



Spermatophore implantation in *Rossia moelleri* Steenstrup, 1856 (Sepiolidae; Cephalopoda)

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ABSTRACT

The small sepiolid cephalopod *Rossia moelleri* Steenstrup, 1856 transfers sperm by implantation of spermatangia into female tissue. Although this is a common sperm transfer and storage strategy in cephalopods, the mechanism behind implantation of spermatangia is poorly understood. In the lab, we artificially induced the spermatophoric reaction and spermatangia implanted into female tissue. The force necessary to penetrate the mantle was measured using a needle attached to a force transducer. Taking diameter and bluntness factor into account, this force was estimated to be 0.3 N. Analysis of the spermatophoric reaction showed that the maximum force (1.12 μ N–9.36 μ N) produced as a result of acceleration (1.57–3.59 mm/s²) of the forward moving sperm mass (2.6–7 mg) was insufficient to be solely responsible for the penetration of the spermatangia into tissue. Scanning electron microscopy revealed no structures that could have facilitated the implantation of the spermatangium. Histological sections of the implanted spermatangium visualized the cement body being orally secreted from the spermatangium, probably facilitating the implantation either by lysis of the surrounding tissue or by acting as a lubricant during implantation. This study shows that the autonomous implantation process of spermatangia of *R. moelleri* does not have a purely mechanical basis but necessitates an additional, probably chemical mechanism or a combination of these two.

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1. Introduction

Cephalopod molluscs are abundant and diverse marine organisms that inhabit the benthic and pelagic environments from coastal areas to the deep-sea (Boyle and Rodhouse, 2005). Reproduction in cephalopods is semelparous, the sexes are separate (Nesis, 1987) and males produce spermatophores that have a unique complexity not found in any other mollusc (Mann, 1984).

In some species, the male transfers sperm in spermatophores using a hectocotylus (a modified arm or arm pair). Alternatively, in species that lack a hectocotylus, a long terminal organ (sometimes referred to as penis) is used (Nesis, 1995). During discharge or inversion (the spermatophoric reaction) of the spermatophore, the sperm mass is enveloped in a different outer casing to form the spermatangium (Fort, 1937). Female cephalopods store spermatangia either in modified sperm receptive tissues (seminal receptacles and bursa copulatrix) or sperm is deeply implanted directly into unmodified tissue (mantle margin, the head, arms etc.) (Nesis, 1995).

Implantation of spermatangia is a widely adopted sperm transfer strategy among males of oceanic and deepwater cephalopods (Nesis, 1995). The mechanism responsible for spermatangia implantation is poorly understood. Because the cephalopods that implant spermatangia often have a long penis, the penis was thought to be responsible for the injection of spermatangia into tissue (Norman and Lu, 1997; Jackson and Jackson, 2004). Recently, it was demonstrated that spermatophores of the deep-sea squid *Moroteuthis ingens* are able to implant into muscle tissue autonomously (Hoving and Laptikhovskiy, 2007). The implantation mechanism of spermatophores is therefore part of the process of eversion of the spermatophore i.e. the spermatophoric reaction.

The spermatophoric reaction is driven by the contraction of spermatophore tunics and by the hydrostatic pressure caused by the uptake of water through osmotic processes (Drew, 1919; Austin et al., 1964; Mann, 1984). Although the spermatophoric reaction of the giant octopus takes up to 2 h, the reaction of decapodiform cephalopod species is known to be fast, taking merely seconds (Drew, 1919; Austin et al., 1964; Mann, 1984; Takahama et al., 1991).

The spermatophores of loliginids have cement bodies that contain a “glue” that allows the spermatangium to attach to the female's body (Drew, 1919). The spermatangia of *Todarodes pacificus* Steenstrup 1880 and *Illex coindetii* Verany 1839 have, besides a cement body,

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spike-like structures on the outer surface, which facilitate superficial implantation in the outer lip of the female (Marchand, 1913; Takahama et al., 1991). In these species, the spermatophoric reaction happens very quickly, allowing the very small spike-like structures on the spermatangium to thrust into the outer lip of the female. For the deep-implanting spermatophores of *Galiteuthis glacialis* it has been speculated that the cement body is caustic and facilitates implantation (McSweeney, 1978; Nesis et al., 1998).

