, Naud and Havenhand, 2006).

In contrast to the in vitro results, several empty spermatangia were found in females in vivo, which suggests that spermatangia might naturally discharge their entire sperm reserve. The flow of the surrounding fluid might influence sperm discharge. In pygmy squids, spermatangia are generally attached near the opening of the funnel and are subjected to the jets of water that are expelled from it.

Most naturally attached spermatangia discharged almost all of their spermatozoa within 24 h after copulation. After 48 h, we could not find any attached spermatangia in females. These results suggest that spermatangia are retained on the female body during the first day but are somehow lost or broken by the following day. Broken spermatangia were also found in the 6-, 12-, 18-, and 24-h treatment groups (Fig. 9). Mated female pygmy squids frequently elongate their buccal mass and remove attached spermatangia by picking at them using the buccal mass (Sato et al., 2013a). However, spermatangia that have discharged almost all of their spermatozoa might become fragile and break easily without any removal behavior by the female. More studies are needed to determine exactly how spermatangia are lost after copulation.

In naturally attached spermatangia in vivo, the volume of residual sperm was highly variable.

This difference might be due to distinct sperm discharge patterns. In vitro, spermatozoa rushed from the opening duct in some spermatangia, but we observed slow sperm discharge in other spermatangia during the first 5 min of sperm release. The volume of the sperm mass inside the spermatophore might create differences in sperm discharge patterns. A greater sperm volume in spermatangia might maintain high internal pressure for a longer duration thus resulting in the release of more sperm.

4.3. Concluding remarks

Spermatozoa rushed from the spermatangia immediately after the spermatophoric reaction, but sperm release became gradually slower with time and sperm discharge was completed after ~1 day in naturally attached spermatangia. This suggests that sperm transfer (from the attached spermatangium to the seminal receptacle) must occur within 24 h after copulation. In the pygmy squid, the spermatangium functions as a transient sperm storage organ. Although spermatangia of loliginid squids are generally larger than those of pygmy squids and store considerably more sperm, their open distal end is broader and sperm discharge seems to be constant for more than 24 h (Drew, 1919). Drew (1919) noted that “spermatozoa escape in a constant cloud which reminds one of the smokes from an evenly discharging factory chimney”. This description suggests that the discharge is rapid, at least at the beginning of sperm release. Therefore, short-term storage of the spermatangia may be common in decapodiforms. To investigate this hypothesis, additional studies of sperm discharge in various species are necessary.

Acknowledgements

We thank J.E.A.R. Marian and Y. Iwata for their critical comments on the manuscript. This work was financially supported by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists (to N.S.) and by a Grant-in-Aid for the Scientific Re-

search from the Japan Society for the Promotion of Science (to H.M.).

Appendix A. Supplementary data

Supplementary data associated with this article may be found in the online version at doi: ##.

References

Austin, C.R., Lutwak-Mann, C., Mann, C., 1964. Spermatophores and spermatozoa of the squid *Loligo pealii*. Proc. R. Soc. London B. 161, 143–152.

Drew, G., 1911. Sexual activities of the squid, *Loligo pealii* (Les.). I. Copulation, egg-laying and fertilization. J. Morphol. 22, 327–359.

Drew, G., 1919. Sexual activities of the squid, *Loligo pealii* (Les.). II. The spermatophore; its structure, ejaculation and formation. J. Morphol. 32, 379–436.

Hanlon, R.T., Messenger, J.B., 1996. Cephalopod Behaviour. Cambridge University Press, Cambridge.

Hanlon, R.T., Ament, S.A., Gabr, H., 1999. Behavioral aspects of sperm competition in cuttlefish, *Sepia officinalis* (Sepioidea: Cephalopoda). Mar. Biol. 134, 719–728.

Hess, S.C., 1987. Comparative morphology, variability, and systematic applications of cephalopod spermatophores (Teuthoidea and Vampyromorpha). PhD Thesis, University of Miami, Coral Gables.

