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Muscle system of the penial complex in three species of Hygrophila (Gastropoda: Pulmonata)

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ABSTRACT

The Hygrophila, a group comprising freshwater lung-bearing gastropods, have a complex copulatory apparatus, whose musculature has recently been shown to provide a number of phylogenetically informative characters that appear to concur with the results of molecular phylogenetic analyses. The number of hygrophilan species for which the male copulatory musculature is known, however, is still relatively small. We examined the muscle arrangement in the penial complex (penis and penis sheath) of three species of Hygrophila: *Aplexa hypnorum* (Linnaeus, 1758) (fam. Physidae), *Kolhymorbis bogatovi* Zatravkin et Moskvicheva, 1985 (fam. Planorbidae, tribe Segmentinini), and *Planorbarius corneus* (Linnaeus, 1758) (fam. Planorbidae, tribe Helisomatini) to explore whether the organization of the male copulatory musculature in these species is consistent with the previously proposed phylogenetic hypotheses. In *A. hypnorum*, the primarily glandular penis sheath has a three-layered musculature with outer and inner circular and intermediate longitudinal layers. The tubular penis also has a three-layered musculature, with the same sequence of muscle fibers as in the penis sheath. In *K. bogatovi*, the sequence of muscle layers in the penis sheath is essentially the same as in *A. hypnorum*. Penial musculature is also arranged in three layers, but the intermediate layer consists of small radial, rather than longitudinal fibers and the inner and outer layers are circular. In *P. corneus*, the boundaries between muscle layers in both penis and penis sheath are somewhat indistinct, but the circular muscles tend to lie close to their outer and inner walls, while the longitudinal fibers are concentrated in the interior. The comparison with other hygrophilan species, in which the copulatory musculature has been previously studied, shows that the male copulatory musculature of *K. bogatovi* is consistent in arrangement with that of other representatives of Segmentinini and that the phylogenetic lineage leading to *P. corneus* has probably diverged before the clade Planorbini + Segmentinini. The musculature of the penial complex of *A. hypnorum* is generally similar to that of another physid, *Physella acuta* (Draparnaud, 1805), but the penis sheath of *P. acuta* lacks an outer layer of circular muscle fibers, which may be explained by reduction.

Key words: confocal microscopy, freshwater pulmonates, muscles, penial complex, penis, penis sheath

Мышечная система пениального комплекса у трех видов Hygrophila (Gastropoda: Pulmonata)

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РЕЗЮМЕ

Пресноводные легочные моллюски (Hygrophila) имеют сложно организованный копулятивный аппарат, чья мускулатура, как было недавно показано, может быть источником целого ряда таксономически информативных признаков, хорошо согласующихся с данными молекулярной филогении, однако к настоящему времени эта мускулатура исследована лишь у относительно небольшого числа видов. Нами было проведено исследование мускулатуры пениального комплекса (пениса и мешка пениса) у трех видов Hygrophila: *Aplexa hypnorum* (Linnaeus, 1758) (сем. Physidae), *Kolhymorbis bogatovi* Zatravkin et Moskvicheva, 1985 (сем. Planorbidae, триба Segmentinini) и *Planorbarius corneus* (Linnaeus, 1758) (сем. Planorbidae, триба Helisomatini), для выяснения вопроса, насколько мышечная организация копулятивного аппарата у этих видов согласуется с предложенными ранее филогенетическими гипотезами. У *A. hypnorum* мешок пениса, состоящий преимущественно из железистой ткани, включает три мышечных слоя: наружный и внутренний кольцевые и промежуточный, состоящий из продольных волокон. Цилиндрический пенис этого вида также имеет трехслойную мускулатуру, с той же последовательностью слоев как и в мешке пениса. У *K. bogatovi* последовательность слоев в мешке пениса сходна с таковой у *A. hypnorum*. Пениальная мускулатура у этого вида также включает три слоя, но промежуточный слой состоит из тонких радиальных, а не продольных, волокон, и внутренний и наружный слои образованы кольцевыми волокнами. У *P. corneus* границы между мышечными слоями в пенисе и мешке пениса выражены плохо, но кольцевые волокна расположены преимущественно вблизи внутренней и наружной стенок, а продольные волокна занимают промежуточное положение. Сравнение с другими видами пресноводных легочных моллюсков, у которых известна организация мускулатуры копулятивного аппарата, показывает, что мышечная система пениального комплекса *K. bogatovi* имеет строение, характерное для представителей трибы Segmentinini, и что филогенетическая ветвь *P. corneus* вероятно обособилась раньше общей клады, включающей Planorbini и Segmentinini. Мускулатура пениального комплекса *A. hypnorum* в основном сходна с таковой у другого представителя физид, *Physella acuta* (Draparnaud, 1805), но в мешке пениса *P. acuta* отсутствует наружный слой кольцевых мышечных волокон, что может быть объяснено его редукцией.

