

## Reproductive strategies of the kangaroo leech, *Marsupiobdella africana* (Glossiphoniidae)

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### ABSTRACT

The Kangaroo Leech, *Marsupiobdella africana*, is a hermaphroditic organism, with insemination taking place by the planting of a spermatophore on another leech. Spermatophores are mostly planted on the anterior of the recipient leech, but not always. Several spermatophores may be planted by different leeches on a single recipient. The spermatophore consists of two side by side lobes. Within minutes from planting of the spermatophore, the contents are squeezed out and into the body of the recipient. Sperm are believed to find the way to the ova by following chemical cues. Kangaroo Leeches display advanced parental care by transferring fertilized eggs from the reproductive opening to a brood pouch on the ventral side. Fully developed leeches may copulate after detaching from the amphibian host *Xenopus laevis*, or from the Cape River Crab *Potamonautes perlatus* with which it maintains a phoretic association.

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### 1. Introduction

Leeches are often viewed as nasty blood sucking creatures and most people display an inherent fear and dislike of them. This perception is illustrated in many phrases in literature. Even the former British Prime Minister, Margaret Thatcher, on occasion, stated “To cure the British disease with socialism was like trying to cure leukemia with leeches”. Despite their close association with medieval medicine, leeches are today widely used in the treatment of various circulatory disorders (Wright and Finical, 2000). In addition to their medical uses, leeches display some amazing behavior and reproductive strategies.

Many leeches are devoted parents, caring for their young in a manner that resembles the care normally associated with higher chordates. Whilst some leeches protect a cocoon containing fertilized eggs (Sawyer et al., 1981; Kutschera and Wirtz, 1986), others deposit encapsulated eggs on the shells of arthropods as a temporary and life stage specific phoretic strategy to protect the eggs from predation by snails (Sawyer et al., 1981). Others build nests for their young or carry eggs or young on their ventral surfaces. The ultimate step in this evolutionary trend is displayed by *Marsupiobdella africana* Goddard and Malan (1913) (Glossiphoniidae), where the parental leech transfers as many as 50

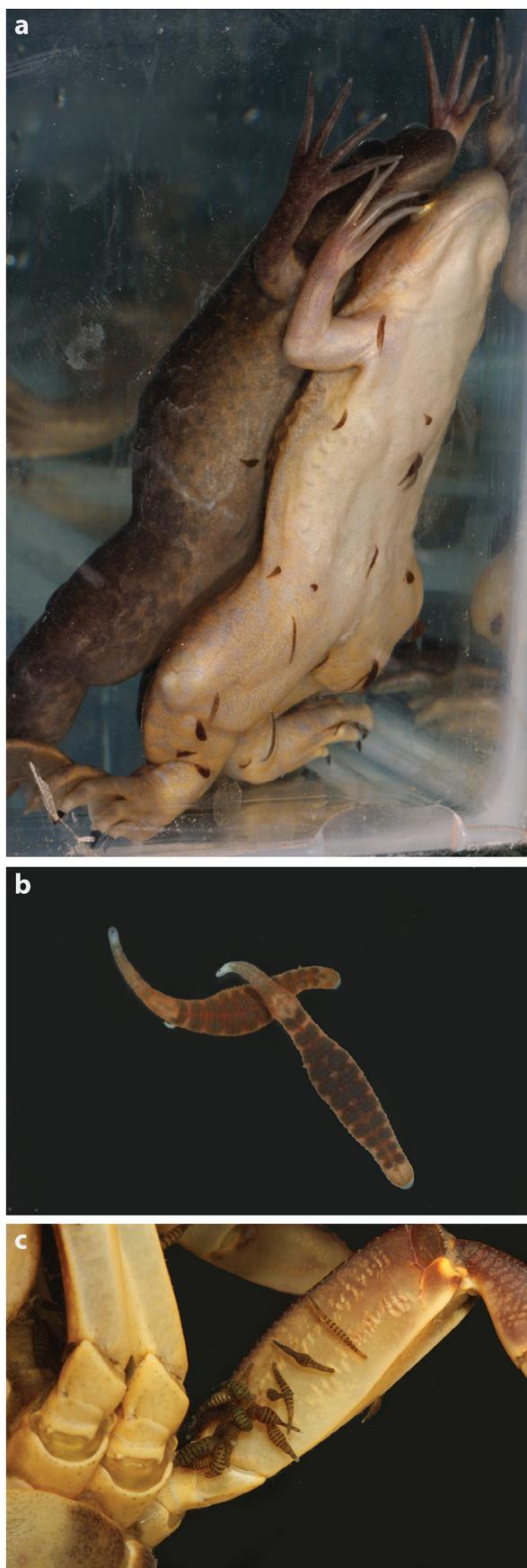
fertilized eggs from the female gonopore to its own ventral brood pouch, incubating them for over a four week period (Van der Lande and Tinsley, 1976; Badets and Du Preez, 2014). This is a very effective form of parental care as the eggs and developing young are safe against predation.

