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Insertion and withdrawal of extremely elongated genitalia: a simple mechanism with a highly modified morphology in the leaf beetle

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A short title: Elongated genitalia in a leaf beetle

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Complicated genital structures are widely known in many animals. It is assumed that

increasing morphological complexity of genital structures would also increase the difficulty of inserting and withdrawing the structures. We examined the insertion and withdrawal mechanisms of extremely elongated genitalia in the Japanese leaf beetle *Lema* (*Lema*) *coronata.* Insertion and withdrawal processes are completed quickly. Investigation of genital morphology showed that there are no protractor or retractor muscles attached to the elongated part. Instead, the elongated part is tightly enveloped by a membrane. Due to the close fit between the elongated part and its surrounding membrane, eversion of the membrane allows for insertion of the elongated part, and retraction of the membrane induces withdrawal of the elongated part. This surrounding membrane also serves as the capacity of the elongated part, with the basal portion of the elongated part extending internally against the entrance. This unusual character state is also observed in other members of the subgenus *Lema*, which have also the elongated part. This condition can be considered to be preadaptation for extreme elongation of genitalia.
ADDITIONAL KEYWORDS: copulation - Chrysomelidae - Criocerinae - endophallus - internal sac - morphology - preadaptation

INTRODUCTION

Animal genitalia are often extremely elaborate in form, especially in species with internal fertilisation (Eberhard, 1985). Elaborate forms can include features such as spines (e.g., Kingsolver, 1970; Mann & Crowson, 1996; Crudgington & Siva-Jothy, 2000; Flowers & Eberhard, 2006; Kamimura, 2008), structures to facilitate sperm transfer by traumatic insemination (e.g., Carayon, 1966; Kamimura, 2007), specialised structures for sperm displacement (e.g., Waage, 1979, 1984; Yokoi, 1990; Kamimura, 2000) and elongated and/or coiled genitalia (summarized in Neufeld & Palmer, 2008; see also Mikkola, 2008 for insects). In particular, elongated genitalia are widely observed throughout the animal kingdom in species from ducks to snails, barnacles, ostracods, spiders and insects (summarized in Neufeld & Palmer, 2008).

It is widely accepted that the evolution of these curious morphologies is driven by sexual selection (Eberhard, 1985; Arnqvist, 1998; Hosken & Stockley, 2004; see also Mikkola, 2008). By contrast, it has been shown that extremely elongated genitalia can

be damaged during copulation due to the fragility of the slender genital tube (Kamimura & Matsuo, 2001; Matsumura & Akimoto, 2009). It means that morphological specialisation can produce not only benefits (increased reproductive fitness) but also costs, such as an increased probability of damage as structures become more complicated. In particular, difficulties in handlings during copulation are thought to increase with the degree of exaggeration in genital morphologies.

How do males with extremely elongated genitalia overcome this problem? For example, a male rove beetle (*Aleochara tristis*) secures its long genital tube between the wing shoulder and the pronotum while holding the tube strained at about one half of its length during genital withdrawal from the female (Gack & Peschke, 2005). In another case, a male fly (*Ceratitis capitata*) folds the basal portion of his distiphallus backward and inserts the distiphallus into a female from the folded portion (Eberhard, 2005). The site of the fold in the distiphallus gradually moves distally deeper into the female due to the increase in fluid pressure in the distiphallus (Eberhard, 2005). These studies are the only published observations about the insertion and withdrawal processes of extremely elongated male genitalia to date.

In both sexes of the Japanese leaf beetle *Lema* (*Lema*) *coronata* Baly, a part of the genitalia is extremely elongated, measuring about twice the length of the body. For males, the median ejaculatory guide (MEG) measures approximately 10 mm in length (Fig. 1A, B); for females, the spermathecal duct is approximately 14 mm long (Fig. 1C) compared to the body length of 6 mm (Matsumura & Suzuki, 2008). MEG is the elongated part of the sclerite on the internal sac, and without any protrusion on the tube (Fig. 1B).

The elongated MEG is inserted into the spermathecal duct during copulation (Matsumura & Akimoto, 2009), but the mechanisms of its insertion and withdrawal are unknown. The mating behaviour is simple in *L. coronata* (Matsumura & Akimoto, 2009), in contrast to the highly specialised mating behaviour of the rove beetle (Gack & Peschke, 2005). To understand how *L. coronata* deals with extremely elongated genitalia, we investigated the morphology of the internal sac, behaviour and genital mesh.

MATERIALS AND METHODS

TERMINOLOGY

In this paper, we adopted the word 'genitalia' for the male intromittent organ because of its common use in evolutionary biology in this sense (e.g., Eberhard, 1985; Hosken & Stockley, 2004). To describe internal sac morphology, nomenclature for the internal sac sclerite must be defined. Beetles of the subfamily Criocerinae (such as *L. coronata*) are considered to be closely related to those of the subfamily Donaciinae (Farrell, 1998; Duckett *et al*., 2004; Gómez-Zurita *et al*., 2007; Gómez-Zurita *et al*., 2008), and it has been reported that the internal sac sclerite of the Criocerinae resembles those of the Donaciinae in shape (Askevold, 1991; Mann & Crowson, 1996). However, positional relationships between the internal sac sclerite and the membrane differ between *L. coronata* and the members of the Donaciinae (pers. obs.). Thus we coined new terms according to the position and/or form of the internal sac sclerite.

REARING AND MATING PROTOCOL

Adults (ca. 120 individuals) were collected in Shiga Prefecture, Japan, on July 13, 2008. To obtain virgin males and females, we reared their offspring to adult and used them for observation (cf. Matsumura & Akimoto, 2009).

Individual pairs were allowed to copulate in 20 ml glass vials at 30°C. To facilitate observation of the mating sequence, mating pairs were immobilised with a cooling spray at eight stages: 1, 1.5, 2, 5, 10, 15, 20 and 25 minutes after the start of copulation (copulation lasted for 34 minutes on average: Matsumura & Akimoto, 2009). We defined the start of copulation as the time when a male mounts a female.

DISSECTION

The mating pairs preserved in a freezer were dissected after thawing. Coupled states of the male internal sac with the female genitalia were recorded in photographs using a binocular microscope (Olympus SZ60) with a digital camera (Sony Cyber-Shot 4.0). Then we made whole slide-mounted specimens of the female spermatheca carrying the male MEG. To measure spermathecal length, photographs were taken under a light microscope (Olympus BX40) with a digital camera (Nikon COOLPIX 995). We also

measured how far MEG was inserted into the spermathecal duct. The lengths of the male and female genitalia in the photos were measured using a curvimeter (Koizumi COMCURVE-9 Junior). Measurement errors in this procedure were 0.40 ± 0.34 (mean ± SD). We also examined the spermatheca for the presence or absence of sperm.