In this study, the process of deep implantation of spermatophores will be studied in *Rossia moelleri*. *Rossia moelleri* belongs to the sepiolid subfamily Rossiinae and inhabits the North Atlantic and Arctic Ocean (Jereb and Roper, 2005). Males of this species have a hectocotylus and implant spermatangia in the heads and mantle of females (Zumholz and Frandsen, 2006). Here, we will describe how spermatophores of this species implant autonomously into tissue under lab conditions. Furthermore, we will assess whether the mechanical force that is generated by the spermatophoric reaction of spermatophores of *R. moelleri* is sufficient to allow the sperm mass to penetrate tissue. Mated females will be examined to determine the position and number of implanted spermatangia. The detailed morphology of the spermatangium will be studied to examine which functional components may facilitate implantation. The spermatophoric reaction (without implantation) will be induced and its kinematics (total duration, maximum velocity and maximum acceleration) will be quantified.

2. Materials and methods

Rossia moelleri were collected by bottom trawl between 2003 and 2006 by RV "Walter Herwig" (Germany) in Greenland waters between 61°38'–66°40' N and 30°34'–58°31' W, at a depths ranging between 91 and 400 m. After capture, the specimens were deep frozen. From

these collections 10 females (ML 32–44 mm) and 10 males (ML 32–41 mm) were used in the present study. Animals were labeled mature when oviducts contained eggs or when spermatophores were present in the males Needham's sacs.

The length of the spermatophores in the Needham's sac of four males was measured and number of spermatophores was recorded. For six additional males, spermatophores were counted only. Also the females were examined and the location and the number of implanted spermatangia were recorded.

To study the spermatophoric reaction, a spermatophore was removed (by forceps) from the Needham's sac from a freshly thawed male, which was kept refrigerated. Removal of spermatophores induces the spermatophoric reaction. The oral end of a spermatophore is attached to the inside of Needham's sac through a 'thread'. During removal of the spermatophore, the thread is pulled, and the tension on the cap, the oral part of the spermatophore increases, causing the initiation of the spermatophoric reaction and the spermatophore starts inverting. Two experiments were performed with inverting spermatophores immediately after removal. In both experiments a high resolution digital video camcorder (JVC GZ-MC500E) was used to record the reaction at 25 frames/s.

The first experiment was performed to confirm that spermatophores of *R. moelleri* were able to implant into tissue autonomously even after kept deep frozen for as long as 4 years. We used a forceps to hold an inverting spermatophore at its aboral end, close to a piece of mantle tissue. Both the spermatophore and the mantle tissue were submerged in artificial seawater (35‰; 8 °C) during this experiment. Using this method, 14 spermatangia were successfully implanted into mantle tissue.

The second experiment involved the recording of the kinematics of inverting spermatophores without implantation. Inverting spermatophores were positioned at the bottom of a Petri-dish filled with