*Marsupiobdella africana* temporarily infests the amphibian host *Xenopus laevis* (see Fig. 1a). If the gravid leech with fully developed embryos makes contact with a *Xenopus*, the young are discharged explosively onto the surface of the host (Van der Lande and Tinsley, 1976). Whilst they remain attached to the host, they feed and develop to maturity over a period of two to three weeks. Initially the testes are rudimentary, but as they reach maturity the testes and genital area in general enlarge. There is no trace of any connection between vas deferens and atria, which makes it typically glossiphoniid in form. The atria are conspicuous structures with globular, thick walls. Inside the atria an aggregation of spermatozoa enclosed in a membrane can occasionally be recognized (Van der Lande and Tinsley, 1976).

After reaching maturity they detach from their hosts to find a mate and copulate (Fig. 1b). The ova start to develop as soon as they are transferred to the brood pouch. Over a period of about a month, the pouch becomes progressively bigger as the young develop and grow on the inside. Such growth occurs until the pouch occupies the majority of the body, thus distorting the organ systems in its vicinity. The nerve cord is forced to fold back double on itself, at both anterior and posterior walls of the pouch, being pushed dorsally against the gut by the pressure of the growing juveniles. The space occupied by the pouch diminishes after the young have been discharged and the pore lengths (Van der Lande and Tinsley, 1976). Leeches then find a Cape River Crab (*Potamonautes perlatus*) that acts as a phoront (Fig. 1c). Leeches

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**Fig. 1.** Light micrographs of (a) two Clawed Frogs *Xenopus laevis* infected with Kangaroo Leeches *Marsupiobdella africana*; (b) leeches on the legs of a Cape River Crab *Potamonautes perlatius*; and (c) two Kangaroo Leeches copulating.

keep to the ventral and lateral sides of the crab body and proximal sections of the legs (Badets and Du Preez, 2014).

Although the deposition of the spermatophore on the surface of the leech has been reported before (Van der Lande and Tinsley, 1976), this study aims to provide information on the copulatory behavior and mechanism involved, as well as the morphology and ultrastructure of the spermatophore.

## 2. Materials and methods

### 2.1. Obtaining specimens

Two adjacent artificial ponds (26,68297S; 27,09519E) in the North-West University Botanical Garden in Potchefstroom, South Africa, sustain populations of Clawed Frogs (*Xenopus laevis*) and the Cape River Crab (*Potamonautes perlatius*). Both ponds are between 0.2 and 0.8 m deep, well vegetated with a variety of aquatic and semi-aquatic plants and contain a muddy bottom with ample organic debris.

Clawed Frogs were collected using baited bucket traps with an inwardly directed funnel (North-West University ethical clearance no. NWU-00005-14-S3 and Department of Development, Environment, Conservation and Tourism of the North-West Province of South Africa collection permit no. 028 NW-11). Traps were baited with chicken liver and left overnight. Crabs were collected at night using a dip net. To avoid leeches detaching, frogs and crabs were minimally handled and scientists wore latex surgical gloves. Leeches were removed from the surface area of the hosts using forceps and placed in a plastic tub containing pond water. The host individuals were released where collected.