RESULTS

INTERNAL SAC MORPHOLOGY

The internal sac of male *L. coronata* is mostly membranous, except for the internal sac sclerite. The membranous part of the sac is largely invaginated into the body cavity until the early stages of copulation (Fig. 2), and thereafter the membrane is largely everted from a limited opening of the internal sac. For convenience, the invaginated and noninvaginated areas of the membrane are referred to here as the 'interior' and 'exterior' internal sac membranes, respectively. Although the areas are continuous without any boundaries, the border is defined here as a constricted neck where the internal sac sclerite is found (Fig. 2, an arrow). A very stout bundle of muscles arises from the median lobe and has a very wide attachment area on the interior internal sac membrane.

We refer to parts of the internal sac sclerite as follows: two lateral lobes (LL), a median ejaculatory guide (MEG) and a basal block (BB) (Fig. 2). BB includes an opening of the ejaculatory duct, and the ejaculatory duct opens inward against the genital cavity (Fig. 2, a red line). We interpreted MEG as an elongated part of a sclerite surrounding the gonopore, thus the position of the gonopore is assumed to the base of MEG.

The basal part of the sclerite, LL and BB, is widely connected to the membrane. In contrast, MEG is unconnected to the interior internal sac membrane but is tidily enveloped by the membrane (Fig. 3A). The interior internal sac membrane grips MEG very tightly (Fig. 3B); therefore, MEG cannot move freely within male genital cavity. The inner wall of the enveloping membrane is covered with many fine spines (the height ranges from approx. 2 to 10 μm; Fig. 3C).

COPULATORY BEHAVIOR

The outline of the insertion process is summarised in Fig. 4. In many cases, insertion of the median lobe did not start until ca. 30 seconds after mounting due to reluctant females. At 1 minute into copulation, the male had inserted its median lobe into the female genital cavity and weakly everted the exterior internal sac membrane in the bursa copulatrix (N=2, Fig. 5A), and pairs were easily separated by a cooling spray. At 1.5 minutes into copulation (N=5), the exterior internal sac membrane was only weakly everted in three males. In two males, the exterior and part of the interior internal sac membranes were everted in the bursa copulatrix, and MEG was partly inserted into the spermathecal duct (Fig. 6). At 2 minutes into copulation (N=4), males had everted the exterior and part of the interior internal sac membranes (Fig. 5B), and MEG was inserted into the spermathecal duct more deeply but by less than half its length (Fig. 6). At 5 minutes into copulation (N=5), males had markedly everted the interior internal sac membrane (Fig. 5C) and had largely inserted MEG into the spermathecal duct (N=3) (Fig. 6). Sperm was observed in the spermathecal capsule of females (N=3) and even in a female that was not carrying MEG. At 10–20 minutes into copulation (N=10 in total), MEG was fully inserted (more than 70–90 % of the whole MEG length) into the spermathecal duct in all mating pairs. The basal part of MEG was never inserted into the spermathecal duct and was always enveloped by the interior internal sac membrane whenever we observed. In some pairs (2/4 pairs), the withdrawal process had been completed at 25 minutes into copulation. No spermatophores were observed in any female genital cavity, so sperm transfer was considered to be completed only through MEG.

WITHDRAWAL PROCESS

The withdrawal process could not be observed directly because it is probably completed in a few seconds, as suggested by the following evidences. First, when we disturbed mating pairs in which MEG was fully inserted (at ca. 5 minutes into copulation), the males dismounted after a few seconds. Even in such cases, the females did not have any fragments of MEG in their bodies (N=2). Secondly, we observed a novel behaviour in three males: the males moved his median lobe back and forth, and his exterior and interior internal sac was inflated and deflated without mounting a

female. The behavioural sequence was completed in a few seconds. Eversion of the interior internal sac membrane indicates that MEG is also largely exposed because MEG is enveloped by the membrane.

DISCUSSION

INSERTION MECHANISM

While the volume of the exterior internal sac increased and the interior internal sac membrane was everted, the inner cavity was filled by blood. This suggests that increasing internal pressure results in eversion of the interior internal sac. In a detailed anatomical study of other leaf beetle *Aspidomorpha miliaris*, it was shown that internal pressure is increased by contraction of the abdomen and that the basal muscle of the median lobe functions to maintain increased pressure (Verma & Kumar, 1972). The basal part of the median lobe of *L. coronata* carries a large bundle of muscles, therefore a mechanism similar to that postulated for *A. miliaris* must play a major role in eversion of the *L. coronata* internal sac and the maintenance of its inflated condition.

The opening of the interior internal sac is too limited to allow the membrane to be everted all at once (Fig. 2), and expanded LL are situated at the ventral border of the interior/exterior internal sac membranes (Fig. 2). Thus, the interior internal sac membrane is extruded through the opening step-by-step from the dorsal margin (Fig. 5, as indicated by an arrowhead). Since MEG is tightly enveloped by the interior internal sac membrane (Fig. 3A, B), MEG is also released step-by-step from its distal tip (Fig. 7A, C). The position of the ostium on the internal sac corresponds to that of an opening of the spermathecal duct into the bursa copulatrix (Fig.1 in Matsumura & Akimoto, 2009). Thus, released MEG inevitably inserts into the spermathecal duct. The insertion process is predicted to take longer than the withdrawal. Probably a non-straight passage of the spermathecal duct (Fig. 1C) and a limited space of the female bursal cavity to evert the internal sac membrane make MEG difficult to insert quickly.

WITHDRAWAL MECHANISM

Verma & Kumar (1972) illustrated a pair of stout retractor muscles attached to the sac whose function was to withdraw the internal sac. Homologous muscles were also

found in *L. coronata*, which indicates that withdrawal of the internal sac is caused by contraction of the muscles attached to the interior internal sac membrane (Fig. 7B). This mechanism explains the withdrawal process of the membrane itself.

When the interior internal sac membrane is withdrawn, a delicate balance of force will arises between the internal pressure and the retractor muscles along the fold of the interior internal sac membrane where the base of MEG is enveloped. The counterforce arising along the fold will guarantees the holding power of the enveloping membrane. As in the insertion process, because expanded LL are situated at the ventral border of the interior/exterior internal sac membranes, the interior internal sac membrane is withdrawn step-by-step through the dorsal margin of the entrance (Fig. 7B, C). In addition, the interior internal sac membrane having a fold in which MEG fits (pers. obs.) and adhesive force of chitin (Vincent & Wegst, 2004) consisting insect body will make MEG easy to return into the male genital cavity.