Hirohashi, N., Alvarez, L., Shiba, K., Fujiwara, E., Iwata, Y., Mohri, T., Inaba, K., Chiba, K., Ochi, H., Supuran, C.T., Kotzur, N., Kakiuchi, Y., Kaupp, U.B., Baba, S.A., 2013. Sperm from sneaker male squids exhibit chemotactic swarming to CO_2. Curr. Biol. 23, 775–781.

Hoving, H.J.T., Laptikhovsky, V., 2007. Getting under the skin: autonomous implantation of squid spermatophores. Biol. Bull. 212, 177–179.

Hoving, H.J.T., Lipinski, M.R., Roeleveld, M.A.C., Durholtz, M.D., 2007. Growth and mating of southern African *Lycoteuthis lorigera* (Steenstrup, 1875) (Cephalopoda; Lycoteuthidae). Rev. Fish. Biol. Fisheries 17, 259–270.

Hoving, H.J.T., Laptikhovsky, V., Piatkowski, U., Önsoy, B., 2008. Reproduction in *Heteroteuthis dispar* (Rüppell, 1844) (Mollusca: Cephalopoda): a sepiolid reproductive adaptation to an oceanic lifestyle. Mar. Biol. 154, 219–230.

Hoving, H.J.T., Nauwelaerts, S., Van Genne, B., Stamhuis, E.J., Zumholz, K., 2009. Spermatophore implantation in *Rossia moelleri* Steenstrup, 1856 (Sepiolidae; Cephalopoda). J. Exp. Mar. Biol. Ecol. 372, 75–81.

Ikeda, Y., Sakurai, Y., Shimazaki, K., 1993. Maturation process of the Japanese common squid *Todarodes pacificus* in captivity. In: Okutani, T., O'Dor, R.K., Kubodera, T. (Eds.), Recent Advances in Cephalopod Fisheries Biology: Contributed Papers to 1991 CIAC International Symposium and Pro-

ceedings of the Workshop on Age, Growth and Population Structure, Tokai University Press, Tokyo, Japan, pp. 179–187.

Iwata, Y., Sakurai, Y., 2007. Threshold dimorphism in ejaculate characteristics in the squid *Loligo bleekeri*. Mar. Ecol. Prog. Ser. 345, 141–146.

Iwata, Y., Munehara, H., Sakurai, Y., 2005. Dependence of paternity rates on alternative reproductive behaviors in the squid *Loligo bleekeri*. Mar. Ecol. Prog. Ser. 298, 219–228.

Kasugai, T., 2000. Reproductive behavior of the pygmy cuttlefish *Idiosepius paradoxus* in an Aquarium. Venus 59, 37–44.

Lum-Kong, A., 1992. A histological study of the accessory reproductive organs of female *Loligo forbesi* (Cephalopoda: Loliginidae). J. Zool. 226, 469–490.

Mangold, K., 1987. Reproduction. In: Boyle, P.R. (Ed.), Cephalopod Life Cycles. Academic Press, London, pp. 157–200.

Mann, T., Martin, A.W., Thiersch, J.B., 1966. Spermatophores and spermatophoric reaction in the giant octopus of the North Pacific, *Octopus dofleini martini*. Nature 211, 1279–1282.

Mann, T., Martin, A.W., Thiersch, J.B., 1970. Male reproductive tract, spermatophores and spermatophoric reaction in the giant octopus of the North Pacific, *Octopus dofleini martini*. Proc. R. Soc. London B. 175, 31–61.

Marian, J.E.A.R., 2011. Perforating potential of loliginid spermatophores. J. Mollusc. Stud. 77, 98–100.

Marian, J.E.A.R., 2012a. Spermatophoric reaction reappraised: novel insights into the functioning of the loliginid spermatophore based on *Doryteuthis plei* (Mollusca: Cephalopoda). J. Morphol. 273, 248–278.

Marian, J.E.A.R., 2012b. A model to explain spermatophore implantation in cephalopods (Mollusca: Cephalopoda) and a discussion on its evolutionary origins and significance. Biol. J. Linn. Soc. 105, 711–726.

