

A comparative study of mating and copulatory mechanics in *Hippeutis complanatus* and *Segmentina oelandica* (Gastropoda: Pulmonata)

Сравнительное исследование спаривания и механизма копуляции у *Hippeutis complanatus* и *Segmentina oelandica* (Gastropoda: Pulmonata)

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Recent studies on mating and copulation in pulmonates have provided a better understanding of a functional relationship between various aspects of their biology and have greatly advanced our knowledge of their reproductive behaviour. Here we describe the mating behaviour, copulatory mechanics and genital anatomy of *Hippeutis complanatus* (Linnaeus, 1758), a Palaearctic freshwater pulmonates, and compare these results with those from *Segmentina oelandica* (Westerlund, 1885). In *H. complanatus*, the individual that initiates mating mounts the shell of the partner and crawls over its basal side to position itself at the shell aperture for intromission. The copulatory connection is achieved by the preputium and its musculo-glandular appendage (preputial organ) that attaches to the shell of the partner. *Hippeutis complanatus* and *S. oelandica* share a number of common traits in genital morphology and mating behaviour, most notably the unilateral copulation and the use of the preputial organ as a holdfast. These species also show some significant differences: the opposite orientation of the partners, chemical stimulation in *S. oelandica* and a significantly longer mating duration in *H. complanatus*. Overall, the mating behaviour of the two species shows a combination of shared and divergent traits that have potential of being useful as taxonomically informative characters.

Исследования последних лет по спариванию и копуляции у легочных моллюсков позволили лучше понять функциональную взаимосвязь между различными аспектами биологии этих моллюсков и существенно расширили представления об их репродуктивном поведении. В настоящей статье описаны спаривание, механизм копуляции и анатомия копулятивных органов у *Hippeutis complanatus* (Linnaeus, 1758), палеарктического вида пресноводных пульмонат, и проведено сравнение с аналогичными данными, полученными для *Segmentina oelandica* (Westerlund, 1885). У *H. complanatus* инициирующая спаривание особь забирается на раковину партнера и ползёт по ее базальной стороне к устью, где занимает положение, используемое при копуляции. Связь между партнерами достигается при помощи препуциума и его мышечно-железистого придатка (препуциального органа), который закрепляется на раковине партнера. *Hippeutis complanatus* и *S. oelandica* обладают рядом общих признаков в морфологии копулятивных органов и половом поведении, наиболее важными из которых являются односторонняя копуляция и использование препуциального органа в качестве органа прикрепления. Процессы спаривания у этих двух видов имеют также несколько существенных различий: противоположная ориентация партнеров при копуляции, химическая стимуляция у *S. oelandica* и значительно более продолжительное спаривание у *H. complanatus*. В целом, спаривание у *H. complanatus* и *S. oelandica* характеризуется как наличием как общих черт, так и отли-

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чительных особенностей, которые могут быть использованы в качестве таксономически значимых признаков.

Key words: mating, reproductive behaviour, copulatory mechanics, genital morphology, *Hippeutis complanatus*, *Segmentina oelandica*

Ключевые слова: спаривание, половое поведение, механизм копуляции, морфология копулятивных органов, *Hippeutis complanatus*, *Segmentina oelandica*

INTRODUCTION

A growing number of studies published recently on mating behaviour and copulatory mechanics of Pulmonata (Boyle & Yoshino, 2000; Vianey-Liaud & Dussart, 2002; Ohbayashi-Hodoki et al., 2004; Koene & Ter Maat, 2005; Facon et al., 2008; Norton et al., 2008; Jordaens et al., 2009; Soldatenko & Petrov, 2009, 2012, 2013; Wethington et al., 2012) shows a resurgence of interest in various aspects of the mating process in this group of gastropods. Major attention in these studies was focused on stereotypic behavioural patterns, interactions between mating partners and their copulatory structures and within-group variation of mating behaviour. This approach has established a functional relationship between a variety of morphological, physiological, behavioural and ecological characteristics of pulmonates and helped in the assessment of correlation between different morphological characters. It has also provided evidence that differences in mating behaviour may have some utility as phylogenetic markers.

The present study uses this approach to investigate the mating behavior and copulatory mechanics in *Hippeutis complanatus* (Linnaeus, 1758), a Palaearctic freshwater pulmonates (Hygrophila) from the family Segmentinidae Baker, 1945 (= Segmentininae Baker, 1945; = Segmentinini Baker, 1945). The resulting data are compared with those from *Segmentina oelandica* (Westerlund, 1885), because the genus *Hippeutis* Charpentier, 1837 is closely related to *Segmentina* Fleming, 1818 (Baker, 1945; Hubendick, 1955; Starobogatov et al., 2004; Bouchet et al., 2005; Albrecht et al.,

2007; Kijashko et al., 2016) and the mating behaviour of *S. oelandica* has been studied previously (Soldatenko & Petrov, 2009, 2012). The mating process of *S. oelandica* is characterised by a distinct set of behavioural patterns: during precopulation, the active individual seeks and mounts a larger individual (passive partner) stimulating it with tentacular secretion; active individuals are often very persistent in their mating attempts; the copulation is always unilateral; it is very brief and assisted by a specialised musculo-glandular organ (preputial organ) that aids in positioning the partners during intromission. This behaviour is markedly different from that described previously in 15 other genera of Hygrophila (Nekrassow, 1928; Precht, 1936; Bondesen, 1950; Abdel-Malek, 1952; Frömming, 1956; Pace, 1971; Kruglov & Berezkina, 1978; Rudolph, 1979a, 1979b, 1983; Berezkina & Starobogatov, 1988; Trigwell et al., 1997; Boyle & Yoshino, 2000; Vianey-Liaud & Dussart, 2002; Ohbayashi-Hodoki et al., 2004; Koene & Ter Maat, 2005; Facon et al., 2008; Norton et al., 2008; Wethington et al., 2012; Soldatenko & Petrov, 2009, 2012, 2013) and may represent a high degree of specialization. The study of mechanical and behavioural aspects of mating in *H. complanatus* and their comparison with those of *S. oelandica* may provide a better understanding of the diversity of these mechanisms and processes within Hygrophila.