We speculated that one of key factors for insertion and withdrawal of MEG is produced by the fine spines on the inner wall of the enveloping membrane (Fig. 3C). Since such large arrays of fine spines are very similar to those of gecko setae (feet hairs) which yield re-attachable adhesive (e.g., Autumn *et al*., 2000; Autumn *et al*., 2002; Geim *et al*., 2003; Huber *et al*., 2005; Niewiarowski *et al*., 2008), it might produce adhesive force for the membranes. A structure that is fundamentally the same is well known in insect feet (e.g., Gorb & Beutel, 2001; Gorb & Gorb, 2008; Gorb *et al*. 2008) and in mouth parts (e.g. Betz, 1996; Karolyi *et al*., 2009). However, further studies are required to understand the function of these fine spines in *L. coronata*.

EVOLUTION OF ELONGETED GENITALIA

The present study revealed that the tight relationship between MEG and the interior internal sac membrane (Fig. 2) makes it possible to handle extremely fine and long tube effectively and also to store such an elongated tube within a small cavity of the median lobe.

In other subgenera and genera of the subfamily Criocerinae having no elongated part, the opening of the ejaculatory duct into the internal sac sclerite faces to the opening of the internal sac (Mann & Crowson, 1996). This condition is also observed in their

sister taxon Donaciinae (see Askevold, 1987, 1991, Hayashi, 2004, 2005). Alternatively, in *L. coronata*, the opening of the ejaculatory duct into the internal sac sclerite faces on the opposite side (Fig. 2). Other species of the subgenus *Lema* have the elongated part even though the length is short, and the same character state is observed (Mann & Crowson, 1996; Matsumura & Suzuki 2008). This indicates that MEG first extends internally with catching the membrane, which makes it possible to store long MEG within the small cavity of the median lobe and to effectively insert and withdraw the elongated MEG. Therefore, the novelty of the character state can be considered to be a preadaptation for the elongation of MEG.

In addition, our results showed that sperm transfer is completed only through MEG. The outer diameter of MEG is too small (less than 2μm; Matsumura & Akimoto, 2009) to go through MEG for plural spermatozoa at one time. As shown by Werner et al. (2007) for the rove beetle *Drusilla canaliculata*, such the small diameter of the extremely elongated MEG can make spermatozoa possible to push themselves off the tube wall and migrate into the apex of the spermathecal duct, spermathecal capsule. If true, as shown in the leaf beetle *Chelymorpha alternans*, the males with longer genitalia might be favored in copulation Rodriguez, 1995). Previous studies showed that cryptic female choice was a strong hypothesis for evolution of genital length (Gschwentner & Tadler, 2000; Rodriguez et al., 2004; Tadler, 1999), so we need to test the hypothesis for *L. coronata*.

The origin and function of such morphological preadaptation are also important points in considering in the evolution of genital elongation. Genital elongation is also known in leaf beetle subfamilies Eumolpinae (e.g. Flowers 1999; Flowers and Eberhard 2006) and Cassidinae (e.g. Rodriguez et al. 2004) which are distantly related to the Criocerinae (e.g. Gómez-Zurita *et al*., 2008). Furthermore, their elongated part is not homologous with that of the Criocerinae. By resolving their phylogenetic relationship and comparing the function and morphology among them, the Chrysomelidae could become a model taxon in understanding the origin of elongated genitalia.

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FIGURE LEGENDS

Fig. 1 Male and female reproductive organs. A, Male genitalia in lateral view (the internal sac is everted). B, Internal sac sclerite (the lateral lobes have been removed). C, Female (Dorsal view).

Fig. 2 Schematic drawings of the internal sac, lateral view. The frontal surface of the interior and exterior internal sac membrane was removed. The grey area indicates the INSIDE of the body cavity. A border between the interior and exterior internal sac membranes is indicated by the arrow.

Fig. 3 Close fit between MEG and its surrounding membrane. A, Internal sac (lateral view) at 2 minutes into copulation; the exterior internal sac membrane is partly removed to show the inner structure; IS: internal sac, MEG: median ejaculatory guide. B, Schematic drawing of a sagittal section of MEG and the enveloping membrane. C, The enveloping membrane.

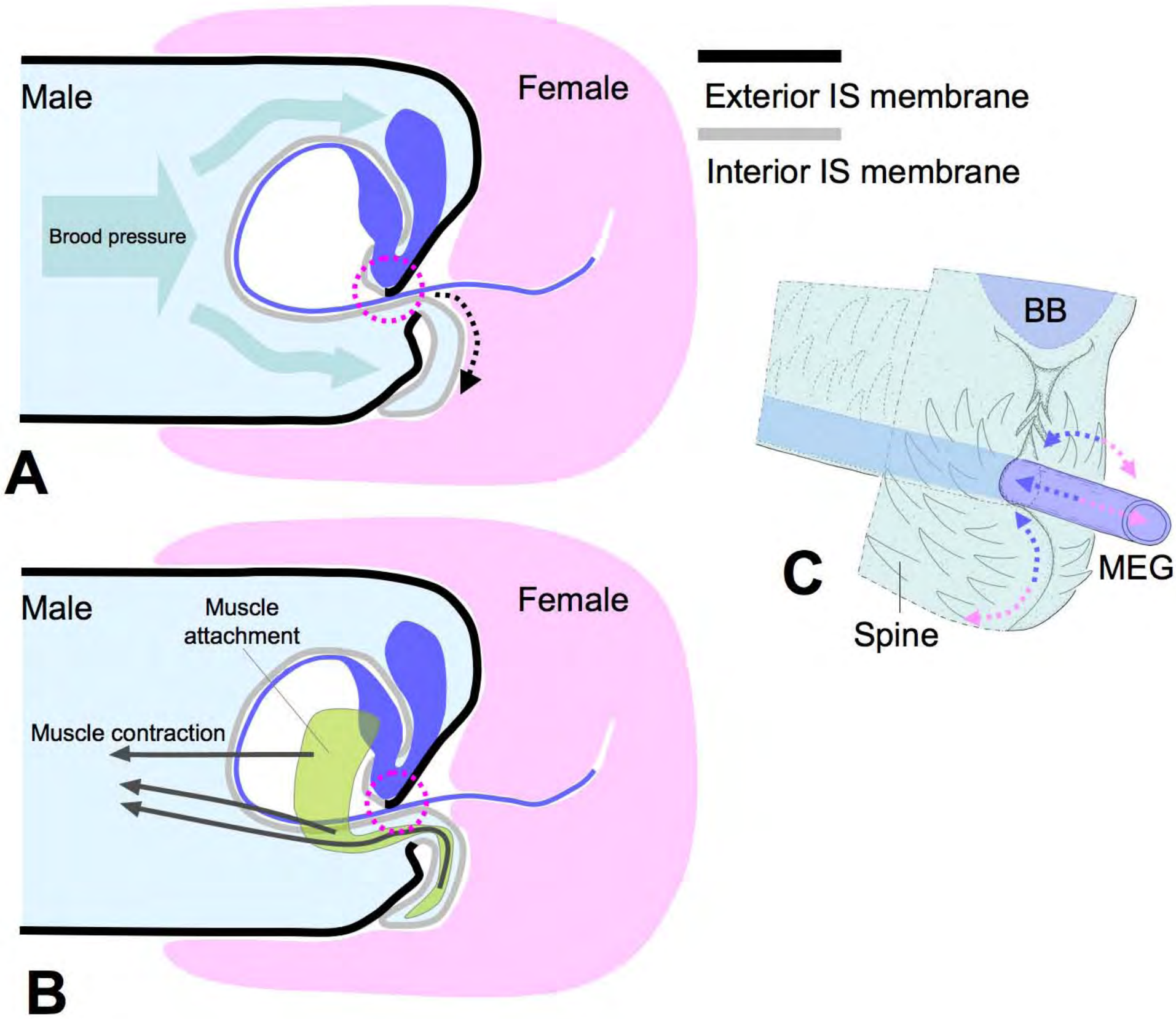
Fig. 4 Mating process.