Ключевые слова: конфокальная микроскопия, пресноводные легочные моллюски, мышцы, пениальный комплекс, пенис, мешок пениса

INTRODUCTION

Characters of the male copulatory organs in gastropods tend to be phylogenetically conserved and have been widely used in gastropod taxonomy, especially at the familial and generic levels (see, e.g., Taylor 2003; Golding et al. 2008; De Mattia et al. 2021). The characters traditionally used in taxonomy are almost exclusively gross anatomical traits of the copulatory apparatus such as the presence or absence of certain structures or the arrangement, shape and relative sizes of organs. The microanatomical traits, on the other hand, have played essentially no role in taxonomy, which is primarily due to our insufficient knowledge of gastropod copulatory organs at the microscopic level.

The microanatomy of the muscle system of the Hygrophila, a molluscan clade comprising freshwater lung-bearing snails, has recently been examined in different representatives of this group (Soldatenko and Petrov 2012, 2016, 2019) showing that the organization of the male copulatory musculature can characterize several major hygrophilan taxa and provide a set of familial and tribal-rank synapomorphies. The copulatory apparatus, or the penial complex, of the Hygrophila begins proximally at the opening of the vas deferens into the penis sheath. The penis sheath is a hollow muscular tube that houses the penis extending as an outgrowth from its proximal end. The penis sheath leads to the terminal part of the penial complex, called the preputium, which is an eversible muscular sleeve opening to the exterior through the genital pore. Microscopic examination of the hygrophilan penial complex has shown that the most promising source of taxonomically informative characters is provided by the musculature of the penis sheath and penis, which vary significantly across taxa (Soldatenko and Petrov 2019).

In the presumed plesiomorphic condition present in the families Acroloxidae (*Acroloxus lacustris* (Linnaeus, 1758)), Physidae (*Physella acuta* (Draparnaud, 1805)), and Lymnaeidae (*Radix auricularia* (Linnaeus, 1758)), the penial musculature includes the outer and inner circular layers and the intermediate

longitudinal layer. In the Ancylidae (*Ancylus fluviatilis* O. F. Müller, 1774) and *Biomphalaria glabrata* (Say, 1818) (fam. Planorbidae), the interior of the penis between the inner and outer circular muscle layers has a more complex muscle arrangement: it is crossed by radial muscle fibers and there are two widely separated longitudinal layers, rather than one. In other planorbids comprising the sister tribes Planorbini and Segmentinini, the penial musculature is essentially reversed in arrangement compared to other Hygrophila: the outer and inner layers are longitudinal in orientation, while the intermediate layer consists of radially oriented fibers. It has been suggested (Soldatenko and Petrov 2019) that this arrangement has evolved from that seen in *Biomphalaria*, in which the outer and inner circular layers have been lost, so that the two initially intermediate layers of longitudinal muscle fibers assumed the inner and outer positions.

The organization of the penis sheath muscles shows variation consistent with that of the penial musculature (Soldatenko and Petrov 2019). In the likely plesiomorphic arrangement found in the Acroloxidae, Lymnaeidae, Physidae, and *Planorbella duryi* (Wetherby, 1879) (fam. Planorbidae), the penial sheath musculature is two-layered consisting of outer longitudinal and inner circular layers. In Segmentinini and Planorbini, three distinct layers are present: the outer circular, the intermediate longitudinal and the inner composed of either circular or diagonal muscle fibers. Similar, although probably convergently evolved, three-layered musculature is also present in the Lymnaeidae (*R. auricularia*). In *B. glabrata*, the penis sheath musculature becomes even more complex consisting, from outside to inside, of a longitudinal, circular, another longitudinal, and diagonal layers.

The distribution of synapomorphies proposed for hygrophilan taxa based on the sequence of penial and penis sheath muscles is in good agreement with existing molecular phylogenetic evidence (Albrecht et al. 2007; Soldatenko and Petrov 2019; Saadi et al. 2020), but in most cases the muscle arrangement is known only for one or two species in each hygrophilan taxon of familial or tribal rank and it is still unclear whether this arrangement in each case characterizes the whole group or only some of its representatives. The aim of the present paper was therefore to examine the penial and penis sheath musculature in three other representatives of previously studied hygrophilan groups. This may provide a better understanding of the extent of variation in copulatory musculature that could be observed within hygrophilan families or tribes and verify the validity of the proposed synapomorphies.