An additional motivation for studying copulation in *H. complanatus* comes from the substantial differences in size of the copulatory structures between this species and *S. oelandica*, despite considerable similarity in shell morphology (shell size and

shape, whorl expansion rate, parameters of the shell aperture, etc.). The study of how morphological differences in copulatory structures might be reflected in behavioural differences during mating may help interpret the functional role of these structures.

The aim of the present study was to examine the mating behaviour and copulatory mechanics in *Hippeutis complanatus* (Linnaeus, 1758) and supplement our previous observations of mating in *S. oelandica* (Soldatenko & Petrov, 2009, 2012) with a description of the copulatory process. A further goal was to compare mating processes and assess the similarities and differences in reproductive characters between these species. This study also uses phalloidin staining and confocal microscopy to examine the musculature of some copulatory structures in *H. complanatus* and describes the gross morphology of genitalia of both species taken from individuals killed during intromission. We also provide here a description of the penial musculature of *S. oelandica*, which adds some important details to those presented in our earlier publication (Soldatenko & Petrov, 2009).

It should be emphasized that the taxonomy of species and families discussed in this paper is beyond the scope of the present study and that their taxonomic status still remains to be resolved: *Segmentina oelandica* (according to Kruglov & Soldatenko, 1997; Starobogatov et al., 2004; Kijashko et al., 2016) = *S. nitida* (Müller, 1774) (according to Glöer, 2002); family Segmentinidae Baker, 1945 (according to Kijashko et al., 2016) = Segmentininae Baker, 1945 (according to Baker, 1945) = Segmentinini Baker, 1945 (according to Zilch 1959–1960; Starobogatov, 1967; Hubendick, 1978; Bouchet et al., 2005).

MATERIAL AND METHODS

Collection sites

Sexually mature individuals of *Hippeutis complanatus* (Linnaeus, 1758) and *Segmentina oelandica* (Westerlund, 1885) were col-

lected by the senior co-author in 2012 and 2015 from the following localities in European Russia (Smolensk Prov., Demidovskiy Distr., Smolenskoe Poozer'e National Park, Przhival'skoe town):

1) *H. complanatus* – inundated shore of Lake Glubokoe (N 55.3082 E 31.4784, altitude 160 m), 29 June 2012;

2) *H. complanatus* – inundated areas around a channel between Lakes Krugloe and Dolgoe (N 55.5076 E 31.8072, altitude 149 m), 25 June 2015; swampy areas on the right-hand side of the road to Baklanovo vill. (N 55. 5076 E 31.8116, altitude 157 m), 29 July 2015;

3) *S. oelandica* – inundated areas on the right-hand side of the road to Baklanovo vill. (N 55. 5076 E 31.8116, altitude 157 m), 29 July 2015.

Behavioural study

Sexually mature individuals of *H. complanatus* and *S. oelandica* were placed in Petri dishes filled with 1 cm of water in groups of 10–12 molluscs per dish. Overall, 30 groups of *H. complanatus* (306 individuals in total) and six groups of *S. oelandica* (60 individuals in total) were observed throughout the study period. Mating was induced by heating the water (Duncan, 1975; Smith, 1981) with fluorescent lamps to 25–28 °C. Nineteen passive individuals of *H. complanatus* were mechanically agitated during copulation, which made them float to the surface in copulo and flip over with the basal sides of their shells facing upwards, allowing an observation of normally concealed behaviours (12 of these pairs continued with mating, Fig. 1b–d). Mating pairs of *H. complanatus* were photographed and time intervals were recorded for all phases of their mating process. The time for the start of precopulation was determined with a fair degree of accuracy, because *H. complanatus* shows a distinct behavioural transition from grazing to pursuit of partner. A total of ten mating pairs and nine courtship rejections were observed in the groups of *S. oelandica*. These