Fig. 5 Degree of development in the internal sac at 1 minute (A), 2 minutes (B) and 5 minutes (C) into copulation. IS: internal sac. Grey lines and broken black lines indicate the outline of the exterior and interior internal sac membranes, respectively.

Fig. 6 Schematic drawings of the MEG inserted into the spermathecal duct during copulation. Lengths of the total spermatheca (□) and MEG inserted in the spermathecal duct (■). The length of MEG inserted corresponds to (A) % of whole length of MEG, and (C) whether or not females had sperm in the spermatheca. ?:

sperm existence could not be determined, ×: sperm did not exist, ♠: sperm existed.

Fig. 7 Schematic drawing of insertion and withdrawal mechanics. A, Insertion. B, Withdrawal. C, Internal sac opening corresponding to the area enclosed by the pink circle in A & B; movements of MEG caused by the eversion and retraction of the interior internal sac membrane is shown.


Male
Female
Brood pressure
A
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Muscle attachment
Female
Muscle contraction
B
Exterior IS membrane
Interior IS membrane
BB
C
MEG
Spine

Fig. 7 A, B, C

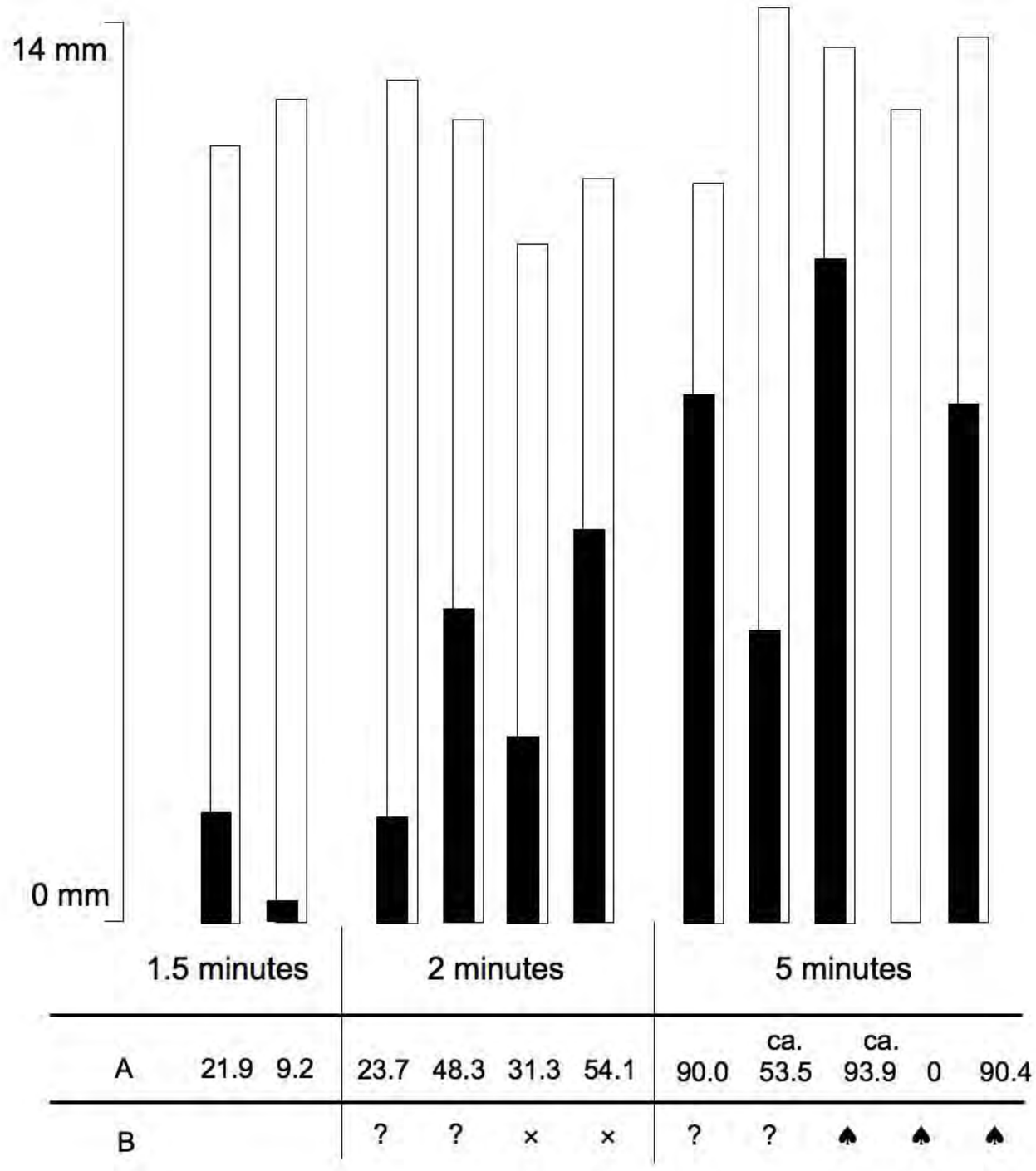

14 mm
0 mm
1.5 minutes
2 minutes
5 minutes
A
21.9
9.2
23.7
48.3
31.3
54.1
90.0
ca. 53.5
ca. 93.9
0
90.4
B
?
?
×
×
?
?
♠
♠
♠