### 2.2. Fixing specimens

Leeches collected were observed using a stereo microscope. Specimens were preserved for whole mount preparations, histological sectioning and/or scanning electron-microscopy. Immediately after collecting, leeches were examined for the presence of spermatophores using a dissection microscope and those with spermatophores were fixed. Leeches without spermatophores were then placed together in a small tub of 100 mm diameter containing pond water and closely observed under the dissecting microscope. As soon as a spermatophore was planted on the surface of another leech it was removed and fixed.

For whole mounts, specimens were fixed in 10% neutral buffered formalin (NBF) and the coverslip was weighted down using a 14 g lead weight. Specimens were stained using acetocarmine and following dehydration in a graded ethanol series and clearing in xylene, specimens were permanently mounted in Canada balsam. Specimens for histological sectioning were fixed in Bouin's fixative, dehydrated, cleared in xylene, impregnated with paraffin wax and imbedded in paraffin wax using a Slee embedding center. Serial sections of 8  $\mu$ m were prepared, mounted on glass slides and routinely stained in Harris hematoxylin and eosin. Sections were examined using a Nikon Eclipse E800 compound microscope, fitted with a Nikon DXM1200 digital camera. For observing surface morphology, specimens were fixed in hot 70% ethanol or NBF, critically point dried and mounted on carbon tape on 12 mm SEM stubs. Before coating, specimens were studied and composite focused images obtained using a Nikon AZ100 microscope fitted with a motorized Z-drive and a high resolution digital camera. Images were captured on a personal computer with Nikon NIS Elements imaging software. Specimens were subsequently coated using a SPI Module sputter coater fitted with a gold-palladium source, and studied using a Phenom Pro-desktop scanning electron microscope at a power of 5 kV.

### 3. Results

#### 3.1. Geographical distribution

Although *X. laevis* is known to occur throughout large areas in southern Africa, *M. africana* has only been reported from Cape Town, Bloemfontein, and Potchefstroom areas. In the Potchefstroom area, where *X. laevis* have been collected at more than 25 sites, *M. africana* has only been detected at two sites, namely, the North-West University Botanical Garden (26,68297S; 27,09519E) and a pond on the outskirts of the city (26,75590S; 27,06762E).

#### 3.2. Reproductive behavior

By the time *M. africana* detach from the definitive host *X. laevis*, the majority are mature or close to maturity. We observed that leeches may copulate as little as one day after detachment. We also observed that leeches may copulate after detachment from the clawed frog host or from the freshwater crab. During copulation the depositor will approach the recipient of the spermatophore from the side or from behind, probing its surface with its oral region (Fig. 1b), or they may raise their bodies more or less upright with ventral surfaces opposed. The anterior region sways rhythmically, entwining alternately to left and right around the anterior part of the other leech. The depositor then lifts its oral region slightly up whilst it presses its genital opening against the recipient. After one or two seconds the depositor pulls back from the recipient and the spermatophore, that is now firmly attached to the recipient, is drawn out from the genital opening of the depositor. The spermatophore does not detach immediately from the depositor and for a few seconds the two leeches are connected via the spermatophore. At this stage both leeches move forward and backward whilst they remain attached to the substrate through their posterior suckers. Eventually the spermatophore breaks loose from the depositor and the two leeches go their separate way with the spermatophore firmly attached to the recipient (Fig. 2a,b). From a sample of seven leeches, five had one spermatophore each, one had two and one had three. Of these 10 spermatophores, three were implanted on the dorsal surface of the recipient, five on the ventral side and two laterally. Five spermatophores were implanted anteriorly, two in the middle section and three posteriorly.

