

MATING BEHAVIOUR AND COPULATORY MECHANICS IN SIX SPECIES OF PLANORBIDAE (GASTROPODA: PULMONATA)

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ABSTRACT

Although Planorbidae are one of the most common and diverse families of pulmonates, their mating behaviour is known only for a few species. This paper describes mating behaviour and copulatory mechanics in four species of the Planorbidae: *Anisus vortex* (Linnaeus, 1758), *Bathyomphalus contortus* (Linnaeus, 1758), *Choanomphalus riparius* (Westerlund, 1865) and *Planorbis planorbis* (Linnaeus, 1758), and supplements previously published observations on two other species: *Segmentina oelandica* (Westerlund, 1885) and *Planorbella (Seminolina) duryi* (Wetherby, 1879). Patterns of musculature of the penial complex [in all species except *P. (S.) duryi*] were studied using fluorescent phalloidin dye specific for actin filaments. *Choanomphalus riparius*, *P. planorbis* and *S. oelandica* were shown to mate by shell mounting and display an elaborate courtship behaviour accompanied by shell-circling. *Planorbella (S.) duryi* mates in a face-to-face position; in this species, courtship is accompanied by mechanical stimulation of the partner's pliae. *Anisus vortex* and *B. contortus* do not exhibit any distinct precopulatory behaviour and mate face-to-face. It is argued that Planorbidae show a relationship between gender expression (functional protandry *vs* simultaneous hermaphroditism) and mating behaviour (unilateral *vs* reciprocal mating). The taxonomic utility of the copulatory behaviour and penial musculature is discussed.

INTRODUCTION

Planorbidae are one of the most diverse and common worldwide families of basommatophoran pulmonates. These freshwater snails are considered to be simultaneous hermaphrodites and have internal fertilization through direct copulation (Hyman, 1967; Minichev & Starobogatov, 1971). Most members of this family are of small size (with shell diameter <20 mm) and their copulatory behaviour typically has to be studied under a light microscope on experimentally paired individuals. This often makes observation a challenging task and so there have been only a few studies dealing with reproductive behaviour in this group. Only a few of these are explicitly focused on mating behaviour and copulatory mechanics (Rudolph, 1979a, b; Trigwell, Dussart & Vianey-Liaud, 1997; Soldatenko & Petrov, 2009b); most studies discuss mating only briefly as part of the reproductive cycle (e.g. Kuma, 1975; Berezkina & Starobogatov, 1988) or explore some of the more specialized aspects of the mating process, such as reproductive physiology (e.g. Kruglov & Berezkina, 1978) or the relationship between reproductive anatomy and copulatory mechanics (Abdel-Malek, 1952; Pace, 1971) or between reproduction and ecological factors (Thomas, Coldworthy & Benjamin, 1974; Boyle & Yoshino, 2000). Some very brief observations on mating can also be found in faunistic (Hazay, 1881) and taxonomic (Baker, 1945; Wu, 1972) accounts.

In recent years, interest in the reproductive processes in freshwater pulmonates has increased and experimental techniques have become more sophisticated (Boyle & Yoshino, 2000; Vianey-Liaud & Dussart, 2002), but most studies (e.g. Koene & Ter Maat, 2005; Facon, Ravigné & Goudet, 2008) are still focused on reproduction in just a few model or parasitologically important species, such as *Biomphalaria glabrata*, *Bulinus globosus* and *Lymnaea stagnalis*. Some recent studies have provided a few preliminary generalizations concerning mating behaviour in pulmonates. For instance, it has been observed that the shell size influences gender role during copulation, i.e. individuals with smaller shells act predominantly in the male role (DeWitt, 1996; Ohbayashi-Hodoki, Ishihama & Shimada, 2004; Norton, Johnson & Mueller, 2008); that flat-spined snails copulate reciprocally and high-spined ones unilaterally (Asami, Cowie & Ohbayashi, 1998); and that all basommatophorans mate by shell mounting with unilateral intromission, and only as an exception in some species is intromission simultaneously reciprocal (Jordaens, Dillen & Backeljau, 2009). Unfortunately, most generalizations have been derived predominantly from observations made for the Stylommatophora; in the Basommatophora, mating behaviour is known only in 15 out of 68 genera and these observations are often anecdotal or fragmentary. Furthermore, there is now a growing body of evidence that challenges some long-held ideas about mating behaviour in

freshwater pulmonates. For example, the notion that planorbids do not display complex ritualized behaviour prior to copulation (precopulation or courtship) (Simroth & Hoffmann, 1908–1928: 1119; Precht, 1936) has now been refuted (Trigwell *et al.*, 1997; Soldatenko & Petrov, 2009b). It is clear that proposed generalizations should be tested on a broader empirical basis, which implies studying copulation in a wider array of basommatophoran species.

The aim of the present paper is to describe mating behaviour and copulatory mechanics in four species of the Planorbidae: *Anisus vortex* (Linnaeus, 1758), *Bathyomphalus contortus* (Linnaeus, 1758), *Choanomphalus riparius* (Westerlund, 1865) and *Planorbis planorbis* (Linnaeus, 1758) and to supplement previously published observations on two other species: *Segmentina oelandica* (Westerlund, 1885) and *Planorbella (Seminolina) duryi* (Wetherby, 1879). All six species live in the Palearctic region, although *P. (S.) duryi* is originally a tropical species and was only recently introduced to the temperate regions through the aquarium trade (Sitnikova *et al.*, 2010). An additional aim is to describe the musculature of the male copulatory organs (penial complex) in these species [except *P. (S.) duryi*] using confocal microscopy and fluorescent phalloidin dye specific for actin filaments.

MATERIAL AND METHODS

Collection sites

The snails (sexually mature individuals) were collected by the senior co-author in 2008–2009 (June through October). Collections were made from the following localities in the European part of Russia: (1) 55°30'N, 31°47'E: Smolensk Prov., Demidovskiy Distr., Smolenskoye Poozerye National Park, near town of Przhevalskoye: lake Rytöye (*A. vortex*, *P. planorbis*); temporary ponds in the outskirts of the Chistik Teaching Centre (Smolensk State University) (*P. planorbis*, *Ch. riparius*, *S. oelandica*); a pool at Kirovka village, temporary ponds (*Ch. riparius*); a pool at Kirovka village, lake Mutnoye (*B. contortus*); a pool at Nikitenki village (*S. oelandica*); (2) 59°58'N, 30°15'E: Leningrad Prov., St Petersburg, ponds on Yelagin Island (*B. contortus*). The individuals of *P. (S.) duryi* were collected in 2009 from an aquarium culture maintained at the Zoological Institute RAS (St Petersburg).

Although the taxonomy of the six species discussed in this paper is a question beyond the scope of the present study, we note that their taxonomic status is still unsettled and that different authors assign these species to different genera: *Bathyomphalus contortus* (according to Glöer, 2002) = *Anisus contortus* (according to Starobogatov *et al.*, 2004); *Choanomphalus riparius* (according to Starobogatov *et al.*, 2004) = *Gyraulus riparius* (according to Meier-Brook, 1983); *Planorbella (Seminolina) duryi* (according to Burch, 1989) = *Helisoma (Seminolina) duryi normale* (according to Baker, 1945). *Segmentina oelandica* (according to Kruglov & Soldatenko, 1997; Starobogatov *et al.*, 2004) is often not accorded full specific status and is synonymized with *S. nitida* (Müller, 1774) (e.g. Glöer, 2002).