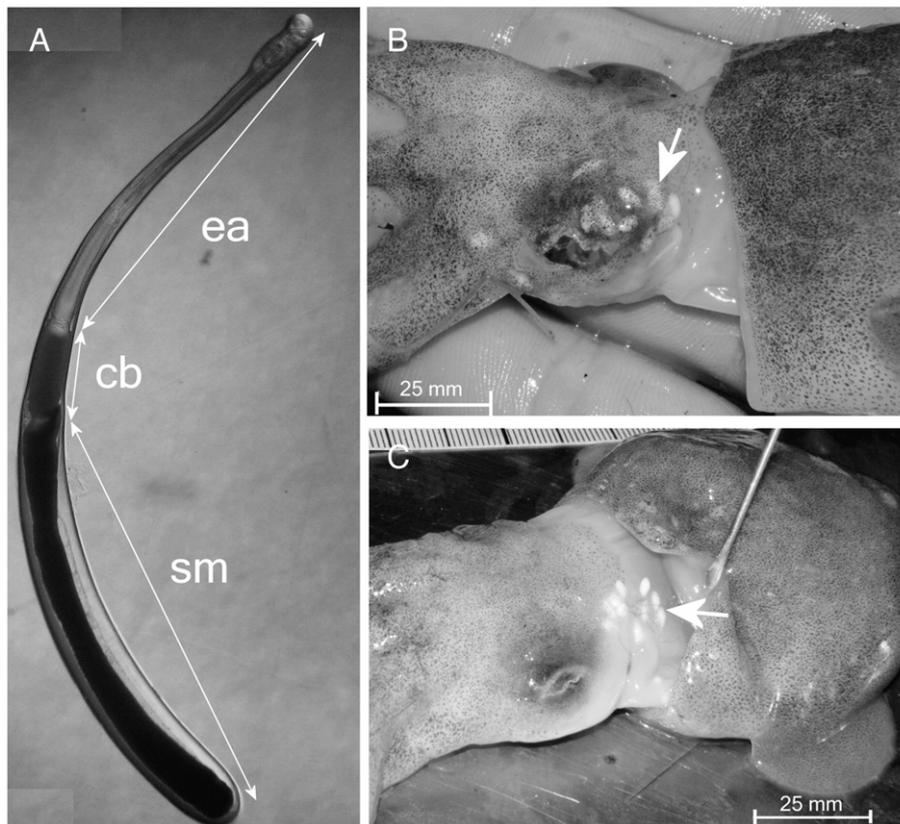


Fig. 1. *Rossia moelleri* (A) Spermatophore with ejaculatory apparatus (ea), cement body (cb) and sperm mass (sm); (B) dorsolateral view of the head and anterior mantle of post-copulatory female with implanted spermatangia above the eye indicated by an arrow; (C) dorsolateral view of a female after copulation with implanted spermatangia in the neck indicated by a white arrow.

artificial seawater (35‰; 8 °C). The reaction of six spermatophores was recorded with the camera in a fixed position to enable correct kinematic analysis.

For six spermatophores, the positions of the oral part of the inverted spermatophore and the moment when the spermatophore content exits were determined manually through frame by frame analysis after point digitisation (Didge 2.0 software). To filter out high frequency noise in the data caused by manual digitization of a discrete pixel pattern, a zero phase shift, fourth order Butterworth filter was applied to the data (cut-off frequency at 1 Hz). The velocity of the spermatophoric reaction was calculated as the first time derivative of the displacement of the front of the inverted contents in the local coordinate system of the spermatophore. The acceleration of the sperm mass was calculated as the time derivative of this velocity.

The force as a result of the forward moving sperm mass was calculated by multiplying the mass of the sperm-mass with the peak acceleration of the sperm-mass during the spermatophoric reaction. The mass of the sperm-mass was measured using a calibrated microscale.

The penetration of spermatophores into the tissue of *R. moelleri* was simulated by pushing needles with different degree of bluntness and attached to a force sensor into tissue of *R. moelleri* while measuring the instantaneous maximal forces at the instant of breaking

through the tissue. The needles were mounted on a single axis traversing system, which moved forward at a constant speed (approximately the same speed as the maximum speed of the spermatophoric reaction). A force transducer connected to the needle measured the force development in time as well as the peak force when the needle penetrated the tissue. This was repeated five times for each needle.

The sharp points of five needles were made blunt using sandpaper and the results were photographed. The bluntness of each needlepoint was determined from its perimeter as measured on the pictures (SigmaScan). The shape factor (surface-to-perimeter ratio standardized to a circle) was kept constant at 0.88. From the perimeter, the radius was calculated using the formula: $\text{perimeter} = 2\pi r$, where r is the radius of the needle point. The radius was used as a measure for bluntness. A repeated measured ANOVA in SPSS was used to test the significance of the correlation between bluntness and peak force.

The oral end of the completely inverted spermatangium was investigated using Scanning Electron Microscopy to search for hard or sharp structures that could facilitate implantation. Tissue samples with implanted spermatangia were stored in 10% formalin. From this solution the samples were dehydrated in a graded ethanol series, cleared in toluene, and embedded in paraffin wax. Longitudinal sections of 3 μm thick were cut using a microtome and stained with haematoxylin and