Fig. 6

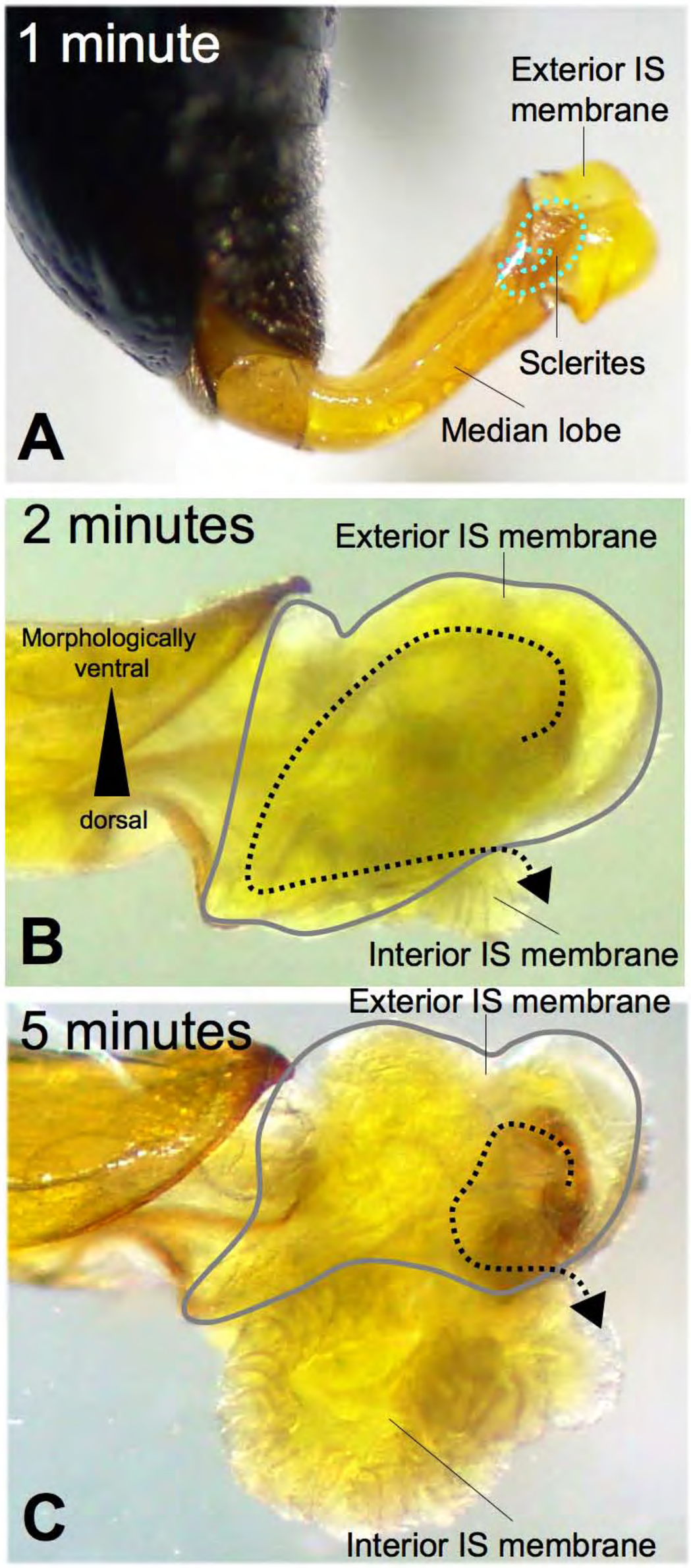

1 minute
Exterior IS membrane
Sclerites
Median lobe
A
2 minutes
Exterior IS membrane
Morphologically ventral
dorsal
B
Interior IS membrane
5 minutes
Exterior IS membrane
C
Interior IS membrane