Behavioural study

Mature snails of each species were placed in groups of 20 individuals in 3-cm deep containers (*B. contortus* and *Ch. riparius*) or in groups of 10 individuals in containers up to 7 cm deep [*A. vortex*, *P. planorbis* and *P. (S.) duryi*]. Some observations of *Ch. riparius* and *S. oelandica* were conducted in Petri dishes, because this setting is more suitable for photography. In Petri dishes the water depth is shallow (<1 cm) and so it is difficult for a snail to assume the initial mating position; the behaviour of mating snails, however, remains essentially the same as in

the larger containers. Mating was induced by an increase in water temperature (Duncan, 1975; Smith, 1981); water was heated with incandescent lamps up to 20–28°C (except for *B. contortus*, which copulates at temperatures not higher than 20°C). The copulating pairs were photographed and time intervals for the phases of the mating process were recorded. For each species the observations were repeated, for at least three to four groups per season.

Some individuals were marked with coloured nail varnish for identification. Several times during the mating season the experimental groups were replenished by freshly collected individuals to sustain mating activity, which starts to decrease after several days in captivity.

A stopwatch was used to time different phases of the mating process; however, the time frames of the phases in some species (e.g. the beginning of precopulation in *A. vortex* or intromission in *B. contortus*) were impossible to determine due to indistinct behaviour patterns (lack of stereotypic precopulatory behaviour) or difficulty of direct observation (copulatory organs are obscured by other structures). In such cases, time intervals were determined only approximately and the duration of insemination was not measured. The time intervals for mating and courtship rejection are summarized in Table 1.

Morphological study

Three to five individuals of each species 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, retaining their original proportions.

For confocal microscopy, two specimens of each species [except *P. (S.) duryi*] were fixed for 1 h in 4% formaldehyde buffered with 0.1 M phosphate-buffered saline (PBS) at room temperature, rinsed in 0.1 M PBS (3 × 15 min), permeabilized for 1 h in PBS containing 0.2% Triton X-100, rinsed again shortly in 0.1 M PBS and then transferred to phalloidin-TRITC (Sigma-Aldrich) for 1 h. After that, the specimens were washed again for 3 × 15 min in 0.1 M PBS, mounted in 80% glycerol on glass slides and viewed with a Leica TCS SP5 microscope.

Terminology

This paper uses the conventional terminology for gender roles (e.g. Smith, 1981; Jordaens *et al.*, 2009). If mating is unilateral, we refer to the individual donating the sperm as 'male' and the individual receiving the sperm as 'female.' If mating is reciprocal, the individual that exhibits a more active mating behaviour and initiates mating is referred to as the 'active partner' and the other individual as the 'passive partner'.

Mating is a complex process which we divide into three consecutive phases (precopulation, copulation and postcopulation) following the terminology of Trigwell *et al.* (1997). Precopulation (courtship) is a set of stereotypic behaviour patterns that immediately precede copulation. In contrast to Trigwell *et al.* (1997), eversion and subsequent movements of accessory structures of the copulatory apparatus (preputium and preputial organ) prior to their attachment are also considered here to be part of the precopulatory phase. Courtship rejection is defined as single or multiple consecutive mating attempts by a suitor that do not lead to intromission due to rejective behaviour of the courted individual. Copulation commences with the penetration of the female genital pore by the penis [e.g. in *P. (S.) duryi*] or everted preputium (e.g. in *A. vortex*), with or without sperm transfer. Insemination is the introduction of sperm into the female genital tract during copulation (which is indicated by swelling and reddening of

Table 1. Durations [mean \pm SD (minimum – maximum), in minutes] for mating and courtship rejection in Planorbidae species.

Species	T (°C)	Courtship rejection			Mating			Copulation	Postcopulation	Mating period
		n	Single mating attempt	n	Total duration	n	Precopulation			
<i>B. contortus</i>	16–20	—	—	—	—	6	12.35 \pm 2.25* (10.00–16.30)*	32.30 \pm 6.30* (25.00–40.00)*	12.35 \pm 4.19 (5.00–17.00)	57.40 \pm 11.03* (41.00–70.00)*
<i>P. planorbis</i>	20–25	—	—	—	—	48	6.57 \pm 2.14 (3.10–15.00)	66.09 \pm 17.27 (40.00–90.00)	0.55 \pm 0.23 (0.10–1.28)	74.01 \pm 17.55 (48.43–103.18)
<i>A. vortex</i>	21–25	—	—	—	—	14	08.49 \pm 3.00* (3.00–14.00)*	66.43 \pm 19.51 (40.00–102.00)	3.06 \pm 1.16 (1.30–6.00)	78.38 \pm 18.39* (50.00–113.00)*
<i>Ch. riparius</i>	23–28	13	2.41 \pm 1.02 (1.38–5.00)	9	4.20 \pm 2.11 (2.56–10.02)	28	5.20 \pm 2.53 (2.32–15.48)	30.45 \pm 7.00 (15.00–42.03)	0.08 \pm 0.02 (0.05–0.12)	36.13 \pm 8.33 (20.00–54.00)
<i>S. oelandica</i>	25–28	21	0.54 \pm 0.16 (0.26–1.20)	27	8.14 \pm 6.19 (2.41–25.00)	43	0.46 \pm 0.15 (0.30–1.20)	1.50 \pm 0.30 (1.03–2.50)	0.11 \pm 0.11 (0.07–0.15)	2.47 \pm 0.37 (1.44–3.50)
<i>P. (S.) duryi</i>	21–24	—	—	—	—	19	10.49 \pm 4.40* (3.00–24.00)*	75.34 \pm 15.12 (45.00–99.06)	3.08 \pm 1.11 (1.46–6.00)	89.31 \pm 16.30* (63.00–120.00)*

Abbreviations: n, number of observed mating encounters; T (°C), temperature range during observations; asterisks indicate durations that were only approximately measured.

the intromittent structures and sometimes accompanied by spasmodic contractions of the copulatory apparatus or even of the whole body). Postcopulation is a set of stereotyped behaviour patterns that follow copulation. The beginning of this phase is marked by penis withdrawal from the vaginal duct [e.g. in *P. (S.) duryi*] or by detachment of the preputium (e.g. in *A. vortex*) and the end is defined by the departure of the male from the female for a distance of more than 1 cm (*Ch. riparius* and *S. oelandica*) or by a complete retraction (inversion) of the penial complex in the female [*A. vortex*, *P. planorbis*, *P. (S.) duryi* and *B. contortus*]. The period from the beginning of precopulation to the end of postcopulation is designated as the mating period.

As the shell of the Planorbidae is essentially discoidal (planispiral), the identification of shell coiling can sometimes be problematic (Baker, 1945; Brown, 1994). All planorbids are anatomically sinistral, with the openings of the reproductive organs on the left side of the body. Planorbid shells are usually described as embryonically sinistral; in adults, they may remain sinistral (orthostrophic) or flip over and reverse the direction of coiling (hyperstrophic) to appear dextral (pseudodextral). In other species, however, the shells are orthostrophically dextral (Checa & Jiménez-Jiménez, 1997). To avoid confusion related to the identification of shell coiling, we use the terms ‘upper’ and ‘basal’ sides of the shell (rather than apical and umbilical sides), referring to the natural way the snails carry their shells during locomotion.