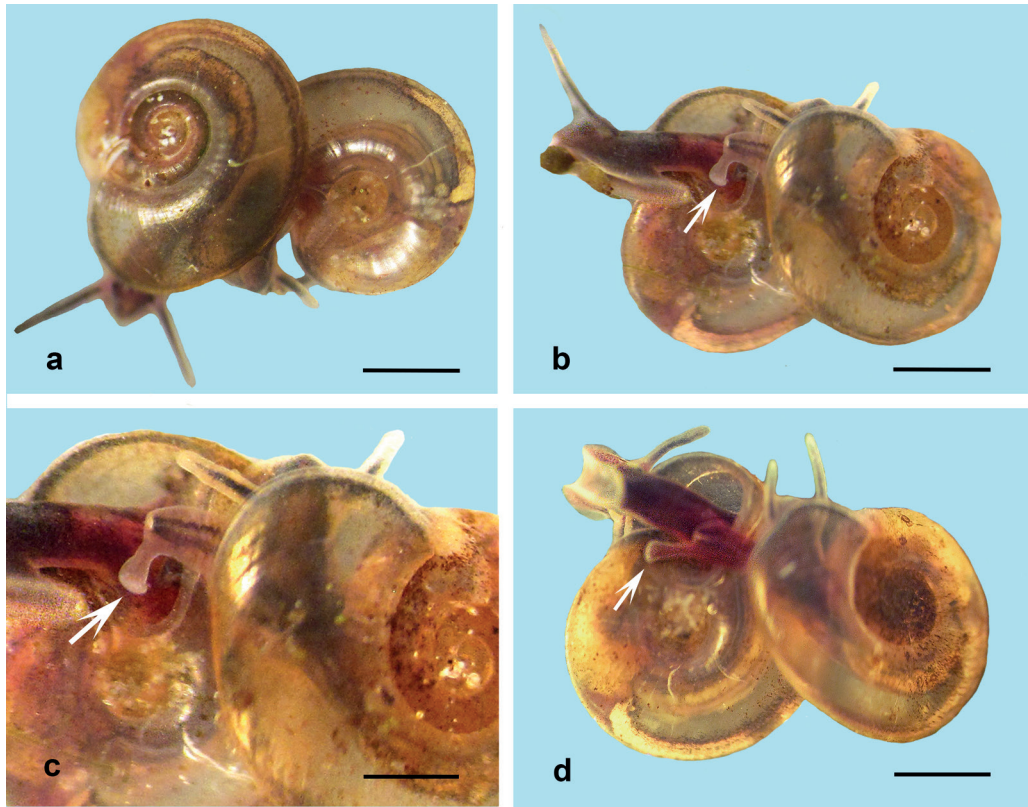


Fig. 1. Copulating individuals of *Hippeutis complanatus*: **a**, precopulation, with the shell of the passive partner viewed from the upper side. The active partner (bottom) crawls over the shell of the passive partner (top) towards the shell aperture; **b**, precopulation, with the shell of the passive partner viewed from the basal side. The active partner extends the preputium to attach it to the female gonopore. The arrow points to the preputial organ of the active partner; **c**, enlarged view of **b** showing the preputium and the preputial organ (arrow); **d**, copulation, the active partner attaches its preputial organ (arrow) to the shell of the partner; the preputium is attached to the female gonopore. Scale bars: 1.0 mm (a, b, d); 0.5 mm (c).

results were pooled together with those reported for *S. oelandica* by Soldatenko & Petrov (2012). The molluscs of both species were kept in laboratory for no longer than one month. All time intervals for the mating process of both species are summarized in Table 1.

Morphological study

Throughout the study, ten attempts were made to fix mating pairs of each species in boiling water. This technique has yielded good results on other planorbid

species: *Anisus vortex* (Linnaeus, 1758), *Planorbarius corneus* (Linnaeus, 1758), and *Planorbella (Seminolina) duryi* (Wetherby, 1879) (Soldatenko & Petrov, 2012, 2013), but in *H. complanatus* and *S. oelandica* it has always resulted in disintegration of the mating pair. Twenty individuals of each species were fixed in 70% ethanol and then dissected; male copulatory apparatuses and distal regions of the female reproductive system removed from these animals were mounted in Canada balsam to study the mechanism of sperm transfer. These preparations were examined and photographed

Table 1. Time intervals (minutes) for courtship rejection in *H. complanatus* and *S. oelandica*.

Species	t °C	n	Single mating attempt	n	Total duration
			mean±SD (min–max)		mean±SD (min–max)
<i>Hippeutis complanatus</i>	25–28	10	6.51±4.32 (2.41–16.35)	11	19.15±7.44 (8.02–37.00)
<i>Segmentina oelandica</i>	25–28	21 + 4	0.53±0.15 (0.26–1.20)	27 + 5	8.14±5.54 (2.41–25.00)

Note: n – number of observed mating encounters.

on a Leica DMLS-2 microscope equipped with a CCD camera. The schematic representations of copulation in *H. complanatus* and *S. oelandica* were made from photographs, retaining the original proportions.

Two specimens of penises of each species were prepared for scanning electron microscopy (SEM). The penises were removed from the penis sheath, dehydrated in ethanol, air-dried for 20 minutes in hexamethyldisilazane (Bock, 1987), coated with platinum in a HITACHI IB-5 ion sputter and viewed on a HITACHI S-570 scanning electron microscope.

For confocal microscopy, *H. complanatus* were fixed for 1 h in 4% formaldehyde buffered with 0.01 M phosphate-buffered saline (PBS) at room temperature, rinsed in PBS (3×15 min), permeabilised for 1 h in PBS containing 0.25% Triton X-100, rinsed again shortly in PBS and then transferred to phalloidin-TRITC (Sigma-Aldrich) for 1 h. After incubation with phalloidin, the specimens were washed again for 3×15 min in PBS, mounted in 80% glycerol or Vectashield (Vector Laboratories Inc.) on glass slides and viewed on a Leica TCS SP5 microscope.

Terminology

This study uses the terminology for the mating process adopted in our previous publications (Soldatenko & Petrov, 2012, 2013) and partly derived from other au-

thors (Smith, 1981; Trigwell et al., 1997; Jordaens et al., 2009). The mating of both *H. complanatus* and *S. oelandica* is unilateral, and an individual that exhibits a more active mating behaviour is referred to as “active partner”, while the other individual as “passive partner”. Mating is divided into three consecutive phases (precopulation, copulation, and postcopulation). The period from the beginning of precopulation to the end of postcopulation is designated as the mating period. *Hippeutis complanatus* and *S. oelandica* present no difficulty in determining the direction of shell coiling; both species are dextral. As in our previous publications (Soldatenko & Petrov, 2012, 2013), we use the terms “upper” for the apical side of the shell (facing up in a normal crawling position) and “basal” for the opposite umbilical side.

RESULTS

Copulatory apparatus of *Hippeutis complanatus*

The male copulatory apparatus comprises two morphological regions. The proximal region consists of a penis sheath that houses the penis and carries two short glandular appendages (flagella) (*fl*, Fig. 2a) (Baker, 1945; Hubendick, 1955; Kijashko et al., 2016). The flagella are equal in length (up to 0.3 mm) and open into the proximal end of the penis sheath near the entrance of the