#### 3.3. Spermatophore morphology

Spermatophores were observed to consist of two cream colored side-by-side elongated ellipsoid lobes that were firmly glued together (Fig. 2c). Each lobe was 642 (422–826)  $\mu\text{m}$  in length and 65 (39–84)  $\mu\text{m}$  in diameter with a shiny smooth surface. The outer chitinous covering layer of each lobe was 2.4 (2.2–2.5)  $\mu\text{m}$  in diameter (Fig. 2c). The proximal end of the spermatophore was firmly attached to the surface of the leech with a disk 83 (72–97)  $\mu\text{m}$  long and 86 (73–98)  $\mu\text{m}$  wide (Fig. 3a). Associated with the disk was a fibrous network of hair-like structures that spread from the dermal wall covering the proximal region up to the germinal region of the spermatophore (Fig. 3b). The distal part of each spermatophore tube was seen to consist of a single short, thin horn 84 (63–102)  $\mu\text{m}$  long and 17 (17–18)  $\mu\text{m}$  wide (Fig. 3c).

The contents from the spermatophore are discharged through the epidermal layers into the body of the recipient (Fig. 2d,e). Spermatozoa were observed in coelomic sinuses of the leech body. Spermatozoa find their way through the body to the ova and fertilization takes place. Fertilized ova are then probably transferred via the genital opening to the brood pouch where they develop. The brood pouch enlarges as the embryos develop and leeches with developed embryos clearly show fully swollen bodies (Fig. 2f,g).

Developing young attach inside the brood pouch with the posterior sucker (Fig. 2h). At this stage gravid leeches seek a host and when the leech finds a Clawed Frog the young are released explosively on the surface of the frog where they attach and start feeding.

### 4. Discussion

#### 4.1. Geographical distribution

Although the southern African distribution of *M. africana* is not known, it would appear that it has an overdispersed distribution. Van der Lande and Tinsley (1976) reported that they obtained *X. laevis* infected with *M. africana* from Cape Town. They mentioned that *M. africana* was first noted in 1967 and thereafter sporadically until November 1973, after which date none could be found in spite of the careful screening of thousands of Clawed Frogs. Author LdP collected *M. africana* in 1998 in a pond on the outskirts of Bloemfontein, South Africa (29,05367S; 26,21636E). As the leeches are not present on the frogs throughout the year, but also spend a considerable time on the phoront (*P. perlatus*), it is quite possible that infestations can be easily overlooked.