RESULTS

Mating behaviour of Bathyomphalus contortus

When two prospective partners meet, they stop feeding and start crawling over each other’s shells, touching with their tentacles. The snails do not display any distinct precopulatory behaviour. A pair is usually formed on a horizontal surface (plant leaves or the bottom of the container) (Fig. 1A). The active individual approaches the prospective mate from the front and its foot adheres to the outer (palatal) side of the partner’s body whorl, near the aperture. At the same time the active individual everts the preputium, which penetrates into the space under the partner’s shell and attaches it to the vaginal opening. The shells of both partners are orientated with their upper sides away from the substrate (Fig. 1A). Once the active individual is in position for mating, the other partner repeats its manoeuvres: it first grasps the partner’s shell with its foot, near the outer edge of the aperture, and then attaches the copulatory organ to the vaginal opening of the active individual. The partners remain motionless for the duration of the copulatory phase, except for waving of tentacles and pulsation of the copulatory organs. The movements of the penis sheath and penis are impossible to observe directly due to certain aspects of morphology and mating behaviour of *B. contortus* (shells are rarely translucent, shell aperture is very narrow, the vaginal opening is located deep under the shell and the preputium is firmly attached to the vagina). The active individual stops copulating first, retracts the copulatory organ and removes the foot from the partner’s shell, but does not perform any active movements for the next 3–5 min, until the passive partner stops copulating. The passive individual typically withdraws the preputium only when the pair has separated. Despite the high overall activity of these snails in the laboratory, copulation was observed only for six pairs throughout the period of the study and only when the optimal conditions for mating were determined (Table 1). The mating of *B. contortus* occurs in nature in spring and early summer, when the water has not yet fully warmed. In the laboratory, copulation never occurs if the water temperature exceeds 20°C.

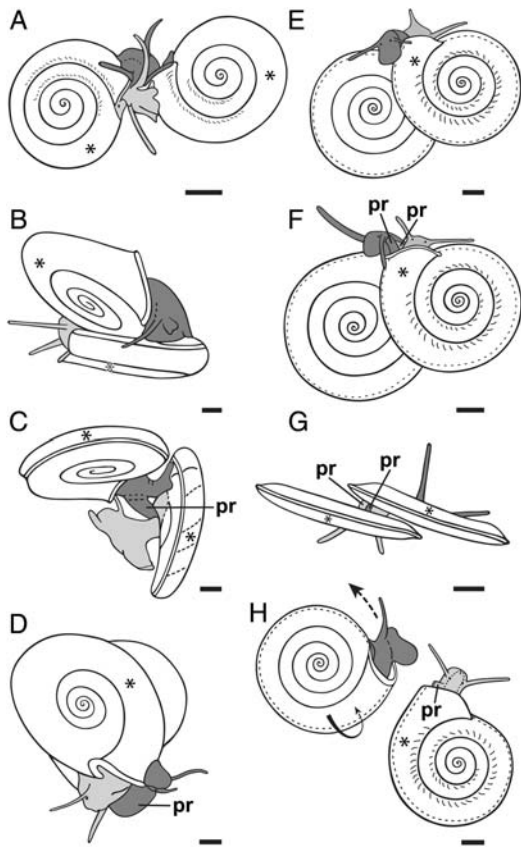


Figure 1. Schematic drawings from photographs showing mating snails. **A.** *Bathymphalus contortus*. The snails are facing away from the substrate. **B–D.** *Planorbis planorbis*. Various positions during intromission. **E–H.** *Anisus vortex*. The active individuals (*A. vortex*, *B. contortus*) and the snails mating in the male role (*P. planorbis*) are shown in dark grey. The solid arrow indicates the direction of shell rotation in the active individual. The dashed arrow shows the direction of movement of the active individual. Asterisks indicate the upper sides of the shells. See text for further details. Abbreviation: pr, preputium. Scale bars: **A–H** = 1.0 mm.

Normally, copulation can be induced when the snails are maintained for several days at low temperature (10–12°C) and then transferred to higher temperature (18–20°C).

Mating behaviour of *Planorbis planorbis*

During the precopulatory phase, *P. planorbis* show distinct stereotyped behaviour. The potential male finds a partner, crawls upon its shell and circles several times along the circumference of its upper side. The male then moves over to the basal side and crawls almost a full circle around the body whorl towards the shell aperture, at the same time slowly everting the preputium and pivoting the shell in such a way that its basal side becomes orientated towards the basal side of the female's shell (Fig. 1B). At this point, the female stops feeding and crawling, and attaches its foot to the male's shell or to the side of the container. When the female's foot is firmly fixed it extends the body, exposing the female gonopore under the shell. Once the female gonopore is fully exposed, the male attaches the preputium to the partner's vaginal opening and the intromission occurs (Figs 1C, 2A). During copulation, the pair is either attached to the substrate or floats on the water surface, with their shells tightly pressed together (Figs 1D, 2B). The male finishes insemination with spasmodic body

contractions and then retracts the preputium and dismounts the female. We observed only unilateral copulations. Successive copulations with sex-role reversal (nonsimultaneous reciprocal copulation, recognized by Kruglov & Berezkina (1978) as a distinct type of copulation in pulmonates) were not observed.

Mating behaviour of *Anisus vortex*

Although these snails do not display any distinct precopulatory behaviour, both prospective mates stop feeding when they meet. When the active individual approaches and attaches to the prospective mate, the latter may float upside down at the surface, hover in the water column or crawl over aquatic vegetation. Mating occurs at the water surface, between clumps of water plants or in detritus, but the passive partner almost never clasps the surrounding objects with its foot. The active individual grips the outer edge of the partner's shell aperture with its foot and everts the preputium. The basal sides of the partners' shells are always facing each other, regardless of the orientation and position of the pair in the water column (Fig. 1E–G). The passive individual repeats its partner's manoeuvres and, after mutual intromissions, the snails usually lose their foothold and remain connected only by their copulatory organs (Fig. 1F). The shell of *A. vortex* is almost always translucent, the aperture is wide and, therefore, the attachment of the copulatory apparatus to the vaginal opening is easily observable. The everted preputia are initially white, but during copulation they become reddish, which indicates the inflow of haemolymph to the copulatory organs and their readiness for sperm exchange. The vaginal penetration is shallow and, if the partners are disturbed by mechanical agitation or irritating substances, the pair readily breaks apart. There are no vigorous contractions of the body or copulatory organs during intromission. The active individual stops copulating first, retracts the copulatory organ and removes the foot from the partner's shell. At this point, the active partner is usually orientated upside down, with its shell facing downwards, while the other partner keeps holding it with its foot. The active individual remains motionless for several minutes, during which time the passive individual finishes intromission and then detaches its foot from the partner's shell and removes the preputium from the vaginal opening. Once the preputium is removed, the active individual flips over by turning its foot downwards and then pivoting the shell in the same direction, and leaves the partner (Fig. 1H).