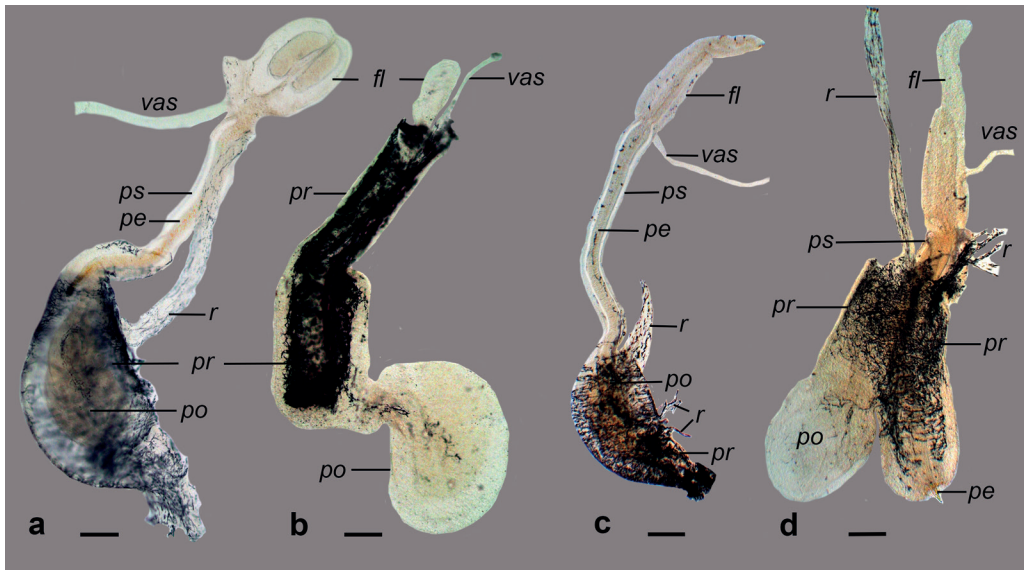


Fig. 2. Whole mounts of male copulatory organs: **a**, inside the body of a non-mating individual in *Hippeutis complanatus*; **b**, in the everted position during copulation in *Hippeutis complanatus*; **c**, inside the body of a non-mating individual of *Segmentina oelandica*; **d**, in the everted position during copulation in *Segmentina oelandica*. Scale bars: 100 μ m (a, b); 200 μ m (c, d). Abbreviations: *fl*, flagellum; *pe*, penis; *po*, preputial organ; *pr*, preputium; *ps*, penis sheath; *r*, retractor muscle; *vas*, vas deferens.

vas deferens. The muscular wall of both flagella consists of two or three rows of wide strap-like muscles. Most of these muscles are oriented around the circumference of the flagellum (*cf*, Fig. 4a, b), but some of them have circular orientation only in their central portions; their distal portions turn in a diagonal (*df*, Fig. 4a, b) or longitudinal direction and lie above or beneath the circular row of muscles. The muscles of the vas deferens have a similar arrangement, with predominantly circular muscles (*cv*, Fig. 4a) that may run distally in a diagonal or longitudinal direction, but the wall of the vas deferens is more densely muscular and comprises four to six rows of muscles. The penis sheath has three layers of muscles in its muscular wall: the outer (*ocs*, Fig. 4c, d) and inner layers (*ics*, Fig. 4c, d) are composed of one to three rows of circular muscles, and the intermediate layer (*ls*, Fig. 4c, d) consists of two to three rows of wide longitudinal muscles. The inner circular muscles have a characteristic L-shaped profile

in cross section (Fig. 4d). Some of the muscles in both outer and inner circular layers can shift to the intermediate layer to lie interspersed between the longitudinal muscles (*arrows*, Fig. 4c). The penis lacks a stylet; the ejaculatory opening is subterminal. The muscular wall of the penis from its base to the ejaculatory opening (penis shaft) consists of outer (*olp*, Fig. 4c, d) and inner (*ilp*, Fig. 4d) longitudinal and intermediate radial (*rp*, Fig. 4c, d) muscle layers. The longitudinal muscles of both layers are few and are widely spaced around the circumference of the penis and the ejaculatory duct. The radial muscles of the intermedial layer are very wide and each is composed of numerous thin myofibres. The terminal portion of the penis distal to the ejaculatory opening (penial papilla) is short (40 μ m) (Fig. 3a); its wall lacks radial and longitudinal muscles and is invested only with one layer of circular muscles.

The distal region of the male copulatory apparatus consists of a preputium that con-

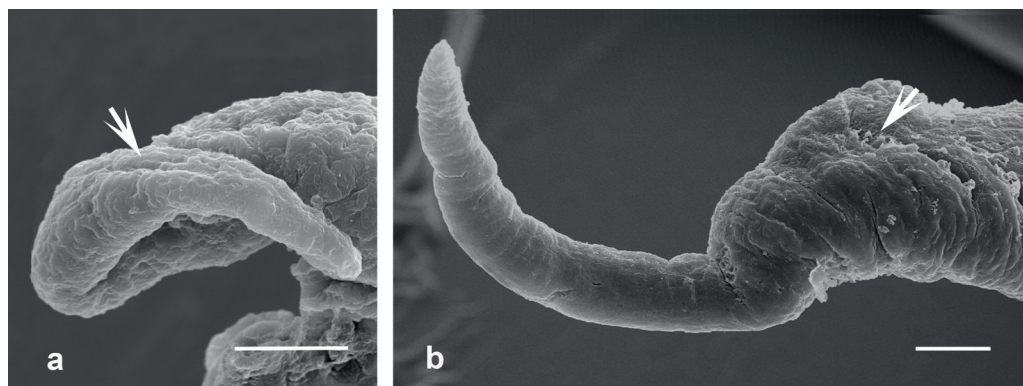


Fig. 3. SEM micrographs of the proximal regions of the penis: **a**, *Hippeutis complanatus*; **b**, *Segmentina oelandica*. Arrows indicate ejaculatory openings. Scale bars: 20 µm (a, b).

tains a large muscular appendage (preputial organ) (*po*, Fig. 2a). The preputial organ is essentially an invagination of the muscular wall of the preputium and lies inside the preputium, when the latter is in its inverted (retracted) position. The preputial organ is highly muscular and its primary function is the attachment to the shell of the partner during copulation.

Penial musculature of *Segmentina oelandica*

The muscular wall of the penis shaft consists of outer and inner longitudinal and intermediate radial muscle layers. The longitudinal muscles of both layers are few and are widely spaced around the circumference of the penis and the ejaculatory duct. The radial muscles (*rp*, Fig. 4e) of the intermedial layer, unlike those in the penis of *H. complanatus*, are not wide and are circular or slightly oval in cross section. The penial papilla is very long (140 µm) (Fig. 3b; *pp*, Fig. 4f); the longitudinal muscles of the penis shaft (*olp*, Fig. 4e) do not extend beyond the ejaculatory opening into the penial papilla and the radial muscles are replaced in the papilla with slender circular muscles (*cp*, Fig. 4e). The circular muscles invest the wall of the papilla along its entire length (Fig. 4f).

