High-speed camera observations of copulatory behaviour in *Idiosepius paradoxus*: function of the dimorphic hectocotyli

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In cephalopods, males transfer sperm to females via a very complex process. Males package sperm in transparent sheaths to form spermatophores that are stored internally in Needham's Sac. Spermatophores are extruded from the internal terminal organ and exit the mantle through the funnel. Although some squid taxa lack the structure, many squids transfer spermatophores to females using an arm specialized to grab the spermatophores, called the hectocotylus (Drew, 1911; Hanlon & Messenger, 1996). During transfer to females, the spermatophore undergoes the spermatophoric reaction to release a spermatangium which is a sac that contains a sperm mass with a cement body at one end (Hanlon & Messenger, 1996; Marian, 2012). The spermatangium is attached to the female body by the cement body.

Some decabrachians species, such as *Austrossia australis* Berry, (1918), *Sepiola aurantiaca* Jatta, (1896), and *Rossia pacifica* Berry, (1911), have two mirror-image hectocotyli (e.g., both left and right arms I are hectocotylised in the same manner in male *R. pacifica*) (Okutani, 2005). However, males of the pygmy squid (genus *Idiosepius* Steenstrup, 1881) have dimorphic hectocotyli; both left and right ventral arms are modified, but are distinctly different (von Byern & Klepal, 2010) (Fig. 1A, B). The left hectocotylus has two flaps at its tip (Fig. 1C); the right hectocotylus has fleshy ridges on its aboral side (Fig. 1D). Males pass spermatophores to females via the left hectocotylus in *I. paradoxus* Ortmann, (1881) (Kasugai, 2000). Males also insert their right hectocotylus into the arm crown of the female during copulation (Kasugai, unpublished data), but the role of the right hectocotylus is unknown. In some cuttlefish species, males are reported to use their arms to remove/scrape out spermatangia attached by rival males (Wada *et al*., 2005, 2006, 2010).

In the present study, we recorded the copulatory behaviour of *I. paradoxus* using a high-speed camera. We observed the entire process of sperm transfer to examine the role of the right hectocotylus during copulation.

Pygmy squids were collected from small stocks of the seagrass *Zostera marina* in the nearshore waters of the Chita Peninsula, central Honshu, Japan (34°43’N, 136°58’E). Mature pygmy squids were collected using a small drag net (1 × 2 m, mesh size: 1.5 mm) on 30 March 2010. Live specimens were placed in well-aerated seawater and transported to the laboratory of the Documentary Channel in Saitama, Japan (35°48’N, 139°44’E). Pygmy squids were maintained in two aquaria (24 × 19 × 27 cm) with a closed circulation system. Seagrasses were planted on the sand at the aquarium bottom to squids could adhere. Two males and two females were introduced into each aquarium. Their sex can be readily confirmed by the presence white testis in males and ripe eggs, nidamental glands and the larger body in females. Lighting provided artificially a 12 h light/12 h dark photoperiod, and the water temperature was maintained at 20°C. Pygmy squids were fed live amphipods (*Ampithoe* sp.) twice daily.

Video recording was started a day after introducing pygmy squids to aquariums. Copulation was recorded using a high-speed camera (Photron, Fastcam SA2) fitted with a 105-mm/f2.8 lens (Nikon, Micro-Nikkor) at 250 frames per second. During filming, two spot lights were additionally used to support the recording. We recorded seven copulations, and were able to observe the spermatangia placement and number of passed spermatangia in six copulations.