#### 4.2. Copulation and spermatophore morphology

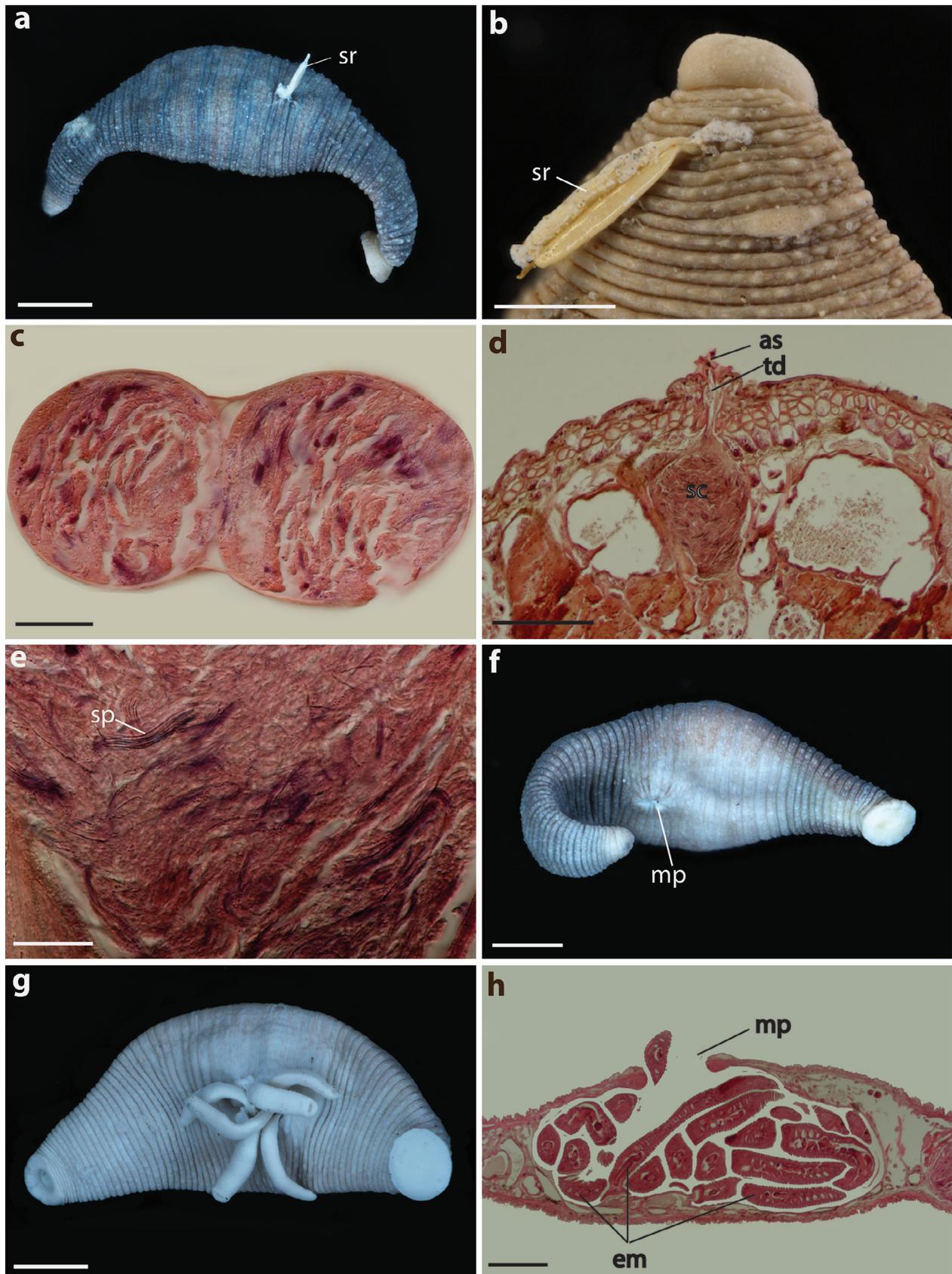
In the present study as many as three spermatophores were observed on a single leech, the majority of which could be found on the antero-ventral surface of the body. This observation is consistent with that reported by Van der Lande and Tinsley (1976) who reported that more than one spermatophore may be deponated and that the majority are deponated in the anterior part of the leech.

The spermatophores are, according to Van der Lande and Tinsley (1976), a white bifid structure almost as long as the leech is wide, which can remain protruding from the body wall for several hours. In the present study it was observed that spermatophores were whitish to cream colored with a shiny surface.

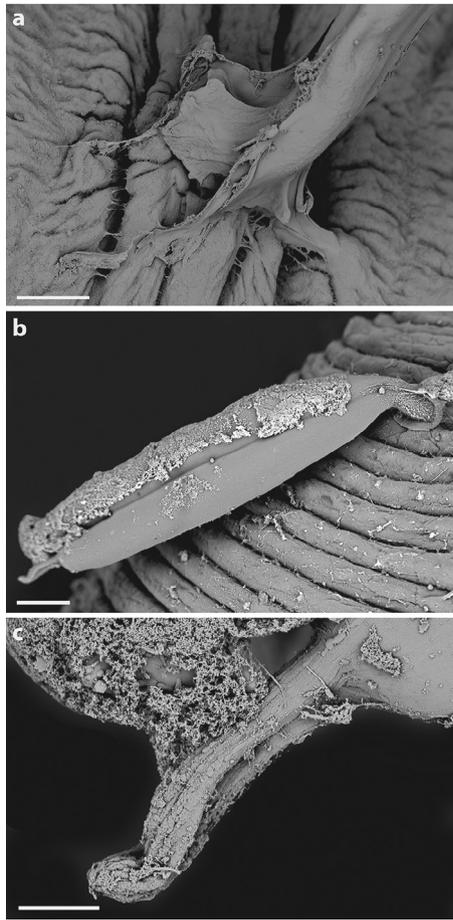
In the present study it was observed that the attachment of the spermatophore was at the surface on the leech and no signs of penetration of the spermatophore itself was observed. This is consistent with an observation by Pennak (1978) who reported for *Placobdella ornata* that the implantation was not deep and that no tissue reaction was observed. In *M. africana* the spermatophore seems to remain attached to the dermal wall of the leech through an adhesive that sets as the spermatophore is pressed against the body surface of the recipient leech, as well as hair-like structures covering the adhesive disk and parts of the central germinal region of the spermatophore.