Mating behaviour of *Hippeutis complanatus*

The precopulation begins when the active individual starts following the prospective partner (“passive individual”) making probing movements with its head and tentacles. The active snail approaches the pursued individual from behind and aligns itself parallel to it, with both snails facing the same direction. When the active individual comes into contact with the partner, it pivots its head and foot so that the sole of the foot is facing upwards and grips the other snail with its foot on the outer side of the shell opposite the shell aperture. In so doing, the active individual squeezes itself under the shell of the partner thereby pushing the shell away from the substrate. Once the active individual is firmly attached to the partner, it flips its shell over so that its basal side is now facing upwards. With the head directed towards the shell aperture of the partner, the active individual starts crawling over the basal side of the body whorl of the partner (Fig. 1a). Making a semi-circle along the whorl, the active partner approaches the shell aperture and begins extending its preputium. The preputium is turned inside out through the gonopore, with the preputial organ being carried to the outside with the everting

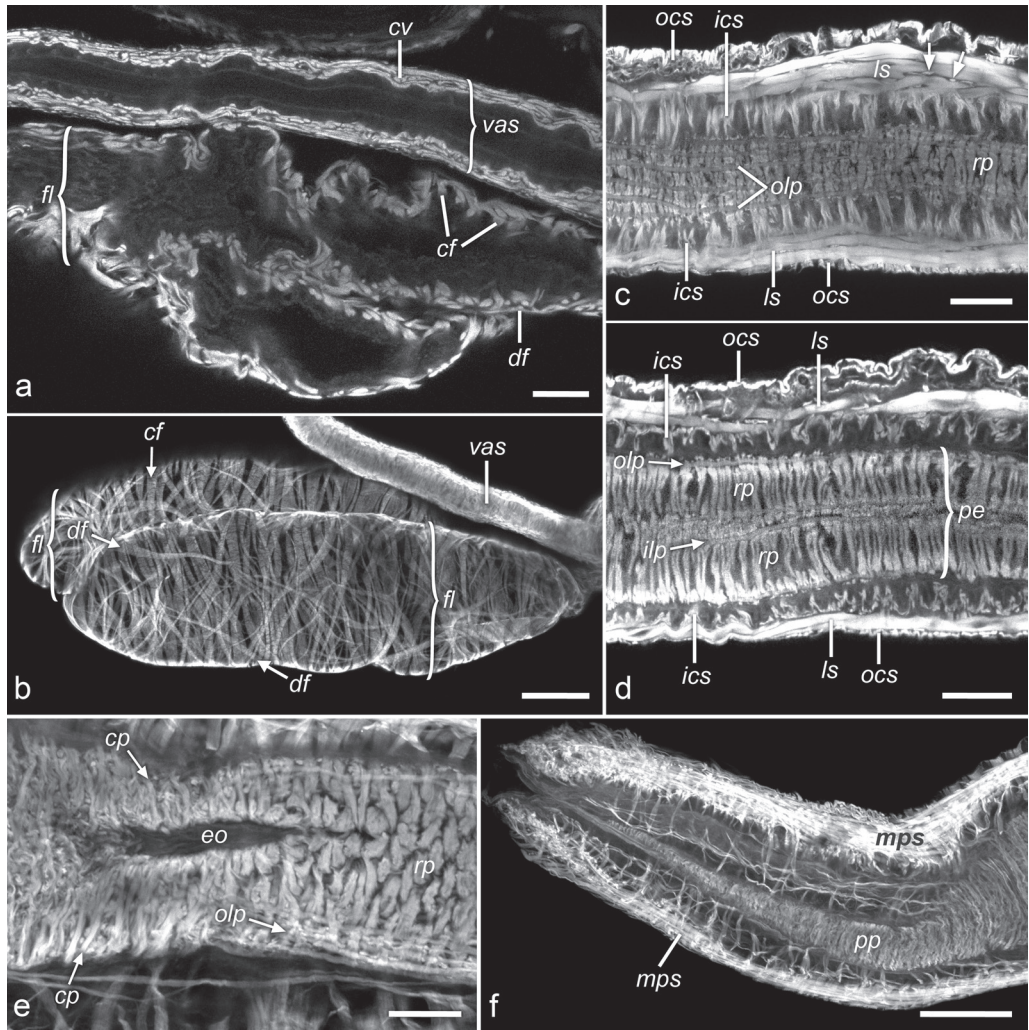


Fig. 4. Musculature of the penial complex of *Hippeutis complanatus* (a–d) and *Segmentina oelandica* (e, f). Maximum intensity projections. **a**, central portions of the flagella (fl) and vas deferens (vas); **b**, projection through the muscular walls of the flagella and vas deferens; **c**, penis sheath and penis, projection through the muscular wall of the penis; arrows show the muscles of the outer circular layer penetrating between the longitudinal muscles; **d**, penis sheath and penis, projection through the central portion of the penis; **e**, penial wall in the region of the ejaculatory opening (eo) showing the transition between penis shaft and penial papilla; **f**, penial papilla surrounded by the penis sheath. Note that the wall of the papilla contains only circular muscles. Scale bars: a, c–e (20 μm); b, f (50 μm). Abbreviations: cf, circular regions of flagellar muscles; cp, circular penial muscles; cv, circular muscles of vas deferens; df, diagonal branches of flagellar muscles; eo, ejaculatory opening; fl, flagellum; ics, inner circular muscles of penis sheath; ilp, inner longitudinal penial muscles; ls, longitudinal muscles of penis sheath; mps, musculature of penis sheath; ocs, outer circular muscles of penis sheath; olp, outer longitudinal penial muscles; pe, penis; pp, penial papilla; rp, radial penial muscles; vas, vas deferens.

preputial walls. After some probing, the active individual attaches the preputium to the female gonopore of the partner (Fig. 1b–c). The whole process of preputium eversion and attachment takes at least several minutes. The eversion of the preputium can only be observed by making the passive partner float to the water surface, with the basal side of its shell towards the observer.