Males seized females from the dorsal side during three copulations and from the ventral side in the remaining four copulations. After grasping the females, males inserted their right hectocotylus into the female’s arm crown (Fig. 2A and Supplementary material 1). The right hectocotylus was expanded over the female’s buccal mass to extend to the opposite side of the arm base. The left hectocotylus was on standby near the funnel opening, and two flaps at its tip were opened. The males oriented the opening of the funnel towards the posterior part of their body, and spermatophores then appeared from the funnel. Spermatophore movement from the opening of the terminal organ to the funnel was observed through the transparent bodies of the pygmy squid. The spermatophores moved to the opening of the funnel very rapidly (during one frame: 0.004 s) and stopped at its opening. The males grasped the spermatophores using the two flaps of the left hectocotylus at the funnel (Fig. 2A). The time between when the spermatophores are seen at funnel opening and the males grasp them was 0.025 ± 0.01s (mean ± SD; n = 16). The fleshy ridges of the right hectocotylus formed a groove into which the left, spermatophore-carrying hectocotylus moved to the tip (Fig. 2B): the spermatophores were conveyed to the site to which the tip of the right hectocotylus pointed (Fig. 2C). The outer case of the spermatophore was extruded while it was still in the left hectocotylus, and a spermatophoric reaction occurred during spermatophore transfer. The spermatangia then quickly attached to the female's body where the tip of the right hectocotylus pointed. The males moved the tip of the right hectocotylus to another arm base after completing a spermatophore transfer, and the left hectocotylus was again on standby near the funnel opening (Fig. 2D). A period of 0.87 ± 0.58 s (n = 11) passed until the next spermatophore ejaculation occurred.

Spermatophore transfer occurred 2.57 times (range = 2 – 3; n = 7) per copulation on average. The mean number of spermatophores passed at each transfer was 1.65 (range = 1 – 3; n = 17). Each time spermatophores were transferred within a copulatory bout, the males changed the site toward which the tip of the right hectocotylus was pointed. Mating duration was 4.03 ± 1.51 sec (n = 7). The average number of spermatophores passed to females during the 6 copulatory bouts we could see in their entirety was 4.33 (range = 2 – 7).

The spermatophoric reaction had already occurred in the left hectocotylus, meaning the spermatangia were transferred to the female. The spermatangium bears a cap thread on the oral end (e.g. Drew, 1919 in *Loligo pealii*; Takahama *et al*., 1991 in *Todarodes pacificus*; Maian, 2012 in *Doryteuthis plei*). The cap thread can trigger the spermatophoric reaction; pulling the thread causes the reaction (Hoving *et al*. 2009; Marian, 2012). The cap thread is deeply entangled inside the Needham's Sac in *D. plei* (Marian, 2012). This entanglement is also observed in *I. paradoxus* (Sato personal observation). Spermatophores stopped at the funnel opening after having been extruded from the terminal organ, the delay could be the function of the cap thread. A potential secondary function of the thread could be to be pulled when the left hectocotylus removes them from the funnel, starting the spermatophoric reaction.

Although octopods have the hectocotylus with the groove which formed by folding the muscle in the tip (Hanlon and Messenger, 1998), they use the groove for transferring the spermatophore from the funnel to the arm tip, and transferring and inserting the spermatophore inside the female (Wodinsky, 2008). Our observations revealed that pygmy squid use the right hectocotylus as a guide for spermatophore transfer by the left hectocotylus. A remaining question is why do *Idiosepius* have dimorphic hectocotyli? Males of the pygmy squid have long spermatophores (2 – 2.5 mm) relative to their body (DML 15 mm) (from Sasaki, 1929). The spermatophore length which is about 20 % of DML, exceed that of other cephalopods (e.g. about 5 % in *L. bleekeri* (Iwata & Sakurai, 2007) and *Sepia pharaonis* or *S. dollfusi* (Gabr *et al*., 1998)). Nevertheless, copulation duration of the pygmy squid is only a few seconds (see also Sato *et al*., 2010, in press). The groove form of the right hectocotylus may allow certain and rapid transport of large spermatangia. Oceanic sepiolids, *Heteroteuthis dispar* have also a huge spermatophore (the length is over 30 % of DML), and their hectocotylus is heteromorphic that have a cushion containing glandular tissues at the base of hectocotylus (Hoving *et al*., 2008). A special function may be needed for hectocotyli to transfer spermatangia in species with a large spermatophore.