Spermatophores penetrate the tegument and internal release of sperm takes place. Male reproductive success is likely to depend on the amount of sperm transferred per spermatophore (Tan et al., 2004).

The spermatophore of *M. africana* is similar to that of *Glossiphonia* spp., which consists of three main regions, namely, a proximal attachment region, a distal horn and the middle germinal region (Amin, 1981). In contrast with *Glossiphonia*, the spermatophore of *M. africana* has a single distal horn on one tube in the germinal region. This distal horn (Fig. 3c) probably plays a role during the transfer of the spermatophore as it was observed that the distal horn remains inside the gonopore of the deponator leech and that the deponator holds the spermatophore for several seconds at a right angle to the body of the recipient. This is most likely to allow the attachment adhesive, probably scleroprotein, to set and keep the spermatophore in position. In *P. ornata*, the attachment organ of more developed spermatophores appeared to have a pronounced subtriangular outline with a rim. Internally, a funnel-like invagination led from the attachment organ to a sperm conveyance tube that branched into two (Amin, 1981).



**Fig. 2.** Microscope images of *Marsupiobdella africana*: (a) mature leech with a spermatophore implanted on the dorsal surface; (b) micrograph of a spermatophore firmly attached to a leech; (c) cross section through a spermatophore showing the two lobes; (d) histological cross section of a leech at the position of the spermatophore attachment; (e) histological section through the spermatophore contents that have been transferred into a leech; (f) light micrograph of a gravid leech with the brood pouch heavily swollen; (g) light micrograph showing young that are being discharged from the brood pouch; (h) sagittal section through the brood pouch containing several developing young. Abbreviations: as, attachment site; em, embryo; mp, marsupial pouch opening; sc, spermatophore contents; sp; sperm; sr, spermatophore; td, transfer duct. Scale bars: a, b, f and g, 500  $\mu$ m; c and e, 20  $\mu$ m; d, 200  $\mu$ m; h, 250  $\mu$ m.



**Fig. 3.** Scanning electron micrographs of a *Marsupiobdella africana* spermatophore: (a) proximal region showing the attachment disk attachment fibres; (b) spermatophore showing smooth surface of the two 'side by side' germinal lobes and; (c) distal horn. Scale bars: a, 50  $\mu$ m; b, 100  $\mu$ m; c, 20  $\mu$ m.

#### 4.3. Hypodermic insemination and reproductive behavior

Clusters of spermatozoa that have been observed in the coelomic space are presumed to migrate through the tissues. Van der Lande and Tinsley (1976) speculate that the ova are probably fertilized by the spermatozoa before their discharge from the ovisacs, as this mechanism is common amongst glossiphoniids. For example, in the leech *Haementeria ghilianii*, the ova are fertilized inside the ovisac before fertilized eggs are expelled from the female pore, itself enclosed in a membranous sac, or cocoon, which remains attached to the ventral body wall of the brooding parent (Sawyer et al., 1981).

Sperm make their way through the coelomic sinuses to the ova (Myers, 1935). Tan et al. (2004) describe sperm transfer of *Helobdella papillornata* (Euhirudinea: Glossiphoniidae) as a dermal implantation of a spermatophore and migration of the sperm through the coelom of the body. Although both reciprocal and unreciprocal fertilization may take place, only reciprocal mating allows spermatophores to be placed near the female gonopores, which may give those sperm a better chance of fertilization (Tan et al., 2004). This is the so called hypodermic implantation found in Glossiphoniidae and Eropobdellidae (Bielecki, 2004). In leeches with indirect insemination, the spermatophores may be implanted anywhere in the body and the mechanism of migration by which the spermatozoa must reach the ovary through the body wall and internal spaces is unknown (Swaitek et al., 2009).