Marian, J.E.A.R., 2014. Evolution of spermatophore transfer mechanisms in cephalopods. J. Nat. Hist. Available online at doi: 10.1080/00222933.2013.825026.

Marian, J.E.A.R., Domaneschi, O. 2012. Unraveling the structure of squids' spermatophores: a combined approach based on *Doryteuthis plei* (Blainville, 1823) (Cephalopoda: Loliginidae). Acta Zool. 93, 281–307.

Marian, J.E.A.R., Shiraki, Y., Kawai, K., Kojima, S., Suzuki, Y., Ono, K., 2012. Revisiting a medical case

of “stinging” in the human oral cavity caused by ingestion of raw squid (Cephalopoda: Teuthida): new data on the functioning of squid’s spermatophores. Zoomorphology 131, 293–301.

Nabhitabhata, J., Suwanamala, J., 2008. Reproductive behaviour and cross-mating of two closely related pygmy squids *Idiosepius biserialis* and *Idiosepius thailandicus* (Cephalopoda: Idiosepiidae). J. Mar. Biol. Assoc. UK 88, 987–993.

Naud, M.-J., Havenhand, J.N., 2006. Sperm motility and longevity in the giant cuttlefish, *Sepia apama* (Mollusca: Cephalopoda). Mar. Biol. 148, 559–566.

Naud, M.-J., Shaw, P.W., Hanlon, R.T. Havenhand, J.N., 2005. Evidence for biased use of sperm sources in wild female giant cuttlefish (*Sepia apama*). Proc. R. Soc. London B. 272, 1047–1051.

Sato, N., Kasugai, T., Ikeda, Y., Munehara, H., 2010. Structure of the seminal receptacle and sperm storage in the Japanese pygmy squid. J. Zool. 282, 151–156.

Sato, N., Kasugai, T., Munehara, H., 2013a. Sperm transfer or spermatangia removal: postcopulatory behaviour of picking up spermatangium by female Japanese pygmy squid. Mar. Biol. 160, 553–561.

Sato, N., Masa-Aki, Y., Fujiwara, E., Kasugai, T., 2013b. High-speed camera observations of copulatory behaviour in *Idiosepius paradoxus*: function of the dimorphic hectocotyli. J. Mollus. Stud. 79, 183–186.

R Development Core Team, 2012. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available at http://www.R-project.org/.

Takahama, H., Kinoshita, T., Sato, M., Sasaki, F., 1991. Fine structure of the spermatophores and their ejaculated forms, sperm reservoirs, of the Japanese common squid, *Todarodes pacificus*. J. Morphol. 207, 241–251.

Takai, H., Morisawa, M., 1995. Change in intracellular K^+ concentration caused by external osmolality change regulates sperm motility in marine and freshwater teleosts. J. Cell Sci., 108, 1175–1181.

van Oordt, G., 1938. Memoirs: The spermatheca of *Loligo vulgaris*. I. Structure of the spermatheca and function of its unicellular glands. Q. J. Microsc. Sci. S2 80, 593–599.

Wada, T., Takegaki, T., Mori, T., Natsukari, Y., 2005. Sperm displacement behavior of the cuttlefish *Sepia esculenta* (Cephalopoda: Sepiidae). J. Ethol. 23, 85–92.