Although *I. thailandicus* and *I. biserialis* males pass spermatangia to females using their tentacles and do not use the hectocotyli (Nabhitabhata & Suwanamala, 2008), they also possess dimorphic hectocotyli (von Byern & Klepal, 2010). Future studies needs to observe how male *I. thailandicus and I. biserialis* use both hectocotyli during copulation to understand the evolution of the dimorphic hectocotyli in *Idiosepius*.

All males moved the right hectocotylus after completing spermatophore transfers, changing the location where they attached spermatangia. Females can elongate the buccal mass, pick up spermatangia, and remove them after copulation (Sato, Kasugai & Munehara, in press). Spermatangia removal by females would decrease a male's chance of fertilizing offspring. However, a previous study also suggested that females likely do not know the exact location of deposited spermatangia because they frequently elongate their buccal mass to sites where no spermatangia were deposited (Sato et al., in press). Males may minimize spermatangia removal by dispersing the locations where they deposit spermatangia.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Molluscan Studies* online.

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FIGURE CAPTIONS

Figure 1. Images of the hectocotyli in *Idiosepius paradoxus*. **A.** The hectocotyli of a living specimen. **B.** Scanning electron microscopic (SEM) images of the distal hectocotyli. Scale bar = 500 μm. **C.** The tip of the left hectocotylus. Scale bar = 100 μm. **D.** A groove made by fleshy ridges of the right hectocotylus on the aboral side. Scale bar = 500 μm.

Figure 2. Description of spermatophore transfer. **A.** The male grasps a female adhered to the seagrass and elongates his right hectocotylus over the female's buccal mass. Spermatophores are held by the left hectocotylus. **B.** The left hectocotylus passes along the groove of the right hectocotylus. **C.** Spermatophores are transferred at the site on the female to which the tip of the right hectocotylus points. **D.** Spermatangia (ejaculated from the spermatophores while in the left hectocotylus) attach to the female. With transfer of spermatangia complete, the male moves the right hectocotylus to another site and the left hectocotylus is again on standby near the funnel.

Figure 1.