Hypodermic insemination has evolved independently in a number of simultaneous hermaphrodites and several functions have been suggested; it may reduce courtship time, allowing an individual to inseminate many mates when contact is brief (Trowbridge, 1995). Most members of the family Piscicolidae have specialized body regions near the gonopores where spermatophores are implanted, and just beneath this copulatory area a specialized form of connective tissue is found, i.e. vector tissue (Swaitek et al., 2009). For Piscicolidae, the route taken by the sperm is well described. When leaving the spermatophore, spermatozoa pass through the vector tissue (probably directed by chemotaxis) to reach the oviducts and ovisacs where the eggs are located and fertilized (Tan et al., 2004; Swaitek et al., 2009). The release of sperm in Piscicolidae is described as a rapid event and at the same time, when the sperm are still inside the spermatophore, other spermatozoa can be observed following through the vector tissue whilst a mass of sperm fills the ovary (Swaitek et al., 2009). The spermatozoa were seen migrating through the vector tissue forming huge aggregations. It was postulated that the sperm of this family is pumped from the spermatophore into the vector tissue at a high pressure, and as a result the vector tissue cells are pushed aside in places where the extracellular matrix fills the gaps between them, and in consequence the spermatozoa can pass rapidly.

Vector tissue is also known to aid in the phagocytosis of defective spermatozoa (Swaitek et al., 2009). This vector tissue has been found in the reproductive system of *Batrachobdelloides moogi* (Glossiphoniidae) and the family Piscicolidae. The presence or absence of this vector tissue in *M. africana* is currently not known and may be a feature of taxonomic relevance. According to Bielecki (2004), vector tissue has been created by a simple hypertrophy of the walls of the oviducts and in general presents a similar structure to oviducts, which can thus be mistaken for such; however, it is usually a vacuolar structure. When individuals of *H. papillornata* were individually isolated, self-fertilization took place and viable offspring were produced (Tan et al., 2004). In *M. africana* only mutual exchanges of spermatophores have been observed.

It is presumed that *M. africana* will follow the same pattern of other glossiphoniids in terms of egg-laying. With the anterior and posterior suckers next to each other the female gonopore and the brood pouch opening could be brought into close apposition for a direct transfer of eggs to the pouch (Van der Lande and Tinsley, 1976). Alternatively, quiescent leeches have been observed closely attached to the substratum for long periods. This could be so that the eggs can be propelled posteriorly through waves of contraction in the space between the body wall and the substratum. Van der Lande and Tinsley (1976) did confirm that both the oviducal pore and the brood pouch are open during egg transfer.

Egg discharge, which is probably rapid, is likely to take place whilst the individual is still attached to the substratum by both suckers. Commonly in glossiphoniids the body arches dorsally during egg-laying and it is not implausible that, in *M. africana*, the female gonopore and pouch opening could be brought in sufficiently close association for direct transfer between the two openings to occur.

#### 4.4. Embryo development

In the case of *Glossiphonia complanata* (Glossiphoniidae), adults have been found to provide their young with nutrients that are passed through the body wall of the parent into the posterior sucker of the young (Thorpe and Covich, 2001). Histological sections through the brood pouch containing developed embryos confirmed that the young attach by means of their posterior suckers (Fig. 2h). It is thus possible that *M. africana* displays similar nutrient providing strategies as *G. complanata*.

#### 4.5. Conclusion

This paper reports on the mating behavior of *M. africana* and it was observed that leeches may reproduce as soon as one day after detachment from both the clawed frog host or the freshwater crab phoront. Scanning electron images of the spermatophore surface and the mode of attachment are provided.

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#### Conflict of interest

The authors declared that there is no conflict of interest.

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