The characteristics of copulation in *Segmentina oelandica* (Westerlund, 1885) (Gastropoda: Pulmonata: Planorbidae)

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The paper examines the precopulatory behaviour and copulation in *Segmentina oelandica*. The morphological traits associated with this process are discussed. The anatomy of the copulatory apparatus is studied using histological and histochemical (phalloidin fluorescence) methods. The copulation is shown to be a complex process determined by a specific set of morphological, physiological, behavioural and ecological characteristics.

Key words: molluscs, morphology, mating behaviour, copulation, Gastropoda, Pulmonata, Planorbidae, *Segmentina*

INRODUCTION

The first description of copulation in orb snails was given in the publication of the field naturalist's observations on the molluscan fauna of the outskirts of Budapest (Hazay, 1881). Subsequent studies (Colton, 1918; Precht, 1936, Geldiay, 1956; Berezkina & Starobogatov, 1988) were mostly focused on other aspects of reproduction in Planorbidae Rafinesque, 1815, while giving little attention to copulation. Precht (1936) examined some aspects of the mating mechanism in *Anisus leucostoma* (Millet, 1813), *A. vortex* (Linnaeus, 1758), *Armiger crista* (Linnaeus, 1758), *Bathyomphalus contortus* (Linnaeus, 1758), *Gyraulus albus* (Müller, 1774), *Planorbis carinatus* (Müller, 1774), and *P. planorbis* (Linnaeus, 1758), and was the first to describe the copulation in *Segmentina nitida* (Müller, 1774) by studying a single copulating pair. Bondesen (1950) summarized somewhat conflicting available data on mating behaviour in freshwater snails and distinguished three major modes of copulation (unilateral, reciprocal and self-copulation); he also posited that some features of mating mechanism in Planorbidae are specific to genera or the groups of

genera. The anatomical studies of the reproductive system, and, especially, of the copulatory apparatus provided some additional insights about the copulation in Planorbidae (Larambergue, 1939; Baker, 1945; Malek, 1952; Pace, 1971) and, specifically, about the correlation between the morphological structure of reproductive organs and the copulatory mechanics.

The early authors (Simroth & Hoffmann, 1908–1928: 1119; Precht, 1936), who described the copulation in freshwater Planorbidae, claimed that a complicated pre-copulatory behaviour (foreplay, epilogues, and mating dances), such as that known, for example, for Stylommatophora (Gerhardt, 1936), is generally lacking in orb snails. Subsequent studies (Pace, 1971; Trigwell et al., 1997), however, challenged these conclusions. Pace (1971), who observed the copulation in the members of the genus *Helisoma,* was the first author who noticed that the snails follow "very similar behaviour patterns" during the copulation. He also observed that the mating is usually initiated by reciprocal tentacular exploration between prospective mates. Trigwell et al. (1997) provided a detailed description of the precopulatory behaviour in *Biomphalar-*

ia glabrata (Say, 1818) and divided the copulatory process into several distinct phases.

Morphologists and taxonomists often have to deal with preserved material and, therefore, can only speculate about the functions of various structures. The study of copulation, however, may help to unify the whole spectrum of morphological, physiological, behavioural and ecological characteristics, to enable the assessment of correlation between various morphological traits, to facilitate our understanding of the relationships between morphological characteristics and biological processes, and to improve the characterization of species and supraspecific taxa. The purpose of the present study is to describe the characteristics of copulation in *Segmentina oelandica* (Westerlund, 1885) and to examine the relationship between the copulation mechanics, on one hand, and the structure of the copulatory apparatus, physiological data and characteristics of the habitat, on the other.

The particular type of copulatory behaviour observed in *S. oelandica* is determined by a specific set of morphological characters of this species (smooth plano-convex shell, wide shell aperture, curved palatal edge of the aperture, lamellae within the outer shell whorl, characters of the copulatory apparatus, relative position of the genital openings, and length of tentacles). Whereas the gross anatomy and the structure of the penial complex in the genus *Segmentina* Fleming, 1818 is relatively well studied (Baker, 1945; Hubendick, 1955; Kruglov & Soldatenko, 1997), the details of the histological structure and the musculature of the copulatory apparatus are much less known. These aspects of the copulatory apparatus are examined in the present paper using histological techniques and phalloidin staining for muscle fibers.