Mating behaviour of *Choanomphalus riparius*

The precopulatory behaviour of the prospective male partner (the suitor) follows a distinct stereotyped pattern. The suitor stops feeding and finds a potential mate (usually a larger individual). The courted snail continues moving and feeding and seems to pay little attention to the suitor. The male approaches the prospective mate (the female) from behind and affixes its foot to the partner's shell, on the lateral side opposite to the aperture (Fig. 3A). The male then raises its shell, moves over to the basal side of the partner's shell (hanging between the shell and the substrate) and turns around so that its head is facing the partner's aperture (Fig. 3B). From this position the male crawls in a semicircle along the circumference of the body whorl on the basal side, heading towards the aperture, and eventually assumes a position near the aperture, to the left of the female's head-foot region (the basal side of the male's shell is orientated upwards) (Fig. 3C). When the male reaches the female's aperture, it affixes itself to the basal lip of the aperture and everts the preputium, attaching it to the female gonopore of the partner (Fig. 3D, E). The shells of *Ch. riparius* are often translucent and the aperture is very wide, but the

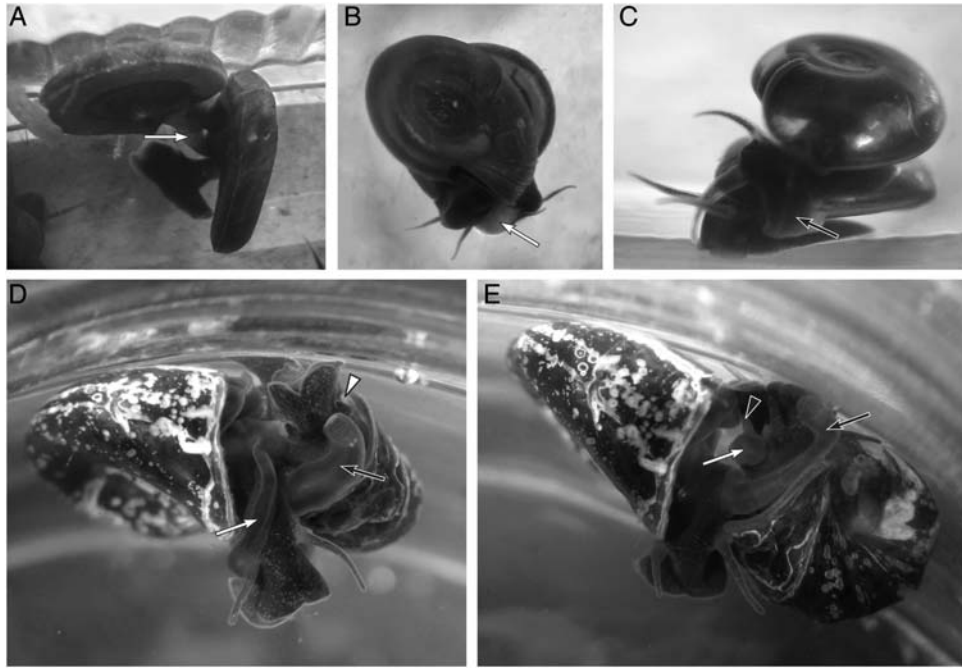


Figure 2. Photographs of copulating snails. **A, B.** *Planorbis planorbis*. Male's preputium (white arrow) is everted and attached to the female's vaginal opening. **C.** *Segmentina oelandica*. Male's preputial organ (black arrow) is everted and attached to the female's shell. **D.** *Planorbella (Seminolina) duryi*. The passive individual makes probing motions with the preputial organ (black arrow) over the partner's head. The preputial organ of the active individual (white arrow) is attached to the partner's plica. White arrowhead indicates the right plica in the active individual. **E.** *Planorbella (Seminolina) duryi*. The active individual removed the penis (black arrowhead) from the partner's vaginal opening and partially withdrew the preputial organ (white arrow). Black arrow indicates the preputial organ in the passive individual.

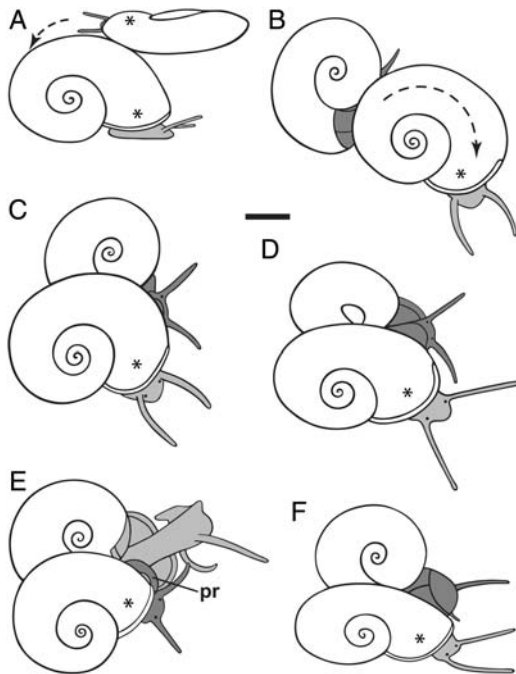


Figure 3. *Choanomphalus riparius*. **A–F.** Schematic drawings from photographs showing successive phases of the mating process (**D, E** are different views of the same position). The individual mating in the male role is shown in dark grey. Arrows indicate the movements of the snails during the precopulatory phase. Asterisks show the upper sides of the shells. See text for further details. Abbreviation: pr, preputium. Scale bars: **A–F** = 1.0 mm.

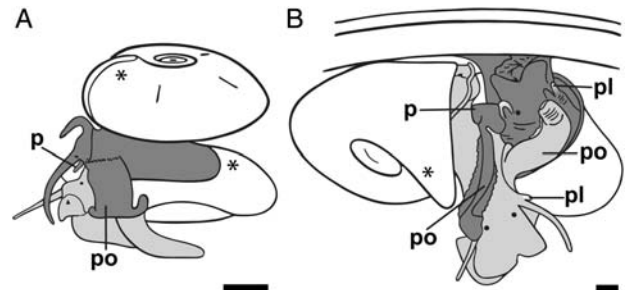


Figure 4. Schematic drawings from photographs showing copulating snails. **A.** *Segmentina oelandica*. **B.** *Planorbella (Seminolina) duryi*. The active individual (*P. duryi*) and the snail mating in the male role (*S. oelandica*) are shown in dark grey. Asterisks indicate the upper sides of the shells. Abbreviations: p, penis; pl, plica; po, everted preputial organ. See text for further details. Scale bars: **A, B** = 1.0 mm.

attachment of the copulatory organs is visible only if the pair is forced to turn upside down or if the female is mechanically agitated, which makes the pair detach from the substrate and rise to the water surface (Fig. 3E). During copulation, there are no spasmodic contractions of the body or copulatory organs; the male moves sporadically over the female's body, while the female continues feeding and crawling. Once the insemination is completed, the male retracts the copulatory organ and removes the foot from the female's shell. Since the male is now orientated upside down, it flips over by turning its foot downwards and then pivoting the shell in the same direction, and leaves the female (Fig. 3F).

Courtship rejection is common in the laboratory. When mounted by an unwanted suitor, the nonreceptive individual

does not exhibit any overtly aggressive behaviour; it stops feeding, slows down, retracts the body into the shell and then remains motionless, signifying to the suitor that it has no intention of mating. The suitors do not seem to be persistent in pursuing nonreceptive individuals and abandon them after a single or a few (up to three) courtship attempts.

Mating behaviour of Segmentina oelandica

Copulation in this species has been described in detail elsewhere (Soldatenko & Petrov, 2009b). Here, we give only a brief description and provide some additional quantitative information (Table 1). *Segmentina oelandica* copulate unilaterally (Fig. 4A). The behaviour of the male partner during shell

mounting is unlike that in all other species studied: the male mounts the upper side of the shell and crawls along the circumference of the body whorl towards the head. The preputial organ of the male is affixed to the shell and is not involved in sperm transfer (Figs 2C, 4A). Courtship rejection is common in laboratory; in this case, the suitors usually undertake several (up to 25) courtship attempts.