Fig. 5A-C

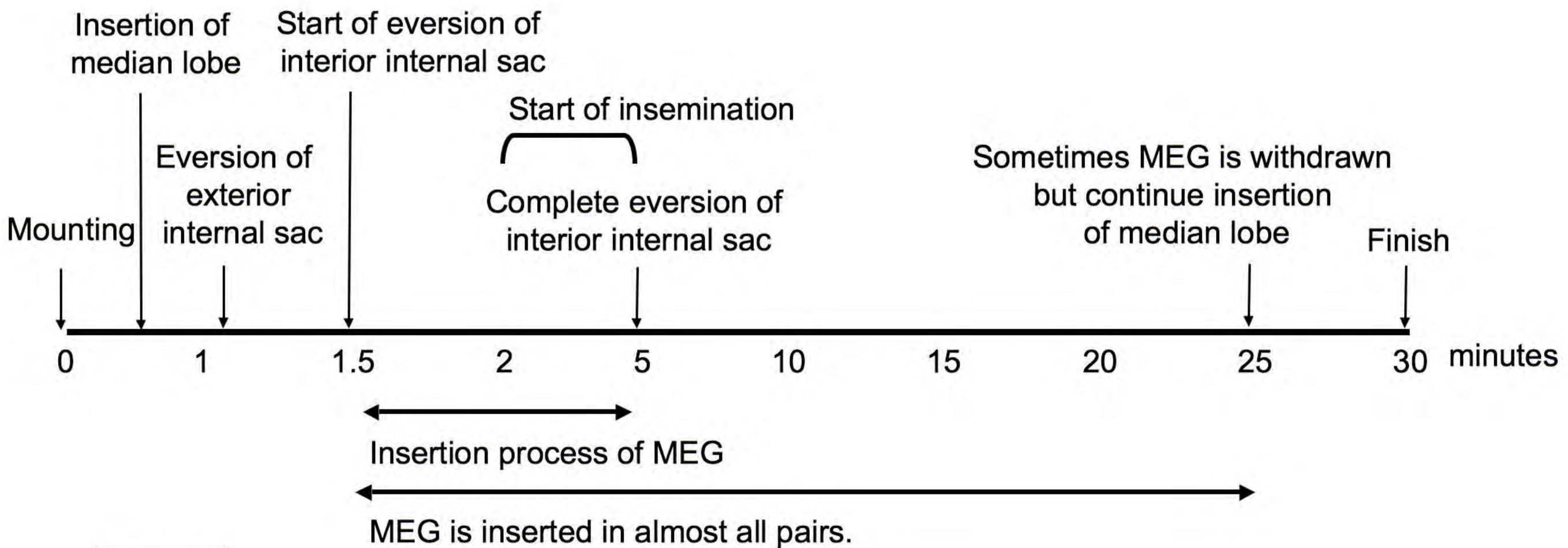

Insertion of median lobe
Start of eversion of interior internal sac
Start of insemination
Eversion of exterior internal sac
Mounting
Complete eversion of interior internal sac
Sometimes MEG is withdrawn but continue insertion of median lobe
Finish
0
1
1.5
2
5
10
15
20
25
30
minutes
Insertion process of MEG
MEG is inserted in almost all pairs.

Fig. 4

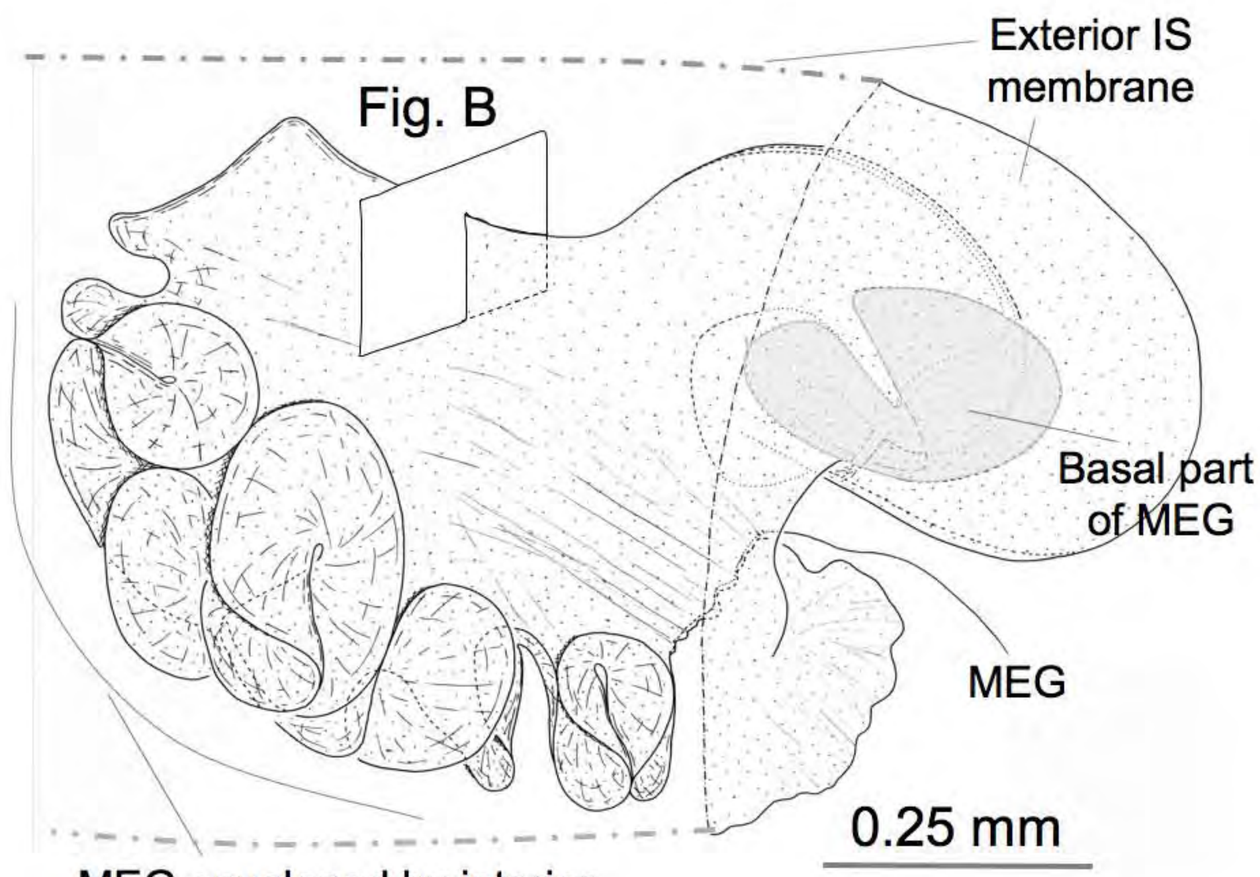


Fig. B
Exterior IS membrane
Basal part of MEG
MEG
0.25 mm
MEG enveloped by interior IS membrane