The whole process of precopulation takes on average about seven minutes (Table 2), during which time the passive individual normally does not show any apparent reaction to the suitor and does not stop feeding and crawling. In some cases, however, the passive individual demonstrates its disapproval to the suitor and molluscs may fail to copulate despite repeated mating attempts by the active individual. In these cases, courted molluscs may exhibit different behaviours. After several (2–5) mating attempts, the nonreceptive snail usually slows down and withdraws its head into the shell; sometimes these snails stop moving entirely, showing to the partner that they do not want to copulate. Some of these nonreceptive snails exhibit an overtly aggressive behaviour, biting the pursuer with its jaw after 7–12 mating attempts (these repeated attempts may take up to 37 minutes, Table 1).

The copulation is always unilateral. During sperm transfer, the active individual affixes its preputial organ to the shell of the partner (Fig. 1d). The body and exposed

parts of the copulatory apparatus of the active partner show no spasmodic contractions, and the passive individual continues crawling and feeding. The copulation lasts on average about 30 minutes (Table 2). Once insemination is completed, the active individual begins retracting its copulatory apparatus and at the same time attaches its foot to the substrate, flips its body and shell 180°, and then crawls away from the partner. This is the shortest phase of the mating process that takes no more than 12 seconds (Table 2). Overall, the whole mating period spans 24.05 to 52.33 minutes (Table 2).

Insemination in *Hippeutis complanatus*

The insemination is always unilateral. The active partner everts its preputium, which grows to about twice its resting size, and then extends its preputial organ (Figs 1b–c, 2b), which it uses to probe the partner and eventually attaches it to the basal surface of the shell of the partner (Fig. 1d). During this process, all of the involved intromittent organs become red in colour by engorgement with haemolymph. Once the preputial organ is affixed to the shell, the active partner attaches the proximal end of the preputium to the body surface around the female opening of the partner (Fig. 5a).

The female gonopore lacks well-developed muscular walls and the copulatory connection is achieved solely by the muscular efforts of the active individual. This con-

Table 2. Time intervals (minutes) for mating in *H. complanatus* and *S. oelandica*.

Species	t°C	n	Pre-copulation	Copulation	Post-copulation	Mating period
			mean±SD (min–max)	mean±SD (min–max)	mean±SD (min–max)	mean±SD (min–max)
<i>Hippeutis complanatus</i>	25–28	23	6.32±3.24 (3.00–15.00)	26.22±4.57 (21.00–40.06)	0.09±0.02 (0.05–0.12)	33.06±7.26 (24.05–52.33)
<i>Segmentina oelandica</i>	25–28	43	0.46±0.15 (0.30–1.20)	1.50±0.30 (1.03–2.50)	0.11±0.11 (0.07–0.15)	2.47±0.37 (1.44–3.50)

Note: n – number of observed mating encounters.