Mating behaviour of Planorbella (Seminolina) duryi

Mating is accompanied by the swelling of the plicae (bulge-like enlargements at the base of the tentacles, according to Yu & Emery, 1991): the plicae expand in size and change colour, becoming bright yellow. During courtship, the active individual

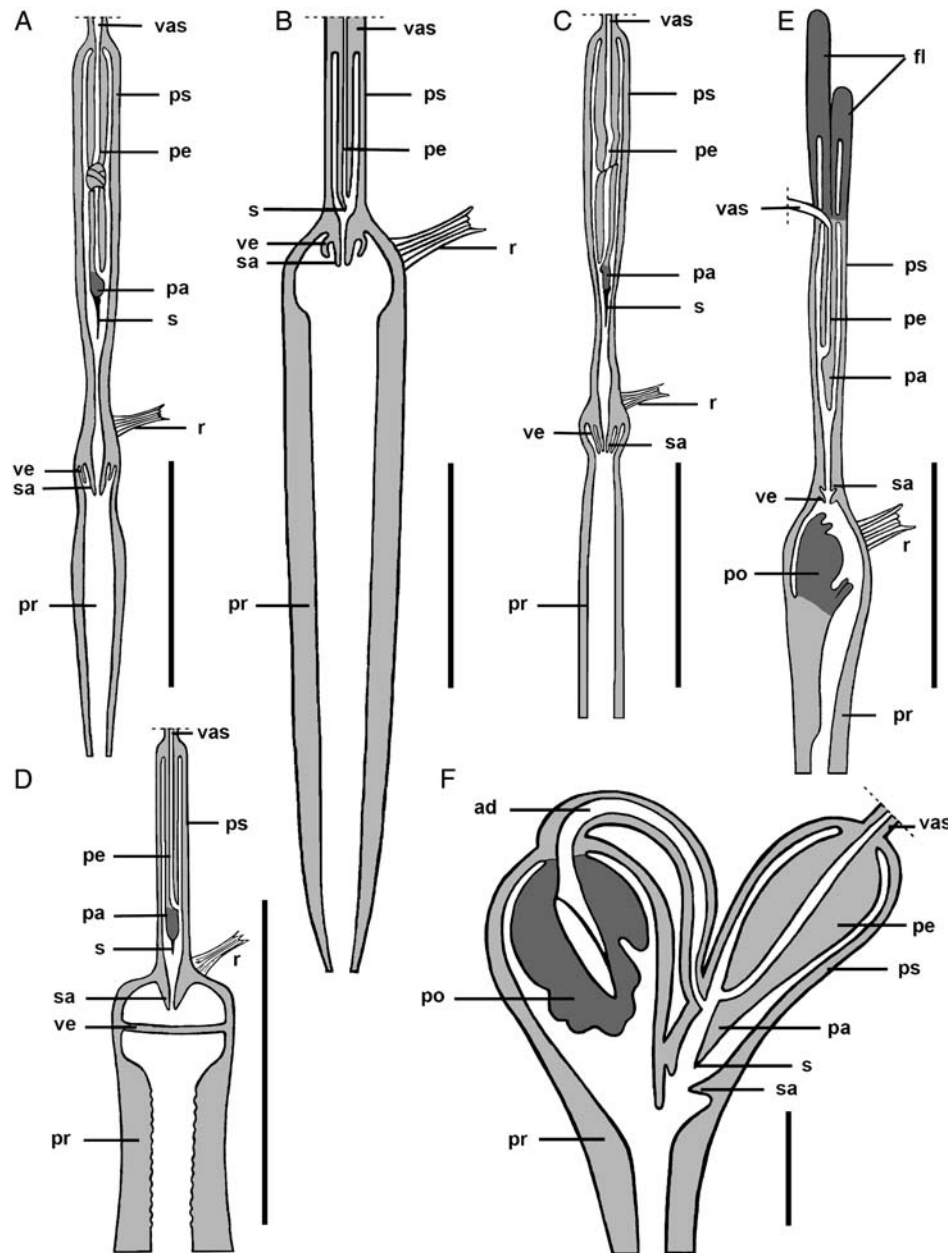


Figure 5. Schematic diagrams of copulatory apparatuses: **A.** *Bathyomphalus contortus*. **B.** *Planorbis planorbis*. **C.** *Anisus vortex*. **D.** *Choanomphalus riparius*. **E.** *Segmentina oelandica*. **F.** *Planorbella (Seminolina) duryi*. Dark grey represents areas of glandular epithelium. Abbreviations: ad, accessory duct; fl, flagellum; pa, papilla; pe, penis; po, preputial organ (gland); pr, preputium; ps, penis sheath; r, retractor muscle (omitted in **F** for clarity); s, stylet; sa, sarcobelum; vas, vas deferens; ve, velum. Scale bars: **A–C** = 1.0 mm; **D** = 0.5 mm; **E, F** = 1.0 mm.

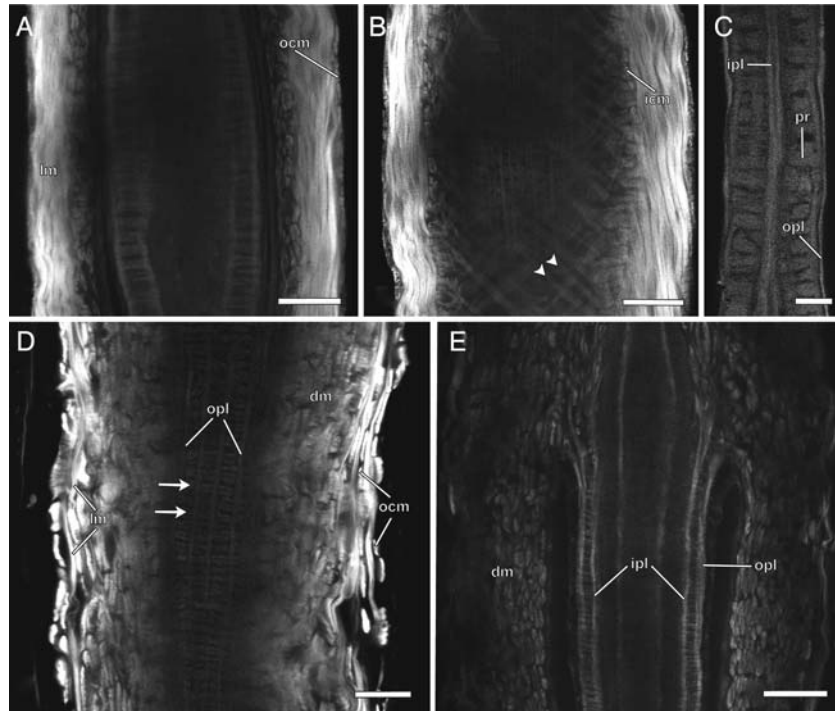


Figure 6. Musculature of the penial complex. Confocal longitudinal sections through phalloidin-stained preparations. **A.** Penis sheath and penis of *Bathymphalus contortus*. **B.** Penis sheath of *B. contortus*. Arrowheads indicate the diagonal muscles of the penis sheath. **C.** Penis of *B. contortus*. **D, E.** Penis sheath and penis of *Planorbis planorbis*. Arrows indicate circular penis muscles. Abbreviations: dm, diagonal penis sheath muscles; icm, inner circular penis sheath muscles; ipl, inner longitudinal penis muscles; lms, longitudinal penis sheath muscles; ocm, outer circular penis sheath muscles; opl, outer longitudinal penis muscles; pr, radial penis muscles. Scale bars: **A, B, D, E** = 20 μm ; **C** = 10 μm .

attaches the distal end of the preputial organ (also yellow in colour) to the plica under the partner's right tentacle, and the surfaces of the plica and preputial organ remain in close contact to the end of copulatory phase (Figs 2D, 4B). In some cases the active individual affixes the preputial organ to the partner's left plica or places it over the frontal side of the partner's head, between the tentacles, grasping its upper lip. The passive individual usually does not hold the partner too firmly and may frequently change the position of the preputial organ; its plicae are smaller and have a more pale coloration than those of the active partner (Fig. 2D, E). If the initial phases of mating were not observed, the size and colour of the plicae can indicate the mating roles of the copulating individuals. Copulation is always reciprocal and is not accompanied by shell mounting. During copulation, the shells are orientated with their basal sides towards each other.