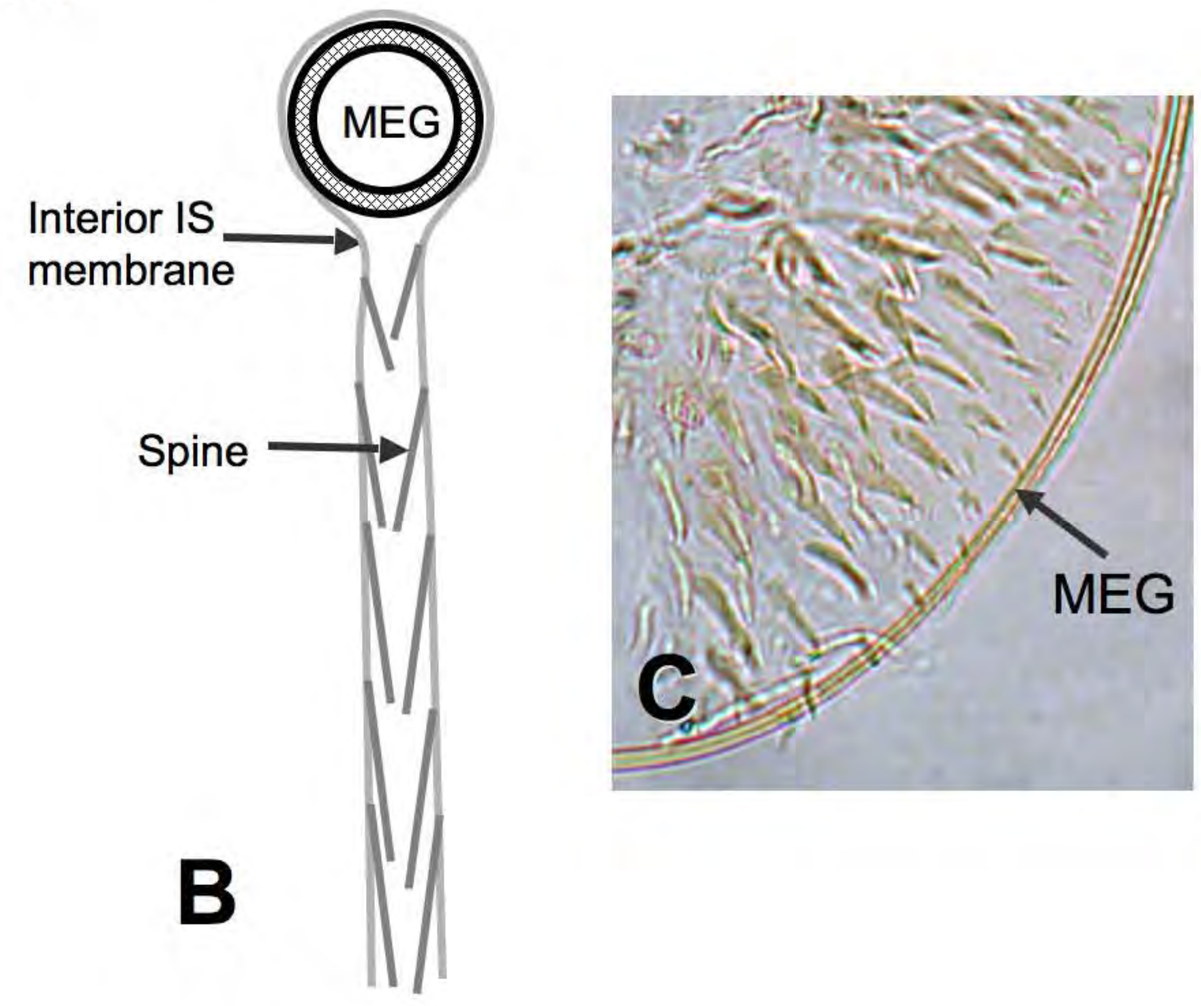

A
MEG
Interior IS membrane
Spine
B
C
MEG

Fig. 3 A, B, C

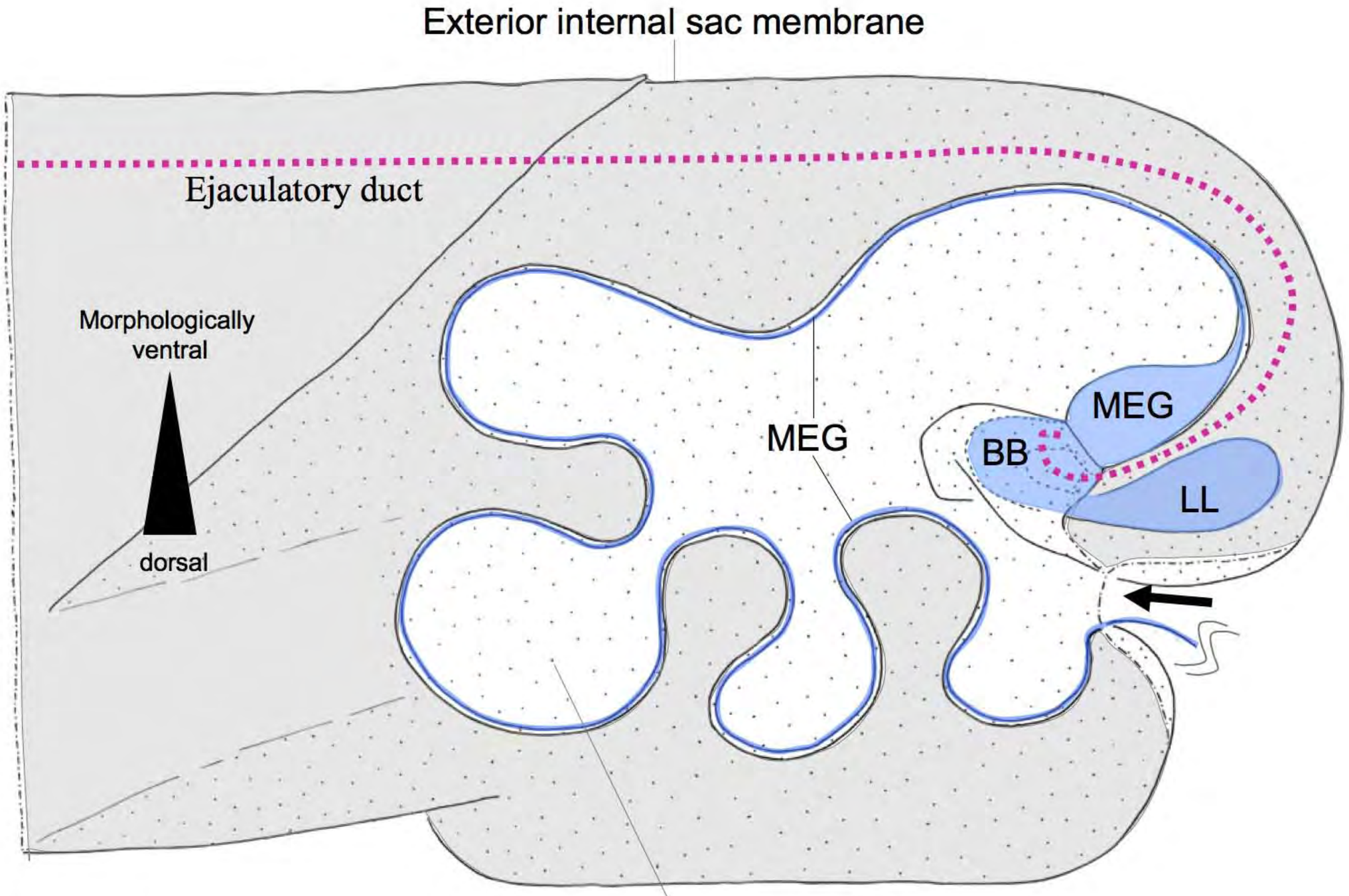
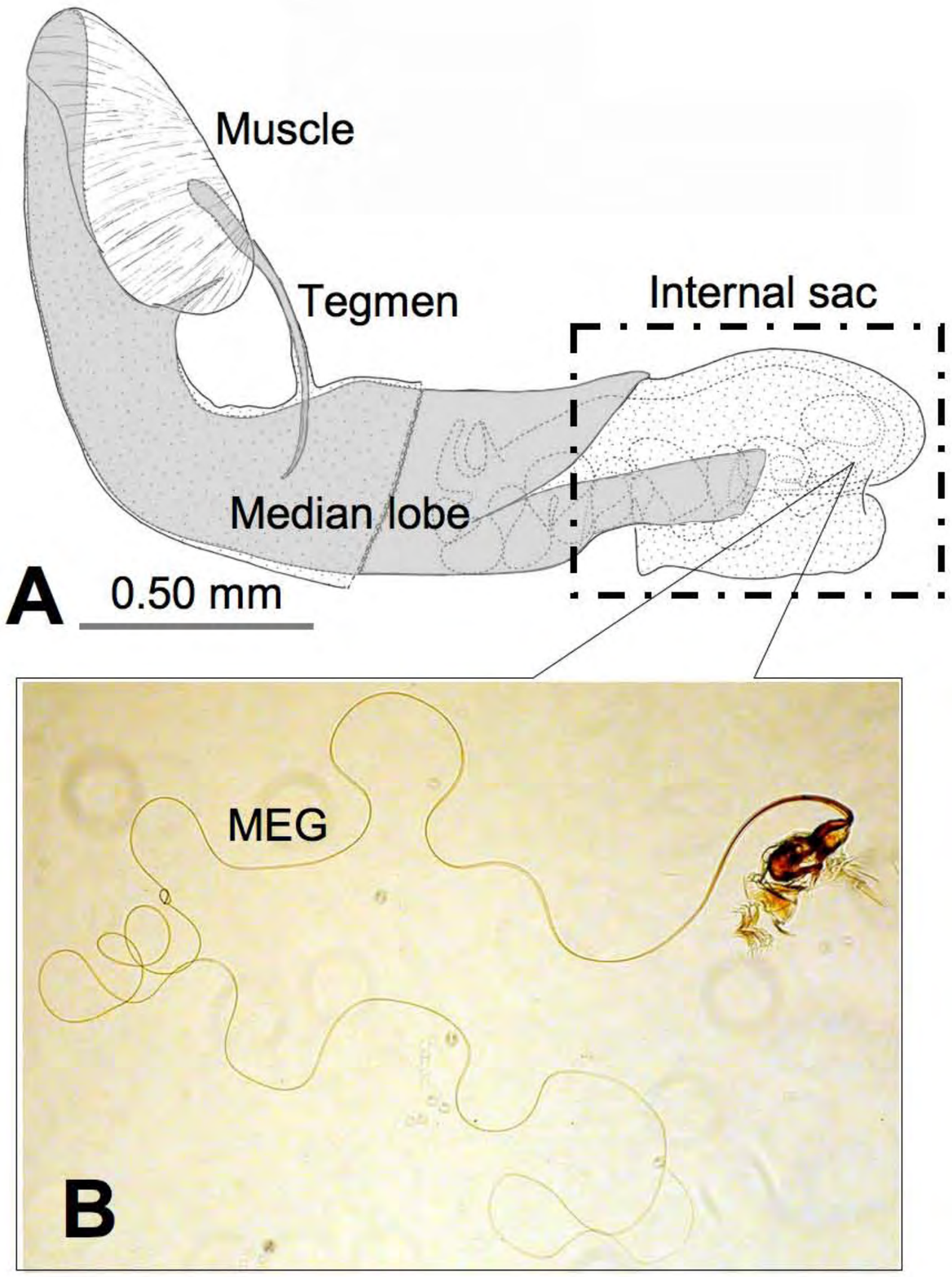