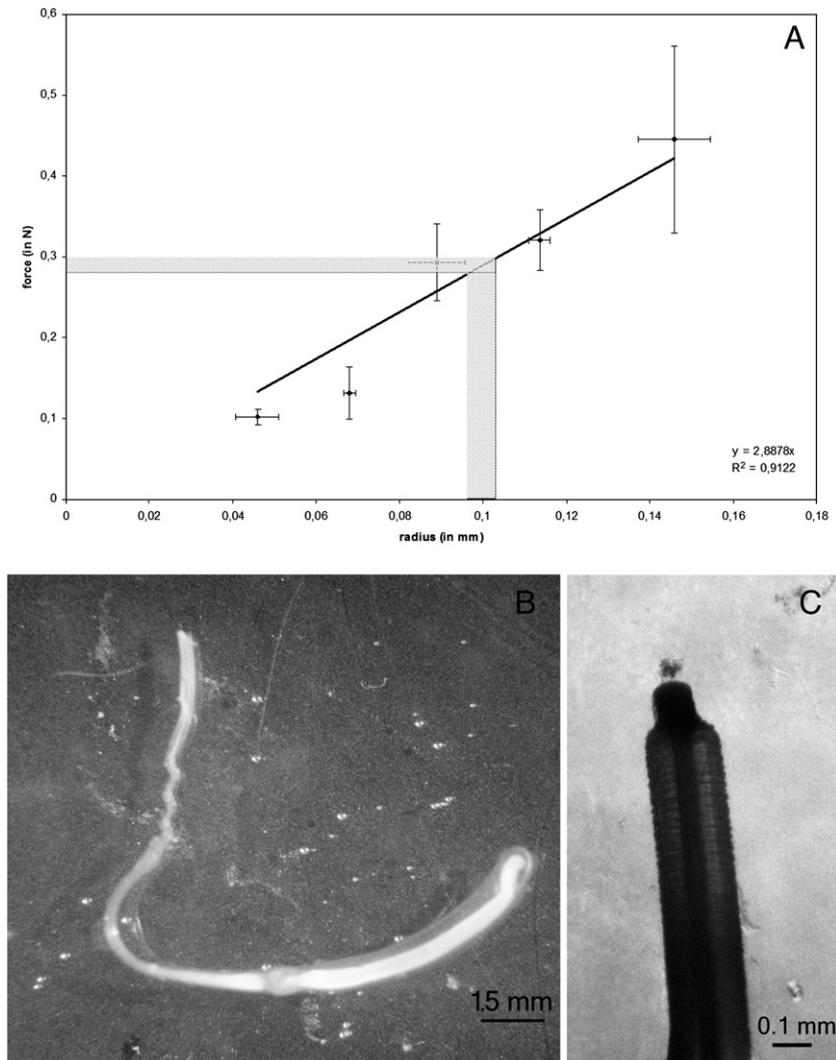


Fig. 2. (A) Relationship between the radius of the needlepoint used for the penetration and the force necessary for penetration of tissue of *Rossia moelleri*. Indicated in grey is the interpolated force value that is required for the inverting spermatophore to penetrate tissue; (B) Spermatophore stage during the spermatophoric reaction with the smallest diameter at the oral end; (C) Detail of the oral end of (B).

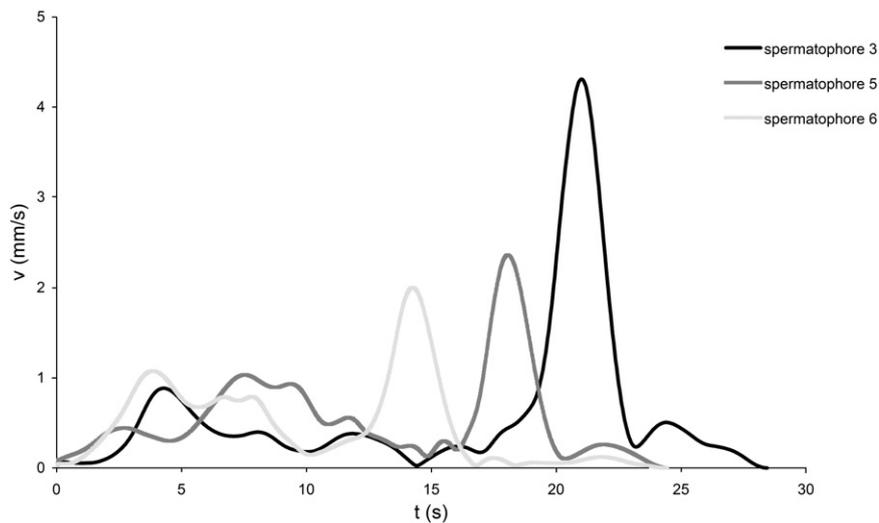


Fig. 3. The development of the velocities of the forward moving sperm masses during the spermatophoric reaction of three different *Rossia moelleri* spermatophores.

eosin. The sections of the implanted spermatangia were examined using a microscope and a calibrated ocular micrometer.

3. Results

3.1. Spermatophore transfer and storage

The presence of relatively small suckers on arm pair I of male *Rossia moelleri* indicates that these arms are hectocotylic. Spermatophores (Fig. 1A) were approximately 9–12 mm long (Hoving et al., 2008a) and consisted of an aboral sperm mass, a cement body (the central component of the spermatophore) and an ejaculatory apparatus, which folded orally in what is known as the cap. From the cap a thread extended (not present in Fig. 1). The spermatophores were situated with their aboral side to the opening of the Needham's sac, which contained between 19 and 56 spermatophores (mean 34 ± 11 spermatophores; $n = 10$) (Hoving et al., 2008a).