MATERIAL AND METHODS

The specimens of *S. oelandica* were collected and the experiments were conducted in 2008–2009 by the first co-author (EVS). The material came from the following localities. EVS; **Russia**, *Smolensk Prov.*, Demidovskiy Distr., outskirts of Przhevalskoye, a pool at Nikitenki Village; EVS; **Russia**, *Smolensk Prov.*, Demidovskiy Distr., at Przhevalskoye, temporary ponds in the outskirts of the 'Chistik' Teaching Center (Smolensk State University).

The groups of sexually mature specimens from the both localities given above were placed in 3 cm-deep containers, 20 specimens in each, for observation on the mating behaviour. The beneficial effect of rising temperature on mating activity in freshwater snails (Duncan, 1978; Smith, 1981) was used to induce mating: the water was heated with incandescent lamps to raise temperature to 20–26°C. The copulating pairs were photographed; the copulation attempts were counted and the time intervals were recorded. The observations were repeated, at least, for four groups per season.

Some of the specimens were marked with colored nail polish to count the number of copulation attempts made by the same individual and to determine whether this individual mates as a male or female, and whether or not the individual alternates sexual roles. Several times during the mating season the samples were replenished by freshly collected specimens (newcomers), because otherwise the mating stops after the end of the active mating period (2–3 days).

Three specimens of *S. oelandica* were prepared as unstained whole mounts. These whole-mount preparations were examined and photographed on a Leica DMLS-2 microscope equipped with a CCD camera; the line drawings were copied from photographs according to their original proportions.

For confocal microscopy, two specimens were fixed in 4% formaldehyde (prepared from paraformaldehyde powder) in 0.1M PBS for one hour at room temperature, rinsed in 0.1M PBS (three times for 15 minutes), permeabilized for one hour in PBS containing 0.2% Triton X-100, rinsed again shortly in the same buffer and then transferred to phalloidin-TRITC (Sigma) for one hour. After that, the specimens were

washed again three times for 15 minutes in the same buffer, mounted in 80% glycerol on glass slides, and viewed on a Leica TSC SP5 microscope.

Seven specimens were taken for histological observations. The dissected copulatory apparatus was fixed in 70% alcohol and after dehydration in xylene was embedded in paraffin using standard histological techniques (Lillie, 1969). Serial sections 6 µm thick were stained with hematoxylin and eosin. Whole mounts and histological sections were examined and photographed on a Leica DM LS-2 microscope.

RESULTS

The copulatory behaviour in *S. oelandica* was initially observed in a wounded specimen that everted the copulatory apparatus in response to shock caused by injury. Although, in this case, the eversion reflex was not accompanied by physiological and behavioural responses, it showed the structural elements of the penial complex and their arrangement, typically obscured during the copulation by the partner (Fig. 1b–c).

Although the individuals of *S. oelandica* are simultaneous hermaphrodites, the reciprocal non-simultaneous copulation, i.e. the alternation of sexual roles in a pair, without any intermittent pauses (recognized by Kruglov, 1980 as a separate type of copulation in pulmonates), was never observed in *S. oelandica*. The only mode of copulation we observed was unilateral copulation. For this mode of copulation we adopt here the conventional terminology (Precht, 1936; Bondesen, 1950; Smith, 1981; Berezkina & Starobogatov, 1988) and refer to the copu-
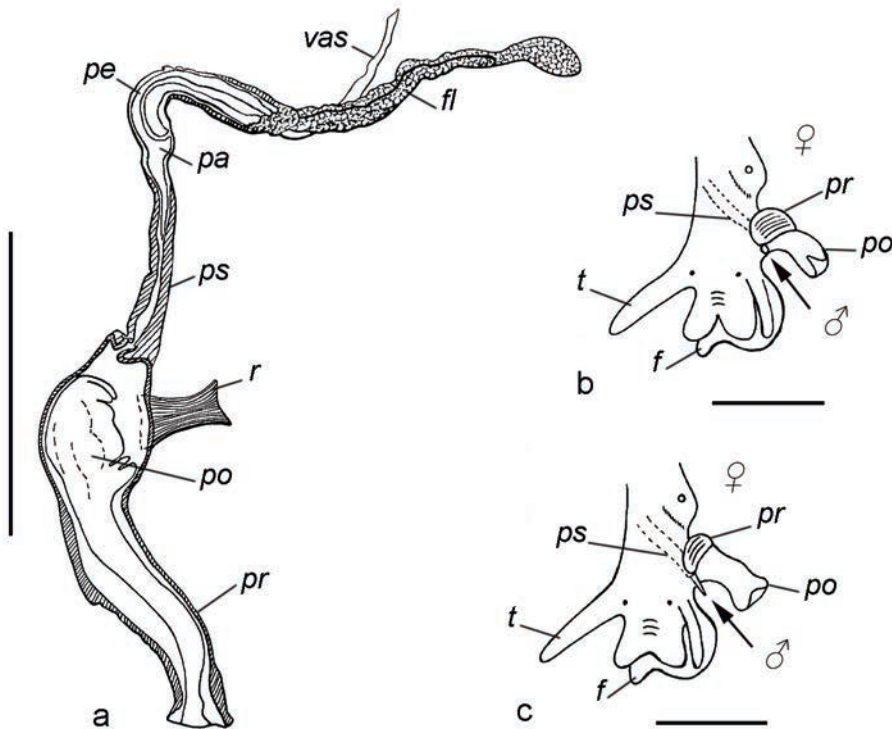