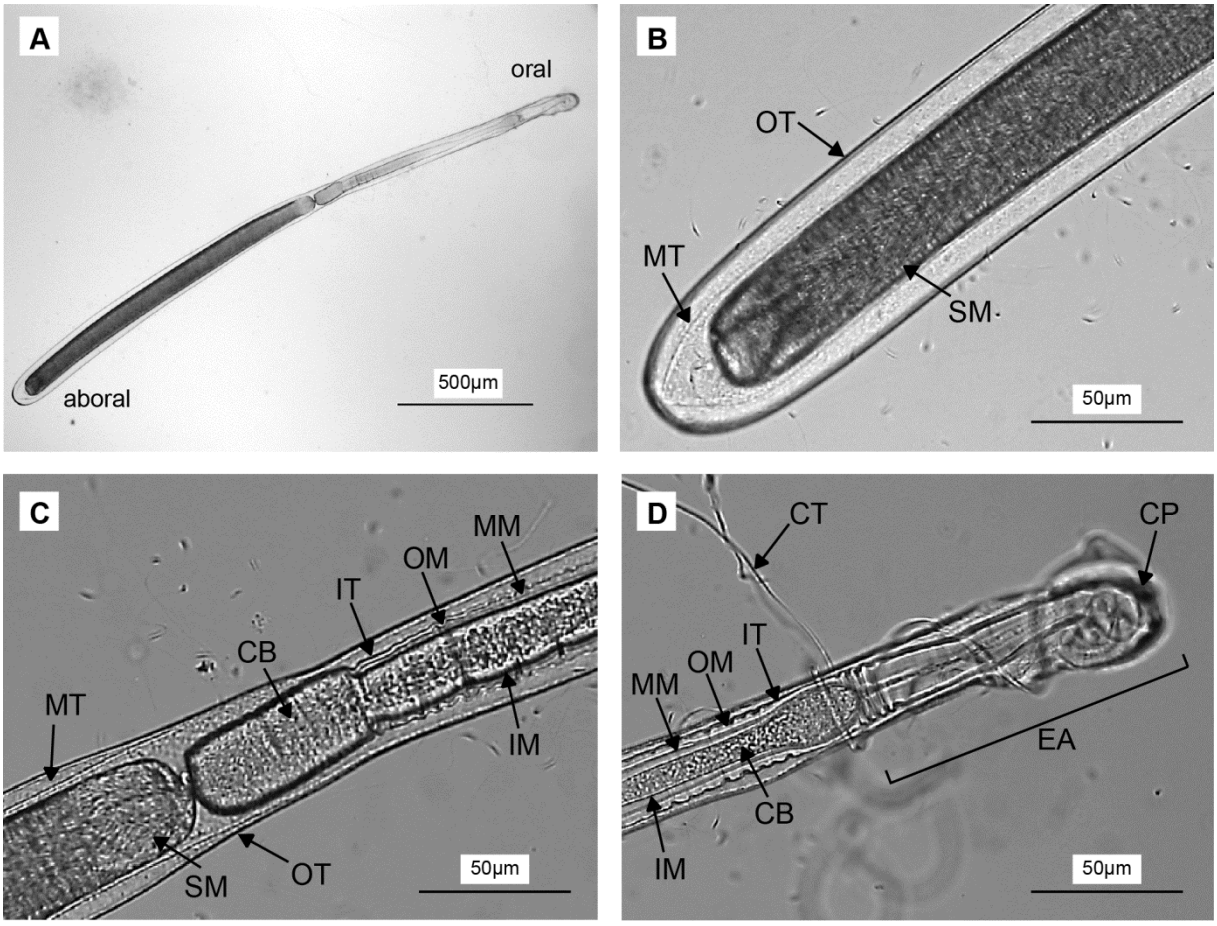


Fig. 1. Spermatophore of *Idiosepius paradoxus*. (A) Whole spermatophore, (B) aboral region of the spermatophore, (C) middle region of the spermatophore, (D) oral region of the spermatophore. Abbreviations: CB, cement body; CP, cap; CT, cap thread; EA, ejaculatory apparatus; IM, inner membrane; IT, inner tunic; MM, middle membrane; MT, middle tunic; OM, outer membrane; OT, outer tunic; SM, sperm mass.