Females did not have a bursa copulatrix. Spermatangia were implanted in the head, neck and anterior mantle region (Fig. 1B and C). The number of implanted spermatangia in females ranged from 6 to 21 (mean 14 ± 5 ; $n = 10$). Spermatangia were mainly implanted on the left side of the females' bodies, the same side as where the distal oviduct opened (mentioned as Hoving et al. *in prep.* in Laptikhovskiy et al., 2008). The majority (64%) of all implanted spermatangia (140 in ten females) were implanted near the left eye, 23% were found implanted on the left and middle anterior mantle margin, 12% were implanted on the left side of the inner mantle and 5 spermatangia were found implanted in the funnel, on the left fin and on the basis of the right first arm.

3.2. Autonomous implantation

When an inverting spermatophore was held just in front of the tissue, the frontal part of the spermatangium penetrated the tissue and moved into it. When the frontal part of the inverting spermatophore did not move further through the tissue, the sperm mass moved from the aboral part of the spermatophore through the inverted ejaculatory apparatus to the oral part of the spermatangium. This resulted in the widening of the implanted oral part of the spermatangium. From the start of the reaction it takes less than a minute for a spermatophore of *R. moelleri* to become implanted into tissue. In successful implanted spermatangia, the empty outer tunic and part of the middle membrane of the spermatophore was extending from the point where the spermatangium had implanted itself. Artificially implanted spermatangia resembled spermatangia found implanted naturally in females.

Implantation attempts were not always successful. The spermatophores sometimes did not start inverting when removed from the Needham's sac. Also sufficiently long and proper contact with the female tissue seemed important factors to ensure implantation.

3.3. Tissue penetration simulation

The relation between the force necessary to penetrate the tissue of a female and the radius of the needle tip was linear and significant ($F = 2.89 \cdot \text{Bluntness}$; $df = 1$; $P = 0.002$) (Fig. 2A). Assuming the spermatophore enters the tissue at the stage where the oral end is the narrowest ($r = 0.1$ mm; Fig. 2B, C), the necessary force to penetrate the tissue is estimated to be 0.28–0.3 N (Fig. 2A). This appeared to be independent of the angle of incidence.

3.4. Spermatophoric reaction without implantation

The inverting spermatophore gradually increased in length by the forward movement of the inverting ejaculatory apparatus. Just prior to reaching its maximum length, the velocity and acceleration of the inverting ejaculatory apparatus showed steep increases and reached their maximum (Fig. 3; Table 1), which ranged between 2–4.5 mm/s and 1.6–3.6 mm/s² (Fig. 3) respectively. This increase in velocity and acceleration probably corresponds with the movement of the inverting spermatophore into and through the tissue (during implantation *in situ*). Maximum velocities and accelerations were reached at $t = 12.4$ – 24.6 s and at $t = 11.8$ – 25.8 s respectively. The total duration of the spermatophoric reaction, without implantation, ranged from 32 to 54 s (Table 1).

The mass of the spermatangia ranged between 0.7–2.6 μg (mean 1.6 ± 0.68 μg ; $n = 10$). The mechanical force as a result of acceleration of the forward moving sperm mass was 1.12 μN –9.36 μN (about 100,000 times less than the force necessary to penetrate the female mantle muscle with a needle of the same shape).

Table 1
Kinematics of the spermatophoric reaction of six spermatophores of *Rossia moelleri*.

Spermatophore	Total duration (s)	max v (mm/s)	t at max v	max a (mm/s ²)	t at max a
1	32	3.2	12.4	2.5	11.8
2	48	2.3	24.6	2.1	25.8
3	45	4.3	21	3.3	20.1
4	52	4.5	21.6	3.6	20.9
5	48	2.4	18.1	2.3	17.2
6	54	2	14.2	1.6	13.5