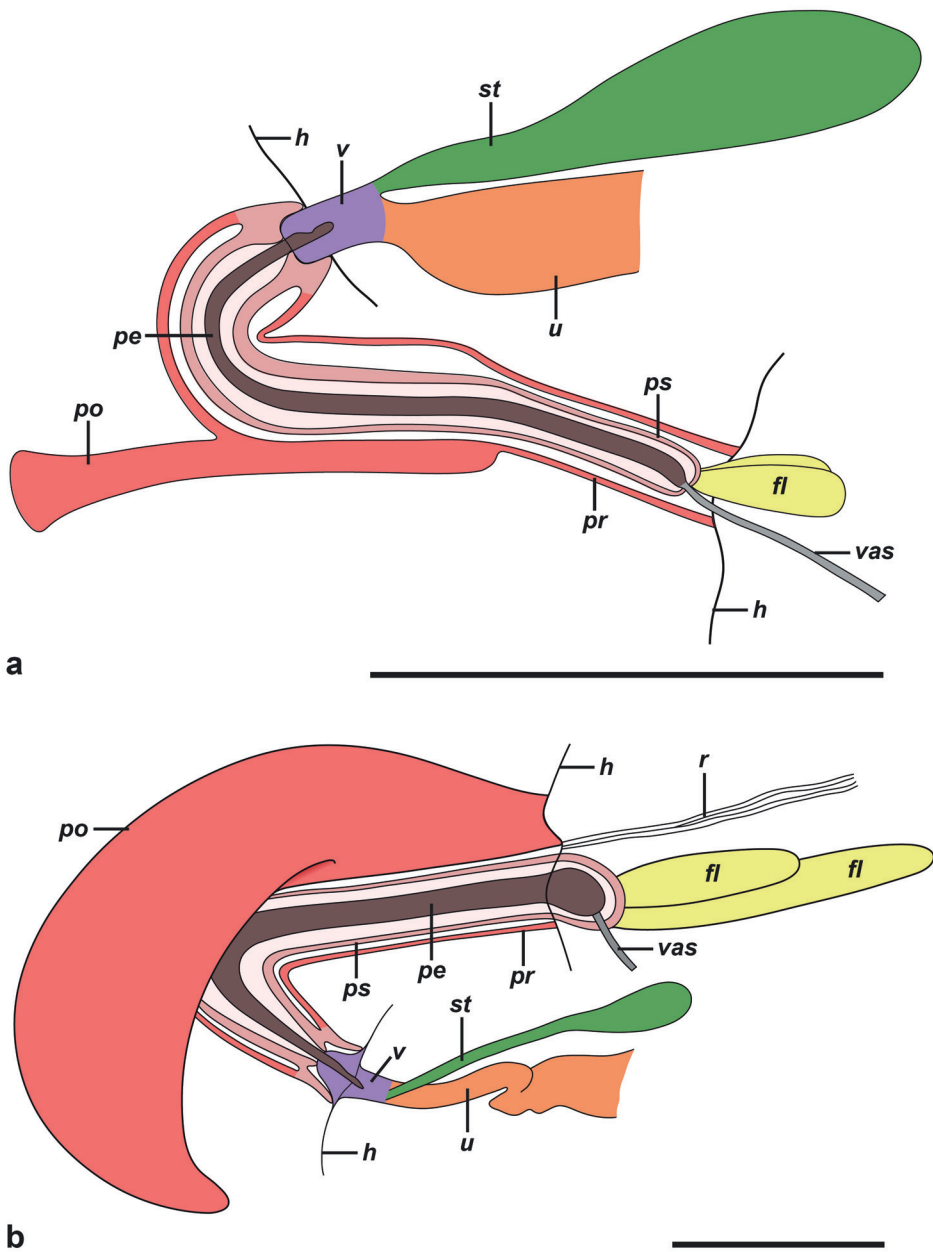


Fig. 5. Schematic representations of the copulatory organs during intromission: **a**, *Hippeutis complanatus*; **b**, *Segmentina oelandica*. Scale bars: 1 mm (a–b). Abbreviations: *fl*, flagellum; *h*, head; *pe*, penis; *po*, preputial organ; *pr*, preputium; *ps*, penis sheath; *r*, retractor muscle; *st*, spermatheca; *u*, uterus; *v*, vagina; *vas*, vas deferens.

nection, however, is rather strong, because the mating pair often remains in copulo, if the passive partner is mechanically agitated. During copulation the penis becomes much longer, but it does not reach deep into the vagina because of the short penial papilla (Fig. 5a). As the penis lacks any additional attachment structures (bulbuses or stylets), the copulants break up immediately upon fixation and the partners cannot be fixed in copulo. The muscular walls of the vas deference and the penis sheath are relatively weakly developed, and the sperm transfer is accomplished primarily by contractions of the penial muscles. As a consequence, the process of sperm transfer (Fig. 5a) requires a relatively long time to complete (up to 40 minutes, Table 2) and is very difficult to observe. The sperm is liquid; the spermatozoa do not coalesce into a single stringy mass and are accumulated in the spermatheca. Once the sperm transfer is finished, the active partner detaches its preputial organ from the shell of the partner, then, several seconds later, removes its preputium from the female gonopore and dismounts the passive individual, often retracting its copulatory organs as it does so. Throughout this process, the passive partner shows no apparent reaction to the active individual.

Insemination of *Segmentina oelandica*

The insemination is always unilateral. The active individual starts everting its preputium only while at the shell aperture of the partner. During this process, the preputium is mostly hidden from view and the observer sees only the massive red cone-shaped preputial organ (Figs 2d, 5b). The active partner uses the preputial organ to grip and raise the shell of the partner; this kind of attachment helps the active individual to maintain its position on the moving partner, forcing it to slow down and extend its head to expose the female opening (Soldatenko & Petrov, 2009). The preputial organ grips the basal surface of the shell of the partner and the proximal end

of the preputium attaches to the body surface around the female gonopore (Fig. 5b). The female gonopore has well-developed muscular walls and, as soon as the penis is inserted into the genital opening, the vagina partially everts to the outside embracing the penis as a muscular ring (Fig. 5b). The sperm transfer is thus achieved by the muscular efforts of both partners, and is very fast (Soldatenko & Petrov, 2012). The connection between the partners is weak and any mechanical agitation breaks up the pair. During insemination the penis becomes much longer and the long penial papilla reaches very deep into the vagina of the partner (Fig. 5b). As the penis lacks any additional attachment structures, the pair breaks up immediately upon fixation and the partners cannot be fixed in copulo. The muscular walls of the vas deferens and the penis sheath are weakly developed, and the sperm transfer is achieved primarily by contractions of the penial muscles assisted by muscular pulsations of the preputium; the spasmodic contractions of the latter are clearly seen during copulation. The sperm is liquid and is accumulated in the spermatheca. The copulation spans only 2.5 minutes (Table 2). Once the sperm transfer is completed, the active partner detaches its preputium and preputial organ and dismounts the partner. If the active individual takes too much time in dismounting, the passive individual may bite the protruding parts of its preputial organ.

DISCUSSION

The study of mating in *Hippeutis complanatus* has expanded our understanding of both common patterns and diversity of reproductive behaviours in Hygrophila. The commonality between *H. complanatus* and *S. oelandica* is manifested in some shared behavioural traits, such as the active pursuit of the prospective mates by the active individuals (courtship), non-involvement of the passive individuals in mating and exclusively unilateral mating

(despite the mechanical possibility of reciprocal mating in *H. complanatus*). The lack of reciprocal mating in Segmentinidae can be explained by functional protandry (Berezkina & Starobogatov, 1988; Trigwell & Dussart, 1998), which is visually reflected in smaller shell sizes of the active partners, in common with other species of freshwater pulmonates (DeWitt, 1996; Ohbayashi-Hodoki et al., 2004; Norton et al., 2008). Another common feature of both species is observed in similar behavioural patterns of courtship rejection, which is always accompanied by an aggressive reaction from the non-responsive individual, if the active partner becomes persistent in its mating attempts. A more specific common trait of the mating process in Segmentinidae is the use of a specialized musculo-glandular structure (preputial organ). Our observations have confirmed that the preputial organ does not participate directly in intromission and does not function as an organ of stimulation, but plays a role of a holdfast. This organ may help the active individual to gain additional purchase on the partner and may assist in positioning the partners relative to each other. However, its primary function is probably to secure the shell of the passive partner during intromission. An occasional movement of the shell might disengage the copulatory connection between the partners and the preputial organ prevents this from happening by keeping the shell a safe distance from the female gonopore.