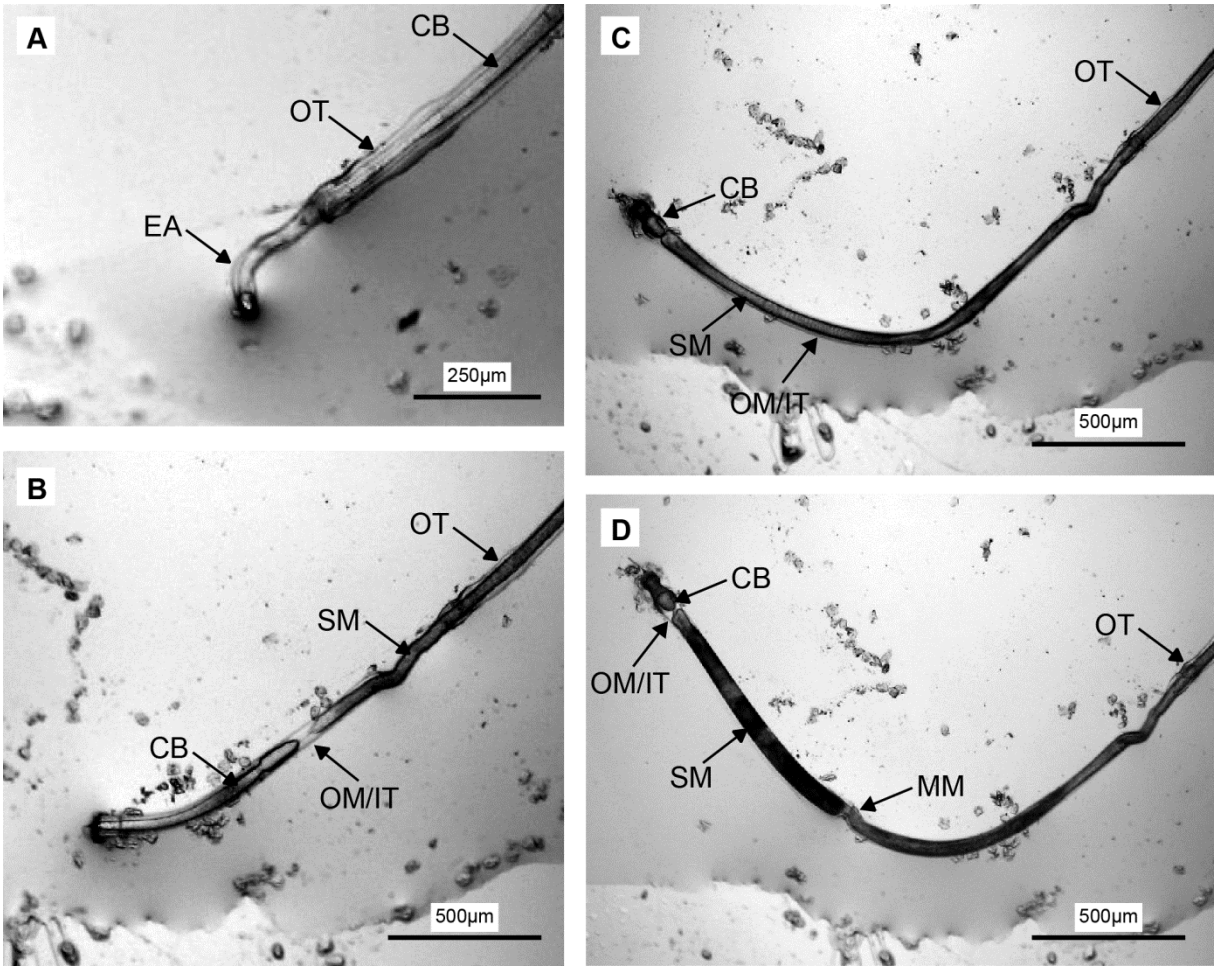
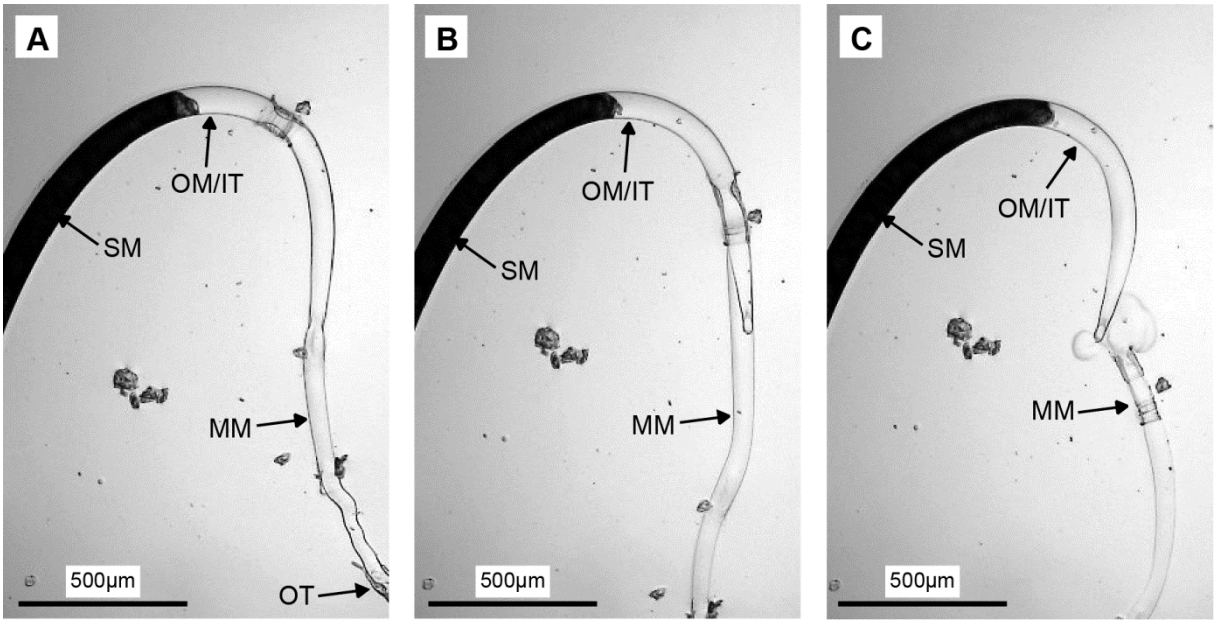
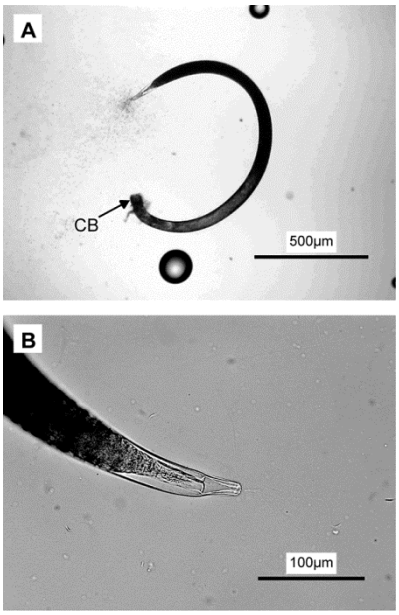