Exterior internal sac membrane
Ejaculatory duct
Morphologically
ventral
dorsal
MEG
MEG
BB
LL
Interior internal sac membrane

Fig. 2


Muscle
Tegmen
Internal sac
Median lobe
A
0.50 mm
MEG
B

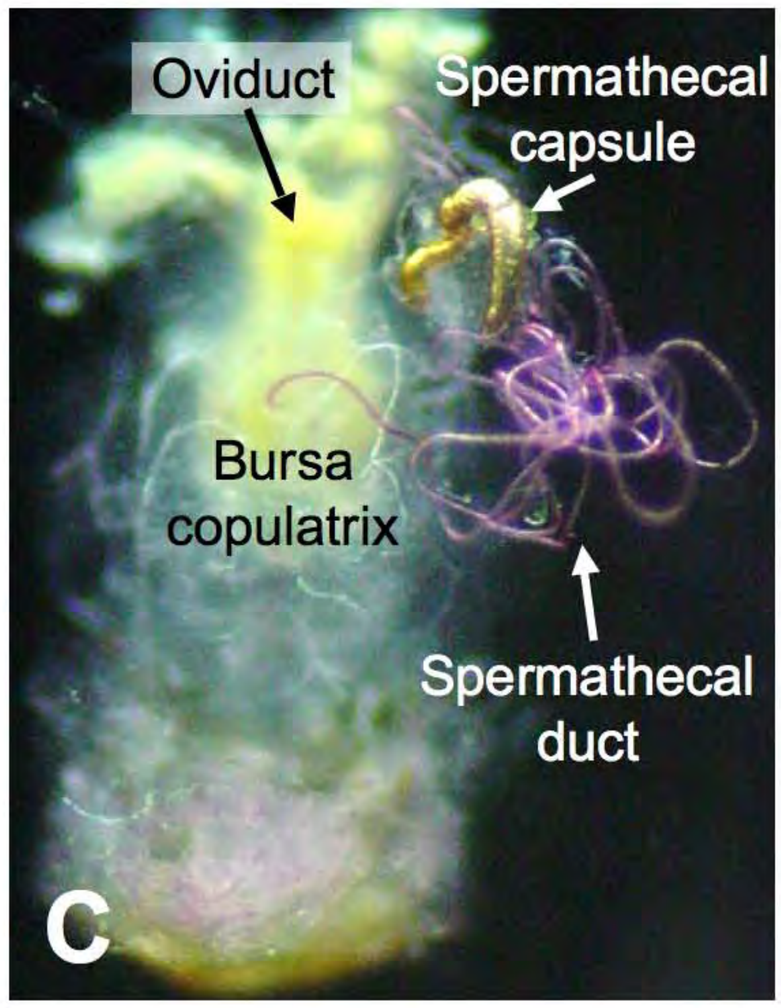

Oviduct
Spermathecal capsule
Bursa copulatrix
Spermathecal duct
C

Fig. 1 A,B, C