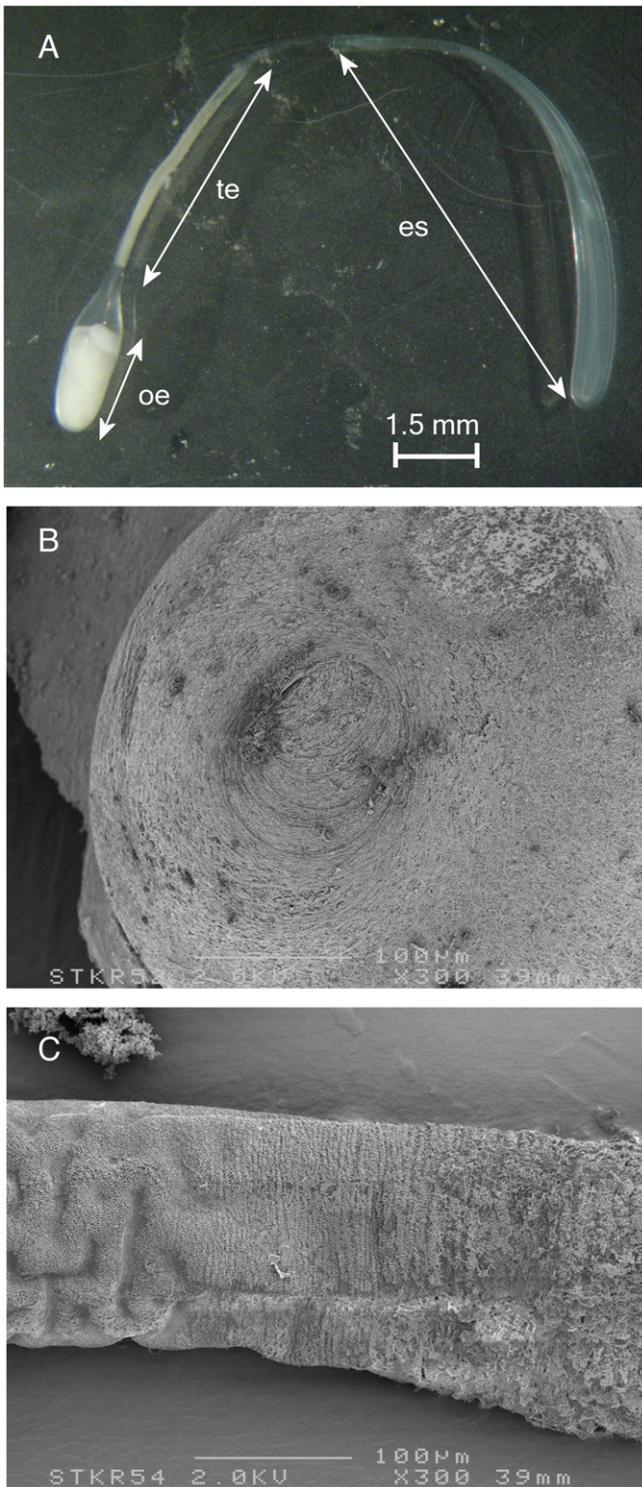


Fig. 4. (A) a completely inverted spermatophore (= a spermatangium) after the spermatophoric reaction was induced in the lab; (B) SEM photograph of the oral end of the spermatangium showing the smooth surface; (C) SEM photograph of the free trailing end (the middle membrane). Indicated are the oral end of the spermatangium (oe), the trailing end (te) and the empty spermatophore sheath (es).

3.5. Morphology of spermatangia

Examination of the external morphology of the spermatangium did not reveal any sharp or hard structures at its oral end, nor an oral opening from the spermatangium to the exterior (Fig. 4). The area between the empty spermatophore sheath and the oral sperm mass (middle membrane) was not smooth but was covered by the stellate

particles probably from the inner membrane of the ejaculatory apparatus (Takahama et al., 1991) (Fig. 4).

The implanted spermatangium consisted of a sperm mass, a cement body and a free trailing end that extended from the point where the spermatangium had implanted itself into the female tissue (Fig. 4, 5).

The inner tunic surrounds the cement body in the intact spermatophore and as eversion occurs during the spermatophoric reaction, the inner tunic also partially everts nearly freeing the cement body while enclosing the sperm mass inside the spermatangium, maintaining separation between the sperm mass and the cement body (Fig. 5). The eversion of the inner tunic causes an oral opening where cement from the cement body was seen to exit the spermatangium (Fig. 5). The middle membrane surrounds the whole spermatangium and extends aborally to form the trailing end, or, in a recently everted spermatophore, to connect with the empty spermatophore sheath (middle and outer tunic) (Fig. 4). In a natural situation, the empty sheath would have been detached from the spermatangium probably shortly after mating. A cross-section of the implanted trailing end shows the middle membrane on the outside, the thicker inner tunic on the inside and some sperm inside the inner tunic (Fig. 5).