Copulatory apparatus of *Bathymphalus contortus*

The copulatory apparatus of *B. contortus* (Fig. 5A) lacks specialized glandular structures; the attachment of the snails to their partners is effected by the thick muscular walls of the preputium which are furnished with two muscular ridges (pilasters). The wall of the penis sheath is relatively thin (Fig. 6A, B) and, from outside inwards, consists of one layer of thin circular muscles (Fig. 6A, ocm), six to seven layers of longitudinal muscles (Fig. 6A, lms), one layer of circular muscles (Fig. 6B, icm) and two criss-crossing layers of diagonal muscles (Fig. 6B, arrowheads). The penis is long and has a thick muscular wall composed of three layers: the outer (Fig. 6C, opl) and inner (Fig. 6C, ipl) layers of thin longitudinal muscles, and the intermediate layer of short radial muscles (Fig. 6C, pr). The penis is tipped with a tubiform stylet 160–175 μm long.

Copulatory apparatus of *Planorbis planorbis*

The copulatory apparatus lacks any specialized glandular structures and consists of a very short and narrow penis sheath enclosing the penis and a massive preputium with two pilasters (Fig. 5B). Attachment to the partner is effected by the preputium muscles. The wall of the penis sheath, from outside inwards, consists of one to three layers of circular muscles (Fig. 6D, ocm), one to two layers of longitudinal muscles (Fig. 6D, lms) and 14–16 layers of criss-crossing oblique (or sometimes circular) muscles (Fig. 6D, E, dm). The muscles of the outermost layer are often interwoven with subjacent longitudinal muscles. The penis is thin and long. The muscular wall of the penis consists of outer (Fig. 6D, E, opl) and inner (Fig. 6D, ipl) layers of longitudinal muscles and an intermediate layer of circular muscles (Fig. 6D, arrows). The penis tip is armed with a small trough-shaped stylet 5–7 μm long (Soldatenko & Petrov, 2009a).

Copulatory apparatus of *Anisus vortex*

The general morphology of the copulatory apparatus in *A. vortex* (Fig. 5C) is nearly identical to that of *B. contortus*. The penis sheath, from outside inwards, consists of a very thin layer of isolated circular muscles, six to seven layers of thin longitudinal muscles (Fig. 7A, olm), four to six layers of criss-crossing oblique (or sometimes circular) muscles (Fig. 7A, cm) and three to four layers of longitudinal muscles (Fig. 7A, ilm). The muscular wall of the penis consists of three layers: outer (Fig. 7B, arrows) and inner (Fig. 7A, ipl) layers of thin longitudinal muscles and an intermediate layer of strong radial muscles (Fig. 7A, B, pr). The penis is armed with a stylet identical to that of *B. contortus*.

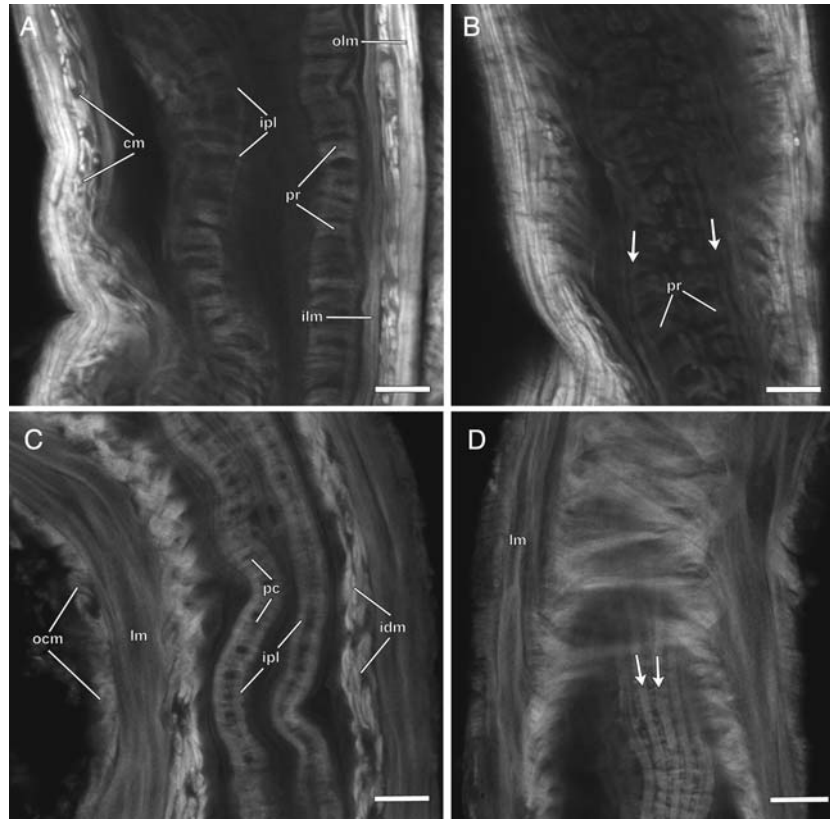


Figure 7. Musculature of the penial complex. Confocal longitudinal sections through phalloidin-stained preparations. **A, B.** Penis sheath and penis of *Anisus vortex*. **C, D.** Penis sheath and penis of *Choanomphalus riparius*. Arrows indicate longitudinal penis muscles. Abbreviations: cm, circular penis sheath muscles; idm, inner diagonal penis sheath muscles; ipl, inner longitudinal penis muscles; lm, longitudinal penis sheath muscles; olm, outer circular penis sheath muscles; ocm, outer circular penis sheath muscles; pc, circular penis muscles; pr, radial penis muscles. Scale bar: 10 μ m.

Copulatory apparatus of *Choanomphalus riparius*

The copulatory apparatus lacks any specialized glandular structures and consists of a narrow penis sheath enclosing a thin penis with a stylet, and of a thick-walled preputium with two pilasters (Fig. 5D). The muscular wall of the penis sheath (Fig. 7C, D), from outside inwards, is composed of one layer of wide circular muscles (Fig. 7C, ocm), four to six layers of longitudinal muscles (Fig. 7C, D, lm) and five layers of criss-crossing oblique muscles (Fig. 7C, idm). The longitudinal muscles diverge to form spaces (possibly lacunae of the vascular system) that contain actin-rich cells. The muscular wall of the penis comprises outer (Fig. 7D, arrows) and inner (Fig. 7C, ipl) layers of relatively thick longitudinal muscles and an intermediate layer of short and wide circular muscles (Fig. 7C, pc). The penis is tipped with a conical stylet 25 μ m long.

Copulatory apparatus of *Segmentina oelandica*

The proximal portion of the copulatory apparatus consists of the long penis sheath (Fig. 5E, ps) that encloses the penis and bears two glandular appendages (flagella, Fig. 5E, fl) of different length. The penis lacks a stylet and ends with a long, thin papilla (Fig. 5E, pa). The distal portion of the copulatory apparatus is composed of an elongated pyriform preputium (Fig. 5E, pr); the wall of the preputium forms a muscular preputial organ (Fig. 5E, po). The proximal enlarged part of the latter contains glandular tissue.