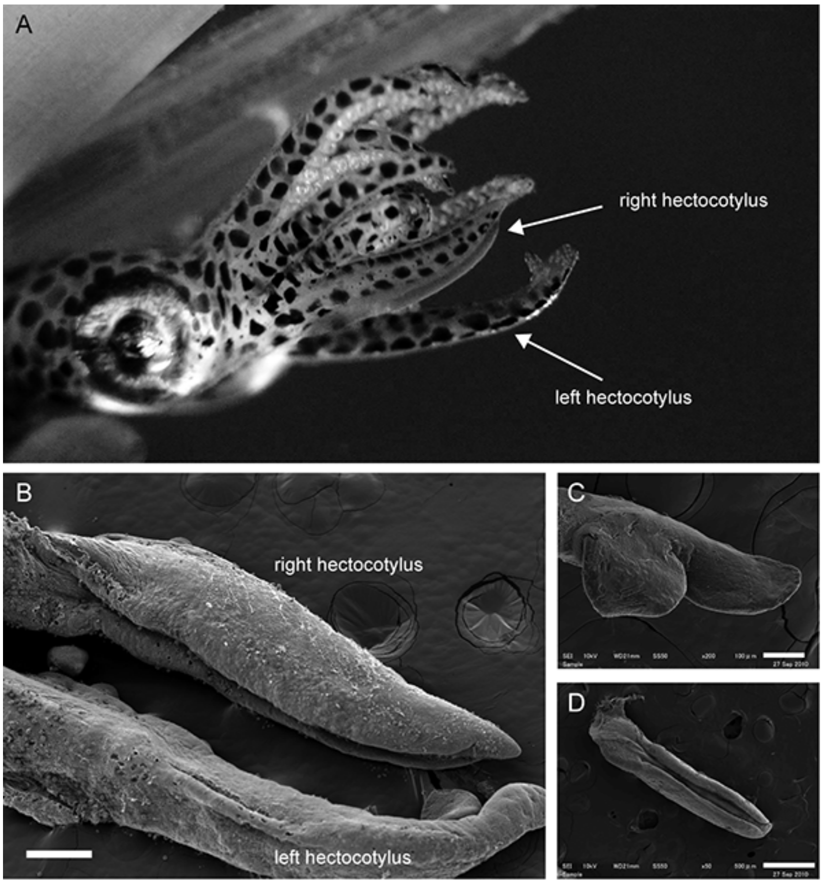
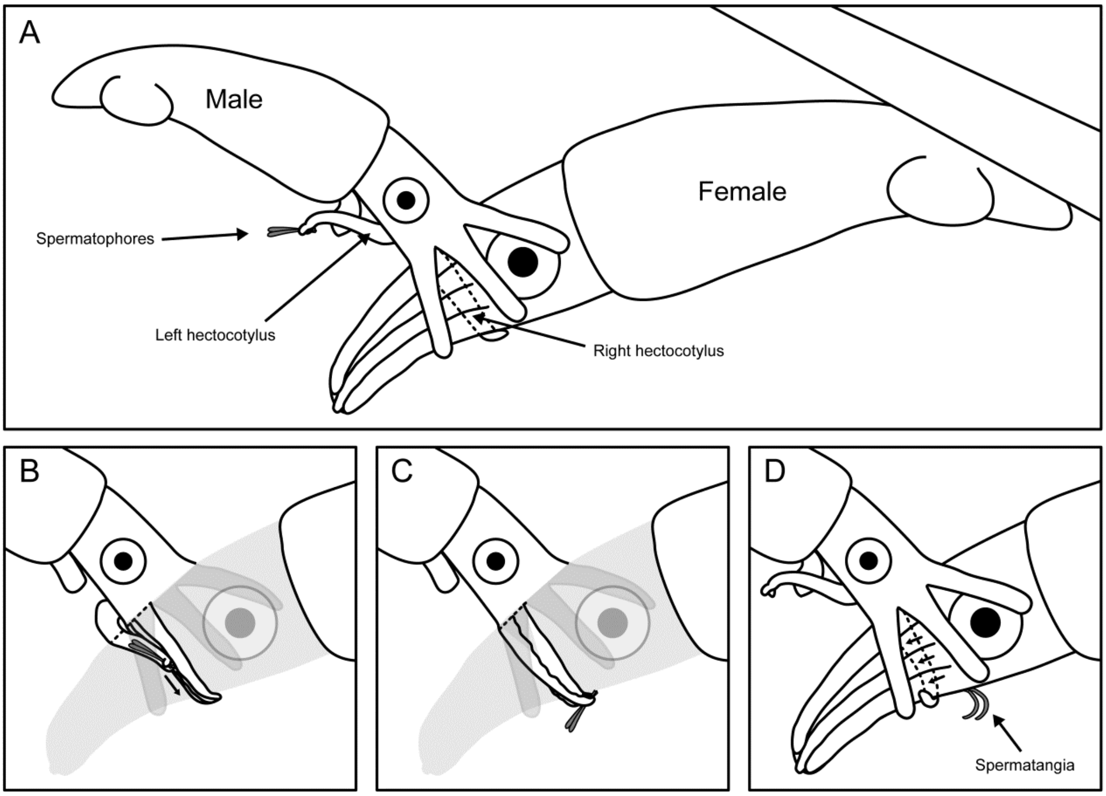

A
right hectocotylus
left hectocotylus
B
right hectocotylus
left hectocotylus
C
D

Figure 2.


A
Male
Female
Spermatophores
Left hectocotylus
Right hectocotylus
B
C
D
Spermatangia