4. Discussion

4.1. Spermatophore transfer and storage

In *R. moelleri* the role of the hectocotylus is to remove spermatophores from Needham's sac, and to position inverting spermatophores on the female's body. There is no evidence suggesting that the hectocotylus of *R. moelleri* would physically break open the spermatophores as observed in *Sepia officinalis* (Hanlon et al., 1999). The spermatophoric reaction is initiated when the spermatophores are removed from Needham's sac because the thread remains attached to the sac and is being "pulled".

The fact that males have more spermatophores in their Needham's sac than females have implanted spermatangia may indicate that not every spermatophore that is transferred to the female is actually implanted. It is also possible that males mate with more than one female. Promiscuity and multiple paternity of spawned eggs is known from other cephalopods (Hanlon and Messenger, 1996; Shaw and Sauer, 2004).

Most spermatangia were implanted on the left side of the female's body, which is the same side as where the oviduct opens. *Rossia moelleri* produces and spawns large eggs continuously after reaching maturity (Laptikhovskiy et al., 2008). How and when the spermatozoa are mobilized is unknown, but perhaps the secretions of the nidamental glands, accessory nidamental glands or oviducal glands initiate sperm mobilization from the spermatangia (Durward et al., 1980). The trailing end of the spermatangium that extended from the point of penetration probably is the exit for the spermatozoa during fertilization of the eggs (Racovitza, 1894; Mann, 1984). Therefore spermatangia implanted on the left side of the female are probably located best for fertilization of the eggs, and males aim to deposit spermatophores in this region.

Implantation of spermatophores, as described for *R. moelleri*, is found in all members of the subfamily Rossinae. All species belonging to the other two sepiolid subfamilies Heteroteuthinae (except *Stoloteuthis leucoptera*: (Villanueva and Sanchez, 1993)) and Sepiolinae (Bello, 1995) have modified tissue for the reception and storage of sperm or spermatangia (for overview see (Hoving et al., 2008a)).

Mating behaviour of *R. moelleri* has not been described. However, mating in the related species *Neorossia caroli* is a rather violent event where males grab females from below and transfer as many spermatophores as possible to the mantle cavity in the shortest possible time before the female escapes (Mangold, 1987; Cuccu et al., 2007).