Fig. 1. Segmentina oelandica, diagrammatic representation of the copulatory apparatus (**a**) and diagrams showing eversion of the copulatory organs (**b**, **c**); *f*, foot; *fl*, flagellum; *pa*, papilla; *pe*, penis; *po*, preputial organ; *pr*, preputium; *ps*, penial sac; *r*, retractor; *t*, tentacle; *vas*, vas deferens. Scale bars (a–c): 1 mm.
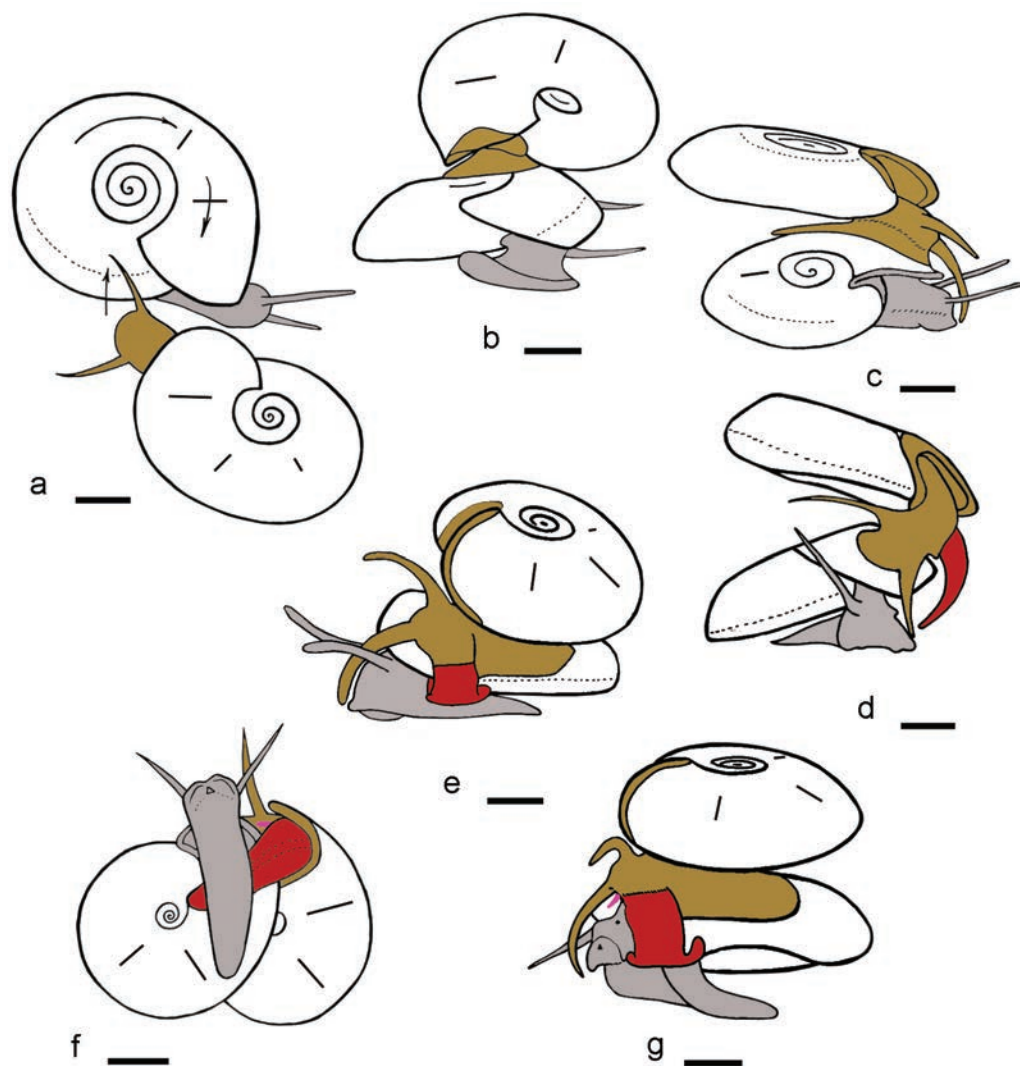
Fig. 2. Copulation in *Segmentina oelandica*. Color codes: male: brown; female: grey; everted preputial organ: red; penis: pink. Figures e–f represent different views of the same stage. See text for further explanation. Scale bars (a–c): 1 mm.
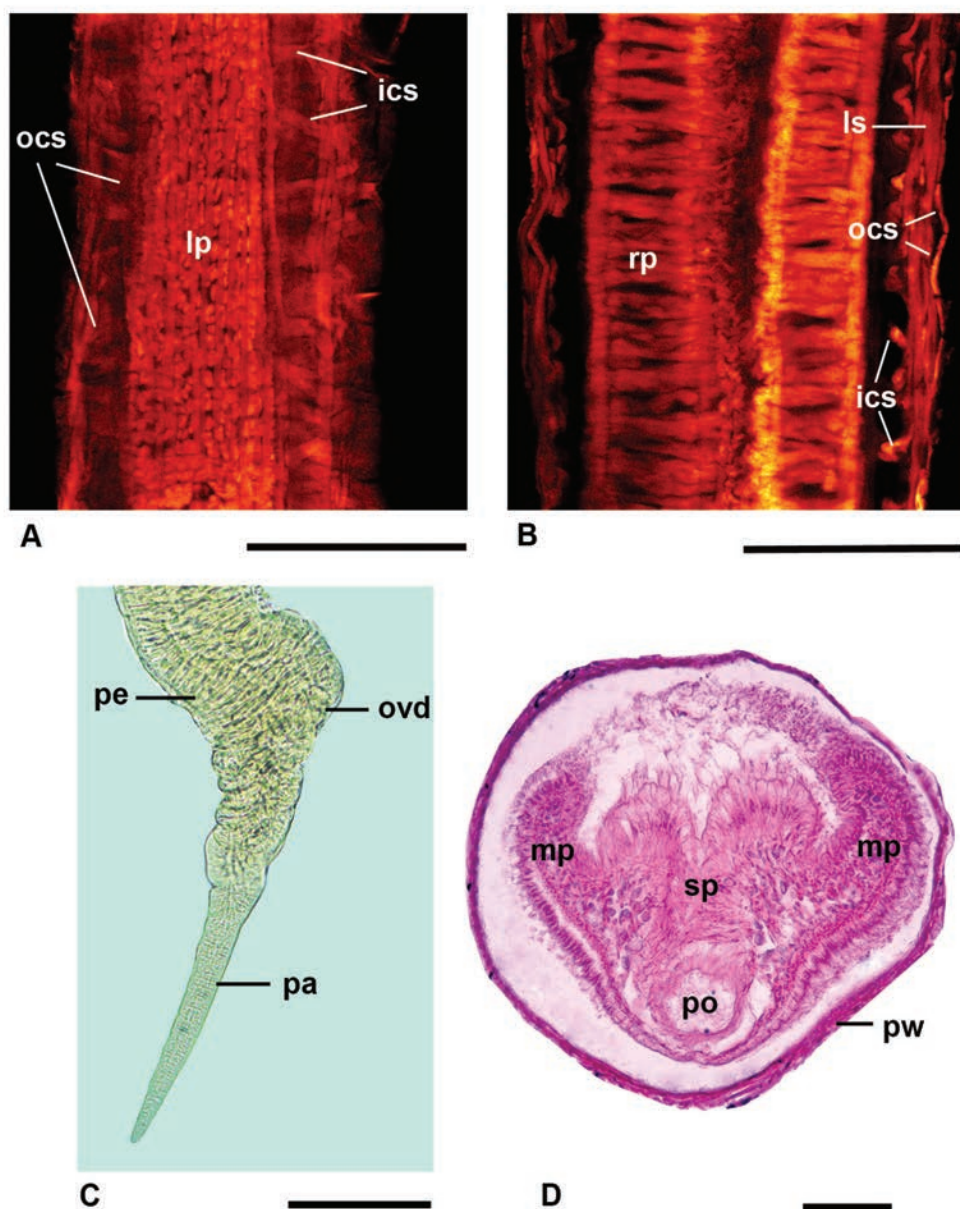
Fig. 3. Anatomy of copulatory apparatus in *Segmentina oelandica*: confocal micrographs of phalloidin-stained whole mounts showing penial sac with penis (**a**, **b**), light micrograph of whole mount of papilla (**c**), microphotographs of histological sections through preputial organ (**d**); *mp*, muscular portion of the preputial organ; *ics*, inner circular layer of the penial sac muscles; *lp*, longitudinal muscles of penis; *ls*, longitudinal penial sac muscles; *ocs*, outer circular layer of penial sac muscles; *ovd*, opening of vas deferens; *pa*, papilla; *pe*, penis; *po*, preputial organ; *pw*, preputium wall; *rp*, radial muscles of penis; *sp*, secretory portion of preputial organ. Scale bars: 50 μm (a–c), 100 μm (d).