Fig. 2. Sequential images of the spermatophoric reaction. (A) Evagination of the ejaculatory apparatus tube. (B) Cement body and sperm mass move to the extruded tube. (C) Compression of the cement body in the oral end of the tube. (D) Sperm mass storage within the space formed by the outer membrane and inner tunic. Abbreviations: CB, cement body; EA, ejaculatory apparatus; IT, inner tunic; MM, middle membrane; OM, outer membrane; OT, outer tunic; SM, sperm mass.



411

412 **Fig. 3.** (A–C) Sequential images of the final stages of the spermatophoric reaction. Sperma-
413 tangium separates from the middle membrane and the reaction is completed. Abbreviations:
414 IT, inner tunic; MM, middle membrane; OM, outer membrane; OT, outer tunic; SM, sperm
415 mass.



416

417 **Fig. 4.** Spermatangium. (A) Whole spermatangium. (B) Open distal end of the spermatangium.
418 Abbreviation: CB, cement body.

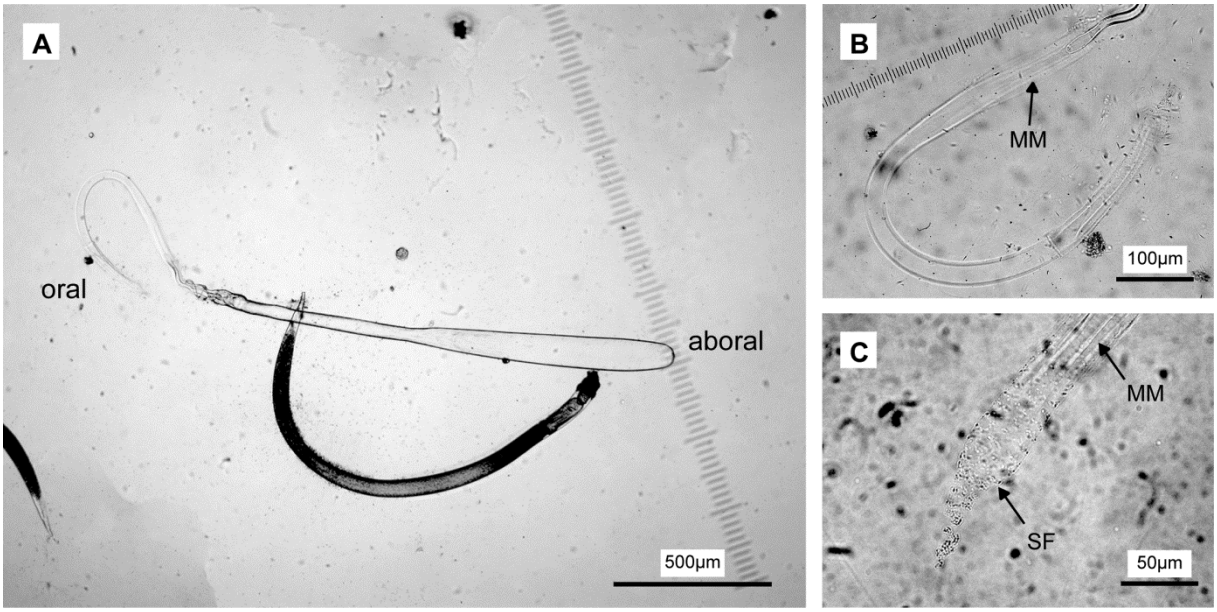


Fig. 5. Remaining empty spermatophore case after the spermatophoric reaction. (A) Whole empty case. (B) Everted ejaculatory apparatus. (C) Oral end of the everted portion, showing the everted spiral filament. Abbreviations: MM, middle membrane; SF, spiral filament.

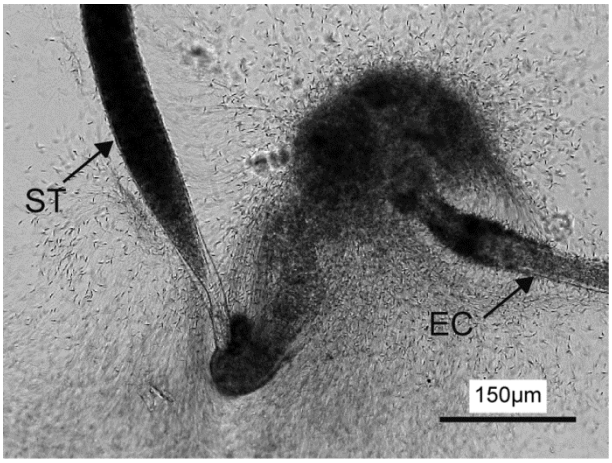


Fig. 6. An incomplete spermatophoric reaction. In this case, the sperm mass was not forced completely into the forming spermatangium, spermatozoa were released from both the spermatangium and the remaining empty case. Abbreviations: EC, empty case of spermatophore; SM, sperm mass.