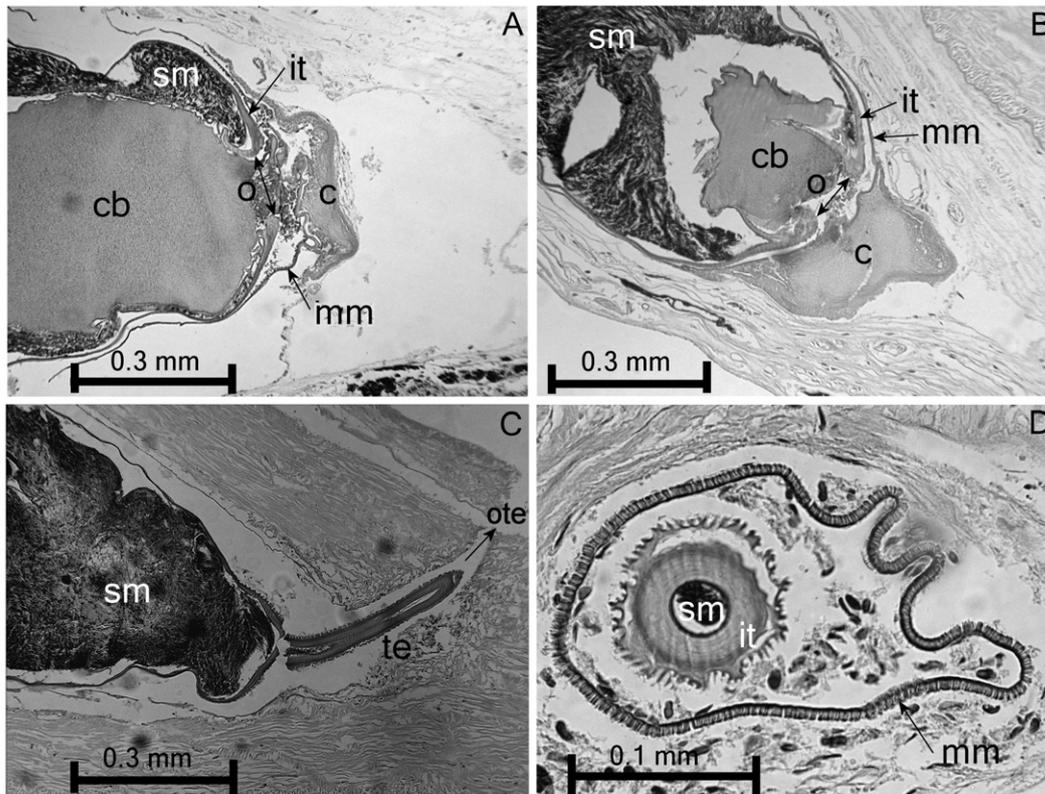


Fig. 5. Histological longitudinal sections of implanted spermatangia of *Rossia moelleri* showing (A) and (B) the oral part of the implanted spermatangium; (C) the aboral part of the spermatangium; (D) a cross-section of the trailing end of the spermatangium. Indicated are the cement body (cb), the sperm mass (sm), the middle membrane (mm), the inner tunic (it), cement (c), oral opening of the spermatangium (o), trailing end (te), opening to exterior (oe).

4.2. Autonomous implantation of spermatangia

This study shows that spermatophores of *R. moelleri* are able to implant autonomously into tissue. Autonomously-implanting spermatangia may benefit the possibly promiscuous behaviour of the male since the less time spent on one copulation, the more females it can mate with in a given time. Since the transfer of spermatophores involves a diversion of attention for both male and female, short mating events may also benefit a reduced predation risk compared to longer mating.

Implanted spermatangia were mostly found around the eyes. Although in this study the physical force to penetrate tissue was only determined for mantle tissue, it is possible that hardness of tissue differs among body regions. The higher number of spermatangia implanted around the eyes may be explained by the fact that the tissue here is softer, probably resulting in a higher implantation success rate compared to other regions on the female body.

The fact that previously frozen spermatophores of *R. moelleri* were still able to implant into mantle tissue may allow greater flexibility in future research on the implantation process of spermatophores of other cephalopods because it will not necessarily depend on the availability of fresh specimens.

4.3. Implantation of spermatangia:

Tissue penetration, spermatophoric reaction and morphology

Our observations on *R. moelleri* suggest that the inverting spermatophore has to touch the female tissue before the forward moving sperm mass reaches its highest acceleration. If so, the male has to deposit the spermatophore on the female 10–30 s after removal from the Needham's sac, or implantation will not take place and the transfer is useless. This time constraint together with the fact that the female may be struggling during mating may explain why the

implanted spermatangia were found in different regions of the female body.

The force necessary for penetration into the mantle tissue of *R. moelleri* exceeds that provided by the forward movement of the sperm mass during the spermatophoric reaction. Additionally, structures on the spermatangium that may have facilitated implantation, like the oral spike in spermatangia of *T. pacificus* (Takahama et al., 1991) or the oral tubular extension in spermatangia of *Octopoteuthis sicula* (Hoving et al., 2008b), were not found in *R. moelleri*. The spermatangia implantation process is therefore not likely to take place purely mechanically through forcefully piercing of the skin. Histological sections of implanted spermatangia visualized the cement body secreted orally from the spermatangium. How exactly the cement is secreted is not clear since there was no distinct opening found. The cement body may aid implantation by lysis of the surrounding tissue (McSweeney, 1978; Nesis et al., 1998), which would reduce the resistance encountered by the spermatangium or create a lumen for the spermatangium to move into. Alternatively, the cement body may act as a lubricant by reducing the resistance during movement through the tissue, although it is difficult to imagine how the actual penetration takes place in that case.

There are no chemical analyses of spermatophore tissues available other than those of the giant Pacific octopus (Mann et al., 1973). The concentration of active glycosidases appeared to be highest in the cement body relative to the other parts of the Octopus' spermatophore. Nesis et al. (1998) proposed a proteolytic function of the cement body during implantation of spermatangia of the Antarctic deep-sea squid *Galiteuthis glacialis*. The histological sections of implanted spermatangia of *R. moelleri* also suggest tissue lysis to have taken place at the oral end of the spermatangium. From this we tentatively conclude that lysis may enable the spermatophore to burrow into tissue. During the spermatophoric reaction, uptake of water into the spermatophore actually causes the sperm mass to move

forward (Drew, 1919; Austin et al., 1964). Therefore the implantation mechanism of cephalopod spermatangia is probably a combination of both mechanical and chemical factors. However, further experimental work is necessary to determine the exact role and consistency of the cement body and to answer the other remaining questions on the implantation of spermatangia in cephalopod tissue.

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