lating individuals as males or females, depending on their behaviour.

The individuals of *S. oelandica* begin copulating copiously, when the water temperature is raised above 20°C. The smaller specimen in a pair is usually mating as a male. The male approaches the female, mounts the apical side of the partner's shell on the left side of the aperture, crawls a full circle along the body whorl toward the aperture and finally takes up the position over the partner's head (Fig. 2a). If the female changes the direction of motion, turning toward the approaching male, the male mounts from the opposite side of the shell (Fig. 2b), immediately turns around in a small circle, and heads toward the partner's aperture. When the male is within a short distance of the shell margin (about 1/8 of the body whorl), he begins to evert the preputial organ and then lowers the pink swollen left tentacle between the female's tentacles (Fig. 2c). The male affixes himself to the partner's shell using the preputial organ as a hold-fast, without ever touching the mate's body, and pulls his shell down toward the foot thereby forcing the partner to slow down (Fig. 2d e). The female continues to move forward, albeit slower than before, and stretches her body to expose the female pore. The distal end of the male's inflated tentacle produces pink secretion, which he probably uses to attract the female. When the male becomes firmly affixed to the partner's shell, he inserts the penis into the female's vagina (Fig. 2f). The penetration is usually very rapid, without seeking movements, and the final phase of the copulation is accompanied by cramp-like contractions of the male's body. The intromission and insemination take up no more than 2.5 min, and then the male quickly leaves the partner. Occasionally, the female can bite the partner's preputial organ with her jaw (Fig. 2g). The courtship ritual lasts no more than 3.5 min, and, during the whole process, the female never stops moving or feeding.

This sequence can be referred to as the true copulation, but we also observed the false copulation, i.e. multiple attempts to copulate without a successful sperm transfer. This phenomenon is very common in laboratory, when the size of the group is increased by adding new individuals after 5–7 days of active mating period. Previously inseminated females refuse to copulate with newcomers; they often resort to biting and avoid true copulation. The male can undertake up to 25 attempts of false copulation with the same partner (by following the courtship ritual, described above), but, without getting an 'approval' from the female, leaves her to pursue another partner. If the false copulation (including several succeeding precopulatory rituals) lasts as long as 15 min to 1 h, a ring of pink secretion is formed on the male's left tentacle.

In some cases, a chain of 3–4 copulating individuals is formed, in which the true copulation between the successive members of the chain almost never occurs simultaneously: as the bottommost individual tries to assume a vertical position on the wall of the container, the upper specimens fail to successfully complete the courtship and the chain disintegrates. Throughout the period of observations, the true simultaneous copulation of all 3 specimens in a chain was observed only once.

After the active spawning period (2–3 days), the individuals cease copulating. Under experimental conditions, the males never attempted copulation with more than 2–3 different females. The females can mate again as females only after 3–4 weeks, and only if the population is replenished with potential males (small-sized specimens collected in cold water); however, over the half of the specimens would die during this period.

The penial sac of *S. oelandica* (Fig. 1a) is furnished with two glandular appendages, whose function is likely to be the production of substances helping the mollusc to attach to the partner's shell during the copulation. The penial sac has relatively thin walls, lined with several muscle layers (Fig. 3a–b). The outermost muscle layer consists

of wide circular (sometimes diagonal) fibers (Fig. 3a–b, *ocs*); underneath this layer are 4–5 layers of longitudinal fibers (Fig. 3b, *ls*); the innermost layer consists of the circular fibers (Fig. 3a–b, *ocs*). The latter are relatively widely spaced and have an L-shaped profile in cross-section. One or two inner layers of longitudinal muscles are partially intertwined with subjacent circular muscles. The penis is long and has a thick muscular wall comprising 2 layers of muscles (Fig. 3a–b): the outer layer composed of longitudinal fibers (Fig. 3a, *lp*), and the inner layer, composed of strong radial fibers (Fig. 3b, *rp*). The penis is tipped with the muscular papilla (Fig. 3c, *pa*). The preputium bears a well-developed preputial organ (Fig. 3d), which is a glandulo-muscular adhesive organ that plays a significant role in fixation of the male on the female's shell during the copulation.