Aside from the apparent similarities in mating behaviours, *S. oelandica* and *H. complanatus* also have some significant differences. The mating process of *H. complanatus* is much longer (24.05–52.33 min) than that of *S. oelandica* (1.44–3.50 min) (Table 2) and closer in duration to the mating process of some other planospiral snails: *Bathymorphalus contortus* (Linnaeus, 1758) (41–70 min) and *Choanomphalus riparius* (Westerlund, 1865) (20–54 min) (Soldatenko & Petrov, 2012). The most notable differences between *H. complanatus* and *S. oelandica* are in the duration of precopu-

lation (by 13 times) and copulation (by 17 times), while the duration of postcopulation differs to a much lesser degree (Table 2). Another major difference is the orientation of the active partner on the shell of the partner: in *S. oelandica* it mounts the upper side of the shell in an upright position, while in *H. complanatus* it sits on the basal side of the shell in an upside-down position. As a consequence, the attachment to the passive partner requires different efforts in *H. complanatus* and *S. oelandica* and this may explain why *S. oelandica* has a larger and more powerful preputial organ. The active partner in *H. complanatus* pushes itself under the shell of the passive individual, which requires more time, but less physical effort. In *H. complanatus*, mating is never accompanied by chemical stimulation observed in *S. oelandica* (Soldatenko & Petrov, 2009, 2012). We have found no evidence of secretion on the tentacles in *H. complanatus*, even after numerous unsuccessful mating attempts. Although the active individuals of *H. complanatus* may spend a substantial amount of time on repeated mating attempts (Table 1), the maximum number of these attempts (12) is less than in *S. oelandica* (25 attempts). In general, although the mating processes in Segmentinidae are essentially similar, the observed differences (Table 3) are sufficiently large and well-defined to justify their use as taxonomically informative characters.

The study of mating has explained some anatomical and histological characteristics of the copulatory apparatuses in *H. complanatus* and *S. oelandica*. All copulatory structures are functionally identical in both species and were correctly identified in earlier publications (Baker, 1945; Hubendick, 1955; Kruglov & Soldatenko, 1997; Glöer, 2002). The dimensions of the copulatory organs, however, are significantly different: the male genitalia in *H. complanatus* (Figs 2, 5) are twice as small as those of *S. oelandica*, even though the shell and the body of the two species are comparable in size. It is clear that *H. com-*

Table 3. Mating behaviour traits and associated morphological characters in *H. complanatus* and *S. oelandica*. Characters 2, 3, and 4 are based on Jordaens et al. (2009).

Characters: (1) copulatory activity of passive individual: NS – continues feeding and crawling. (2) shell orientation: M – mounted, i.e. umbilicus of upper shell is over spire of the lower shell; Re – reverse, i.e. shells are oriented with their umbilicus towards each other. (3) mating position: SM – shell mounting. (4) penis (ultrapenis) usage during copulation: U – unilateral. (5) morphology of the penial complex: po – preputial organ, fl – flagellum. (6) stylet: – absent; (7) copulatory stimulation: * copulation is accompanied by mechanical or chemical stimulation; – stimulation not observed. (8) gender expression: FP – functional protandry.

Species	Characters							
	1	2	3	4	5	6	7	8
<i>Hippeutis. complanatus</i> (present paper)	NS	Re	SM	U	fl+po	–	–	FP
<i>Segmentina. oelandica</i> (Soldatenko & Petrov, 2012)	NS	M	SM	U	fl+po	–	*	FP

planatus has smaller genitalia, because it exerts less efforts in attachment to the partner and in sperm transfer. The vas deferens does not play any important role in sperm transfer and this function is accomplished entirely by the muscular penis. The penial musculature is essentially similar in both species and differs only in secondary characters (significantly wider radial muscles in *H. complanatus*). The penis is subdivided into two functionally different regions: penis shaft and penial papilla. The muscular wall of the shaft is dominated by radial muscles that apparently function to dilate the ejaculatory canal, while the penial papilla is a solid structure invested with circular muscles, whose contraction elongates and extends the papilla.

The muscles in the wall of the penis sheath have a similar arrangement in both species. The muscular wall of the sheath is relatively thin and the function of the penis sheath in copulation is probably secondary, except for its distal portion, whose muscular folds play a crucial role in copulatory connection (Fig. 5). The preputium is a thin-walled structure and, like the penis sheath, has only a passive role in copulation acting as a sleeve, rather than an active muscular pump.

The comparison of mating behaviour in *H. complanatus* with that of the previously

studied *Ch. riparius* (Soldatenko & Petrov, 2012) reveals a number of similarities in the overall duration of the mating process, the courtship ritual of the active partner, the mutual orientation of the partners during copulation and behavioural patterns of courtship rejection. In fact, the analysis of only visual aspects of these processes may lead to an incorrect conclusion that the mating process of *H. complanatus* has more in common with that of *Ch. riparius* than with that of *S. oelandica*. The mating process of *Ch. riparius*, however, is entirely different. *Choanomphalus riparius* has no flagella or preputial organ, the structures that play an essential role in the copulatory process of Segmentinidae. In *Ch. riparius*, the active individual is attached to the shell of the partner only with its foot (not with the preputial organ), one of the main functions of the preputium is that of attachment, and an important role in intromission is played by the penial stylet, which is absent in Segmentinidae. The reason why mating behaviours of *H. complanatus* and *Ch. riparius* are similar is probably due to the fact that there is only a limited number of positions that may lead to successful mating. This example demonstrates that the correct functional and taxonomic assessment of the characters related to mating requires a complete study of all aspects of this process

(copulatory behaviour, mechanism of insemination, and the anatomy of the copulatory apparatus).

The results of our study show that the mating process in freshwater pulmonates is a remarkably complex interplay of morphological, physiological, and behavioural factors. The assessment of these factors and their interactions in different members of this taxonomic group may give a useful insight for understanding of the diversity of their biological processes and may become a valuable source of phylogenetic information.

ACKNOWLEDGEMENTS

This work was supported by the Russian Foundation for Basic Research (grant 15-04-05278).

Electron microscopy was performed at the Taxon Resource Research Centre (Zoological Institute RAS, St Petersburg).

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Received 15 Oct. 2016 / Accepted 5 Dec. 2016

Editorial responsibility: P.V. Kijashko