Copulatory apparatus of *Planorbella (Seminolina) duryi*

The copulatory apparatus (Fig. 5F) lacks glandular appendages. The penis sheath is thin-walled and encloses a massive penis provided with a small conical stylet (Fig. 5F, s). The preputium is somewhat larger than the penis sheath and in its proximal portion bears a cup-shaped preputial organ (Fig. 5F, po). The distal end of the penis sheath and preputium are connected by an external accessory duct (Fig. 5F, ad). A small sarcobelum is formed between the penis sheath and preputium (Fig. 5F, sa).

DISCUSSION

The results of this study (summarized in Table 2) clearly indicate that mating behaviour in planorbids is more diverse than generally recognized. Mating was shown to follow two distinct behavioural patterns: *Ch. riparius*, *P. planorbis* and *S. oelandica* mate unilaterally by shell mounting, whereas *B. contortus*, *P. (S.) duryi* and *A. vortex* mate reciprocally in a face-to-face position. The species mating by shell mounting show complex stereotyped courtship, during which the male crawls along the circumference of the female's shell (shell-circling); in the reciprocally mating species the precopulatory phase is less distinct and is not accompanied by any apparent stereotypical behaviour. None of the six species was observed to mate both unilaterally and reciprocally, but such a possibility cannot be ruled out entirely. Three planorbid species, *Biomphalaria glabrata* (Brumst, 1941; Vernon & Taylor, 1996; Trigwell *et al.*, 1997;

Table 2. Summary of mating behaviour traits and associated morphological characters.

Species	Characters							
	1	2	3	4	5	6	7	8
<i>B. contortus</i>	S	P	FF	R	–	+	–	SH
<i>P. planorbis</i>	S	BB	SM/FF	U/R	–	+	–	FP
<i>A. vortex</i>	S	BB	FF	R	–	+	–	SH
<i>Ch. riparius</i>	NS	BB	SM	U	–	+	–	FP
<i>S. oelandica</i>	NS	M	SM	U	fl + po	–	*	FP
<i>P. (S.) duryi</i>	S	BB	FF/SM	R/U	po	+	*	?

Characters 2, 3 and 4 are based on Jordaens *et al.* (2009). Characters: (1) female copulatory activity: S, stops feeding and crawling; NS, continues feeding and crawling. (2) shell orientation: M, mounted, i.e. the basal side of the upper shell is over the upper side of the lower shell; P, planar, i.e. both shells lie on the substrate, with their upper sides away from the substrate; BB, basal-to-basal, i.e. shells are oriented with their basal sides towards each other. (3) Mating position: FF, face-to-face; SM, shell mounting. (4) Penis usage during copulation: R, reciprocal; U, unilateral. (5) Morphology of the penial complex: fl, flagellum; po, preputial organ. (6) Stylet: +, present; –, absent. (7) Copulatory stimulation: *, copulation is accompanied by mechanical or chemical stimulation; –, stimulation not observed. (8) Gender expression: SH, simultaneous hermaphrodites; FP, functional protandry.

Vianey-Liaud, 1997), *B. tenographila* (Springer de Freitas, Pires Paula & Cariello, 1997) and *Planorbella (Pierosoma) trivolvis* (Abdel-Malek, 1952), have previously been reported to have both unilateral and simultaneously reciprocal mating. In *B. glabrata* reciprocal copulation (in a face-to-face position) is exceedingly rare and occurs only in c. 5% of observed copulations (Trigwell *et al.*, 1997). It is similarly likely that in some of the species discussed in this paper one of the two mating strategies may occur only under certain conditions or in very rare cases and, therefore, was not observed in our experiments. Although we observed only simultaneously reciprocal mating in *P. (S.) duryi*, the occurrence of both types of mating behaviour in closely related *P. (P.) trivolvis* (Abdel-Malek, 1952) suggests that *P. (S.) duryi* may also occasionally copulate unilaterally. Similarly, we observed only unilateral copulation in *P. planorbis*, although according to Precht (1936) these snails mate by simultaneous reciprocal copulation. Unfortunately, Precht does not give any details about mating behaviour in this species, but it stands to reason that in this case partners should mate face-to-face, which is the only anatomically possible position for reciprocal simultaneous copulation. It should also be noted that the position that mating snails assume during unilateral intromission (Figs 1D, 2B) is actually similar to that during face-to-face copulation and that the transition from this position to the reciprocal mating can be easily achieved.

The reciprocally copulating species show two forms of mating behaviour. *Bathyomphalus contortus* and *A. vortex* are essentially similar both in mating patterns and morphology, which may indicate a close phylogenetic affinity between these species (see also Baker, 1945). Both species mate reciprocally; prospective partners stop crawling and feeding simultaneously and begin to copulate in a face-to-face position (Fig. 1A, E–G). Mating is not accompanied by mechanical stimulation of the partner. Copulation is effected by the massive musculature of the eversible preputium; the preputium generates a substantial compression force against well-developed muscular folds (velum and sarcobelum) and against the penis sheath and penis. A strong longitudinal musculature of the penis sheath may prevent penis from being over-

compressed (constricted) during intromission. It seems likely, from morphological examination of the copulatory organs (Fig. 5A, C), that only a short muscular cone formed by the velum and sarcobelum is inserted into the partner's vagina. The penis is greatly extended during copulation by contraction of the radial musculature. The sperm is ejected through a long stylet that might protect the penis tip from being constricted. It is of interest that, despite these similarities, *B. contortus* and *A. vortex* have different temperature optima for mating. The individuals of *B. contortus* spawn in May through June, when water temperature is below 20°C. The remaining species (including *A. vortex*) reach peak reproductive activity in midsummer and in the laboratory prefer to mate at 22–28°C. These differences, however, do not seem to affect mating behaviour, although the mean duration of the copulatory phase is about twice as long in *A. vortex* as in *B. contortus* (Table 1).

Mating behaviour in the third reciprocally copulating species, *P. (S.) duryi*, is quite different from that in the other five species and shows a peculiar mechanism of attachment to the partner accompanied by mechanical stimulation of its plicae. A general description of copulation in *P. (S.) duryi* was given by Pace (1971) and our data supplement his observations. Mating behaviour in *P. (S.) duryi* is very similar to that of a closely related species, *Planorbella (Pierosoma) trivolvis* (Abdel-Malek, 1952; Pace, 1971; Norton *et al.*, 2008; as *Helisoma trivolvis*) and possibly also to those of the other members of the tribe *Helisomini* (*sensu* Burch, 1989). It has been shown that during mating four species of this tribe, including *P. (P.) trivolvis* and *P. (S.) duryi*, use the preputial organ as a holdfast (Pace, 1971); this organ attaches either to the dorsal or to the right side of the partner's head-foot region, or occasionally to the inside of the left lip of the partner's shell. According to our observations, the preputial organ in *P. (S.) duryi* is also sometimes placed between the tentacles grasping the upper lip of the partner. In most cases, however, the preputial organ of *P. (S.) duryi* attaches to the plica under the right (or sometimes left) tentacle of the partner, apparently functioning both as a holdfast and an excitatory organ. This type of mechanical stimulation was first suggested for the subfamily Helisomatinae by Baker (1945). Pace (1971) does not mention that the preputial organ may stimulate the plicae but, judging from the position of the preputial organ in his drawings of *P. trivolvis* (Pace, 1971: 22), this type of copulatory stimulation is also possible in this species.