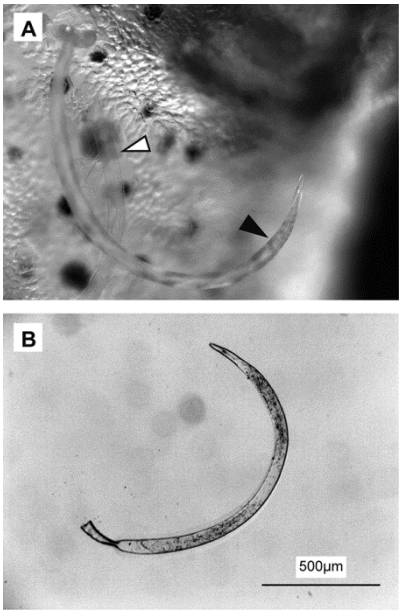


Fig. 7. Spermatangia naturally attached to a female body. (A) Area near the right eye, on the ventral side of the female 18 h after copulation. White and black arrowheads indicate an empty spermatangium and a spermatangium that retained some spermatozoa, respectively. (B) An empty spermatangium collected from a mated female 12 h after copulation.

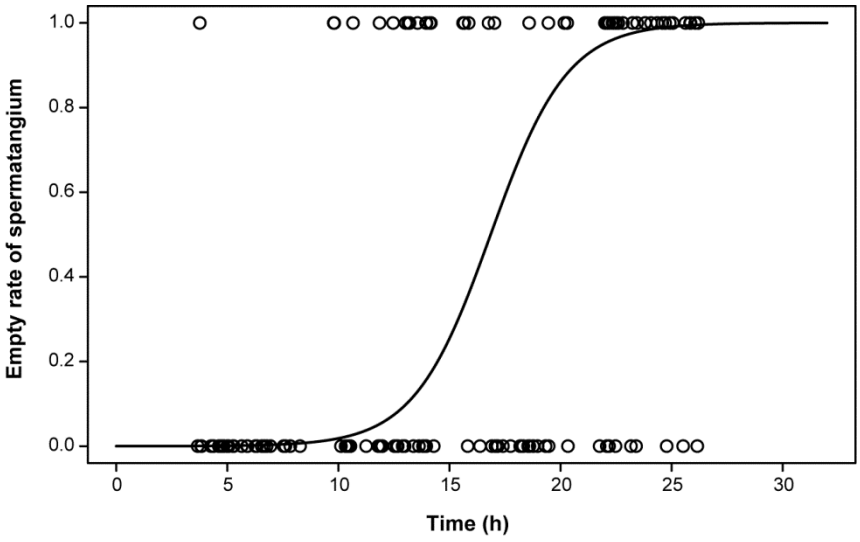
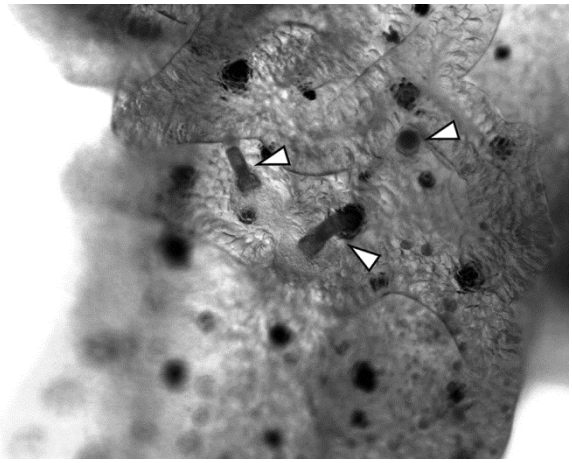


Fig. 8. Probability of empty spermatangia over time. Solid line shows predicted values from the GLMM with a binomial error distribution and a logit link function.



436

437 **Fig. 9.** Broken spermatangia attached to the ventral side of the bases of left arms I and II of a
438 female 24 h after copulation. White arrowheads indicate remaining cement bodies of sperma-
439 tangia.

Table 1
Condition of naturally attached spermatangia over time (6, 12, 18 and 24 h after copulation).

	6h	12h	18h	24h
Observed spermatangia (n)	34	36	28	37
Empty spermatangia (n)	1	14	9	28
Probability of emptiness (%)	2.9	38.9	32.1	75.7

440