DISCUSSION

The comparison of the description made by Precht (1936) for the copulation of *S. nitida* with our own observations shows that this author most likely observed the false copulation, which occurred after an active mating period. The assumption (Precht, 1936: 82) that the members of this genus can copulate in a side-by-side position is incorrect. The misinterpretation of the reproductive morphology of *S. nitida* made by this author can be also easily explained – we suppose that he apparently mistook the preputial organ for the penis due to the extremely small size of the latter (Fig. 2f–g).

The lack of any ritual behaviour (foreplay) during the mating of freshwater snails reported by Simroth & Hoffmann (1908–1928) and Precht (1936) is inconsistent with our observations. It was noted in our experiment, that the "reference point" used by the male to mount on the partner's shell is defined by the position of the female's head and by tentacular contact. The same precopulatory behaviour is exhibited by all individuals. False copulations outnumber true copulations in laboratory, but this is probably not the case for individuals in nature. Copulating chains composed of 3–4 specimens are also likely to be uncommon in nature. The same copulatory phases described by Trigwell et al (1997) for *B. glabrata*, i.e. pre-copulation – copulation – insemination – fertilization, are observed in *S. oelandica* as well.

The morphological characteristics of the shell and the position of the female genital pore necessitate the development of additional muscular (preputial organ) and glandular structures (flagellums). The walls of the preputium are relatively thin, especially in its proximal portion, most likely because they function as a passive sheath and, like the walls of the penial sac, are not subjected to mechanical stress during the copulation. The penis is a highly extensible organ due to a pronounced layer of radial muscles. The pointed soft penial papilla is not covered with the cuticle and the additional protection from the cuticle is not required, because: 1) mechanical stress is minimal (our experiment shows that the males rarely inseminate more than 3 females per season), and the time interval allowed for intromission is very brief; 2) during the copulation, the penis bends over the outer margin of the shell aperture; in this case, a more rigid structure would have been less mobile or sensitive, and, therefore, would have provided less selective advantage.

As the phenotypic traits associated with mating strategies are subject to strong selective pressures, it seems reasonable to assume that the mating behaviour in *S. oelandica* is also affected by the characteristics of the habitat of this species. *Segmentina oelandica* inhabits shallow waters (during the active phase of the lifecycle the individuals of this species are never found deeper than 0.2 m), favouring thick patches of duckweed and coarse plant detritus, as was also reported by Frömming (1956) and Stadnichenko (1990) for other *Segmentina* species. *Segmentina oelandica* is also found abundantly on the underside of floating or submerged

tree branches. It may be concluded that the mating process in *S. oelandica* evolved in sun-warmed habitats (only existing for one to two summer months) that provide enough support for grazing and an abundant supply of food.

There is no accurate information on the post-copulatory management of autosperm in the Planorbidae. It could be suspected judging from the observed behaviour that males cannot mate as females until they discard or resorb the autosperm. It is also known that the members of this family do not practice self-fertilization in isolation (Bondesen, 1950). A one-year life cycle, often with two hibernation periods (in summer and winter), and a brief breeding period (Stadnichenko, 1990), also favour unilateral copulation proceeding in such a way that the female is not distracted from crawling or feeding.

The mating process in the subgenus *Segmentina* of the genus *Segmentina* is similar to that in *B. glabrata* (Trigwell et al., 1997), i.e. the copulation is accompanied by the ritual behaviour (pre-copulation), albeit fairly primitive, compared to that of the land pulmonates. The copulation mechanics of *S. oelandica* is strictly determined by a specific set of characters and cannot proceed in any other way, than described above. All species of the subgenus *Segmentina* have fairly similar shell morphology and the structure of the copulatory apparatus (Kruglov & Soldatenko, 1997), and therefore there should be no significant differences in mating behaviour between various members of this group. Nothing is yet known about the copulation in *S. distinguenda* (Gredler, 1859) that belongs to the subgenus *Parasegmentina* Kruglov et Soldatenko, 1997 and is morphologically different from the members of the subgenus *Segmentina*.

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