In addition to the differences in morphology and copulatory mechanics, the species are clearly distinguished by the duration of the mating phases. Copulation in the Planorbidae is thought to be a relatively long process, taking between 30 min and 5.5 h (Precht, 1936; Abdel-Malek, 1952). Indeed, our study confirmed that mating lasts 1–2 h in four of the species studied [*B. contortus*, *P. planorbis*, *A. vortex* and *P. (S.) duryi*] (Table 1). In *S. oelandica*, however, the mating period is much shorter (no longer than 3.5 min, with all the mating phases present). In fact, this is the first case in the Basommatophora of such a fast sperm transfer between partners. These differences suggest that *S. oelandica* might be phylogenetically distant from the other species, which is corroborated by molecular phylogenetic data (Albrecht, Kuhn & Streit, 2007). Time intervals in *Ch. riparius* are more or less intermediate, but still much closer to the species with a long copulation (Table 1).

Our data suggest a possible correlation in the six species studied between gender expression (simultaneous hermaphroditism *vs* protandry) and mating behaviour (reciprocity *vs* unilaterality of copulation) (Table 2). Planorbidae are generally considered true simultaneous hermaphrodites, but there is now evidence that some species may exhibit functional protandry, i.e. spermatogenesis in these species precedes oogenesis and so sperm differentiate before oocytes (Berezkina & Starobogatov,

1988; Trigwell & Dussart, 1998). Three of the six species discussed in this paper (*Ch. riparius*, *S. oelandica* and *P. planorbis*) show juvenile functional protandry (Berezkina & Starobogatov, 1988). This type of protandry is found only in young individuals at the beginning of the reproductive period; individuals first function as males, but after a relatively brief period (20 days in *P. planorbis*, 3–4 weeks in *S. oelandica*) start functioning as females, thereby becoming simultaneous hermaphrodites (Berezkina & Starobogatov, 1988; Soldatenko & Petrov, 2009b; E. Soldatenko, unpubl.). It is not clear whether or not functional protandry is also present in *P. (S.) duryi*. The remaining two species (*B. contortus* and *A. vortex*) appear to be true simultaneous hermaphrodites (Hubendick, 1978), although additional histological evidence may be needed to identify the form of gender expression in these species with more certainty. It can therefore be seen that protandrous species copulate unilaterally, whereas simultaneously hermaphroditic species have reciprocal mating. Among other species of planorbids, functional protandry has been described for *Biomphalaria glabrata* (Trigwell & Dussart, 1998); this species has also been shown to mate predominantly unilaterally, with reciprocal mating as a rare exception (Trigwell *et al.*, 1997).

Overall, our knowledge about prevalence of protandry in the Basommatophora is too limited to make broad generalizations, but the comparison with the possible outgroups of the Planorbidae indicates that the most primitive planorbids were probably protandrous species with unilateral mating. Functional protandry is not uncommon in outgroups such as Ancyliidae, Physidae, Bulinidae and Lymnaeidae (Duncan, 1959, 1975; Russell-Hunter & McMahon, 1976; Rudolph, 1983; Wethington & Dillon, 1993) and unilateral copulation is the only known mating pattern in all basommatophorans except the Planorbidae (Jordaens *et al.*, 2009). It is therefore likely that simultaneously reciprocal mating has emerged within the Planorbidae and may have led to a simplification of courtship behaviour (loss of shell-circling). Unilateral mating followed by an immediate reciprocation, as observed in some Lymnaeidae and Physidae (e.g. Rudolph, 1979a; Koene & Ter Maat, 2005; Facon *et al.*, 2008), may be viewed as an intermediate step between strictly unilateral mating and simultaneously reciprocal copulation. Another derived feature that is likely to have a recent origin within the Planorbidae is the behaviour patterns accompanied by partner's stimulation during mating: in *P. (S.) duryi* mating snails are mechanically stimulated by the preputial organ, whereas in *S. oelandica* mating is accompanied by chemical stimulation from secretion released by the tentacular glands (Soldatenko & Petrov, 2009b). The tentacular secretion is discharged during the precopulatory phase and becomes clearly visible only if the individual that initiates mating is rejected several times by a potential mate (Soldatenko & Petrov, 2009b). Apparently a small amount of secretion is released at the beginning of each mating attempt, which suggests that it may serve for pheromonal or hormonal manipulation or may be presented as a form of nuptial gift.

A further distinction between unilaterally and reciprocally mating species is observed in size-assortative mating and in the prevalence of rejective behaviour (courtship rejection). In the unilaterally mating protandrous species, *Ch. riparius* and *S. oelandica*, the male usually selects a partner with a larger shell size. Size-assortative mating occurs in various groups of animals, both gonochorists (reviewed in Crespi, 1989) and hermaphrodites (Otsuka, Rouger & Tobach, 1980; Vreys & Michiels, 1997; Lüscher & Wedekind, 2002; Monroy *et al.*, 2005), but its mechanism and causes are still not fully understood. A commonly suggested explanation is that the individuals that initiate mating favour large partners, because fecundity is positively related to size. In protandrous snails, size-assortative behaviour may have evolved as a method to

ensure unilateral mating: the best strategy for a protandrous male is to choose a larger partner, because large size may signify that this individual has reached the hermaphroditic phase and can copulate in the female role. It is possible that complex stereotyped courtship is related to unilateral mating and size-assortative behaviour, and that one of the functions of shell-circling is to help the male actor compare its size with that of the partner, as has previously been suggested for Succineidae (Örstan, 2010).

Rejection behaviour was observed in two protandrous species (*Ch. riparius* and *S. oelandica*), but not in the simultaneous hermaphrodites, which can be explained by more complex gender relations (sexual conflict) in the snails with exclusively unilateral copulation. In *S. oelandica* potential suitors are usually quite persistent in pursuing nonreceptive individuals (up to 25 consecutive mating attempts) and courtship rejection is often accompanied by aggressive behaviour (biting), while in *Ch. riparius* the suitors are less persistent and potential sperm recipients show only a passive rejection.

Muscular architecture of the copulatory organs, as revealed by phalloidin/phalloidin staining and confocal microscopy, has successfully been used in the taxonomy of several invertebrate groups (e.g. Hooge & Tyler, 2005; Marotta *et al.*, 2009). The potential taxonomic importance of penial musculature in the Planorbidae is evident from the comparison of the arrangement of penis muscles among five planorbid species (this paper and Soldatenko & Petrov, 2009b for *S. oelandica*). There are two distinct patterns of penial musculature: in *P. planorbis* and *Ch. riparius* it consists of outer and inner longitudinal layers and an intermediate circular layer; in *B. contortus*, *A. vortex* and *S. oelandica* the intermediate layer is composed of radial muscles. Presumably, one of the two patterns is plesiomorphic for the Planorbidae, but the primitive state cannot be determined until more information is available on the arrangement of penial muscles both in planorbids and in the potential outgroups. In the five species studied, the architecture of penial muscles shows no apparent relationship with the characteristics of mating behaviour.

The results reported in this paper expand our understanding of the richness and diversity of mating patterns in freshwater pulmonates. It is now well established that planorbids display stereotyped sexual behaviours, and in four species [*B. glabrata*, *Ch. riparius*, *P. (S.) duryi* and *S. oelandica*] copulation is shown to be preceded by elaborate stereotyped courtship. Contrary to the assumption of Asami *et al.* (1998), this study shows that flat-spined snails can copulate both unilaterally and reciprocally (face-to-face), although unilateral (*Ch. riparius* and *S. oelandica*) or reciprocal mating [*B. contortus*, *A. vortex* and possibly *P. (S.) duryi*] appears to be the only possible modes of copulation in some species. Planorbids can copulate both by shell mounting and face-to-face, and *B. contortus* and *A. vortex* always copulate in a face-to-face position.

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