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

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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.

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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 Decapodiformes 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 Decapodiformes 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 Laptikhovskiy, 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

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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 process 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 h.

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

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