MATERIAL AND METHODS

The musculature of the penial complex was studied in three species of Hygrophila: *Aplexa hypnorum* (Linnaeus, 1758), *Planorbarius corneus* (Linnaeus, 1758), and *Kolhymorbis bogatovi* Zatravkin et Moskvicheva, 1985. Localities and the number of specimens studied for each species are listed in Table 1. The mature individuals with the fully developed male reproductive system were fixed intact with 4% formaldehyde in 0.01 M phosphate-buffered saline (PBS) for 4–6 h at ambient temperature, then their shells were removed and their bodies dissected to remove the copulatory apparatuses. Most penial complexes were studied as whole mounts, but some of the large penial complexes of *P. corneus* were sliced transversely or longitudinally with a razor blade. The removed copulatory apparatuses were rinsed and kept in 0.01 M PBS containing 0.1% (w/v) sodium azide (Sigma-Aldrich, St. Louis, Missouri, USA) at 4–6˚C. For phalloidin staining, the copulatory apparatuses were permeabilized for 2 h with 0.25% Triton X-100 (Sigma-Aldrich, St. Louis, Missouri, USA) in 0.01 M PBS, stained for 24 h with Alexa Fluor 555-conjugated phalloidin (Life Technologies, Carlsbad, California, USA; diluted 1:150), washed in 0.01 M PBS and then mounted on slides with Vectashield (Vector Laboratories Inc., Burlingame, California, USA). Confocal images were acquired and photographed on a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems GmbH, Wetzlar, Germany) at the Taxon Research Resource Center (Zoological Institute RAS). All confocal images of phalloidin-stained preparations are presented as maximum intensity projections of the Z-stacks.

RESULTS

***Aplexa hypnorum*.** The penis sheath is hyaline and its walls are mostly glandular. The outer muscle layer of the penis sheath consists of loosely spaced, thin circular muscle fibers arranged in a single row (ocs, Fig. 1A, B). The circular muscle fibers make numerous anastomoses with one another to form a single meshwork of fibers. Beneath the circular muscles is a thick layer of glandular tissue, which also contains

Table 1. List of the species studied.

Species	Locality and collection date	Coordinates	Collector	NoS
Aplexa hypnorum (Linnaeus, 1758)	Russia, Smolensk Prov., Demidovskiy Distr., National Park "Smolenskoye Poozerye", near Przhevalskoye town, temporary ponds near "Chistik" Teaching Centre (Smolensk State University), August 6, 2020	55°30′ N, 31°47′ E	E.V. Soldatenko	5
	Pskov Prov., Bol'shaya Budnitsa village, an inundated depression on the left-hand side of the road at the village exit toward Smolensk, July 18, 2020	55°52′54.4″ N, 30°20′11.9″ E	E.V. Soldatenko	7
Kolhymorbis bogatovi Zatravkin et Moskvicheva in Zatravkin, 1985	Russia, Vladivostok, Russky Island, wetland near the mouth of the Russkaya River on the shore of Voyevoda Bay, August 25, 2018	43°00′08.0″ N, 131°48′17.5″ E	L.A. Prozorova	3
Planorbarius corneus (Linnaeus, 1758)	Russia, Smolensk Prov., Demidovskiy Distr., National Park "Smolenskoye Poozerye", near Przhevalskoye town, Lake Rytoye, July 2015	55°30′ N, 31°46′ E	E.V. Soldatenko	5
	Russia, Smolensk Prov., Smolensk, slow moving section of the Dubrovenka River, August 2020	54°47′ N, 31°56′ E	E.V. Soldatenko	4

Abbreviations: NoS – Number of individuals studied.

loosely spaced, anastomosing longitudinal muscle fibers (lps, Fig. 1A, B). Below the glandular cells is the inner (luminal) layer of circular muscle fibers (ics, Fig. 1B). The individual fibers in this layer are much broader than those in the outer layer and much denser spaced.

The tubular penis (Fig. 1C) is slightly longer than half the length of the penis sheath. Distally, the penis is distended into a circular swelling (bs, Fig. 1C), which then tapers to the penial apex to form a conical papilla-like tip that makes up more than one-third of the penis length (pt, Fig. 1C). The penial musculature is arranged in three layers. Both outer (ocp, Fig. 1C, D) and inner (icp, Fig. 1C, D) layers consist of a single row of densely spaced circular muscles. The third, intermediate, layer (lp, Fig. 1C, D) is composed of several rows of muscle fibers oriented primarily in the longitudinal or slightly diagonal direction. In nearly all cases, however, the proximal ends of these muscle fibers attach to the inner circular layer and their distal ends to the outer circular layer and these fibers are therefore topologically radial. At the enlarged base of the penis tip, both endings of longitudinal muscle fibers anchor to the outer wall of the penis, at the proximal and distal sides of the circular swelling.

***Kolhymorbis bogatovi*.** The elongated, tubular penis sheath (ps, Fig. 2A) has a relatively thin wall with musculature arranged in three layers. The outer layer is composed of one row of wide strap-shaped circular fibers (ocs, Fig. 2B, C). The intermediate layer (lps, Fig. 2B, C) is longitudinal and is 2–3 fiber thick. The inner layer (ics, Fig. 2B, C) consists of 1–2 rows of circular muscle fibers that are round or oval in cross section.

The tubular penis (pe, Fig. 2A) is about as long as the penis sheath and at the tip bears a small, trough-shaped stylet. The ejaculatory canal opens in the terminal position at the base of the stylet. A small glandular structure (flagellum; fl, Fig. 2A, B) is located in the invagination of the penis sheath wall at the base of the penis and opens into the penis sheath cavity. The penial musculature consists of the outer (olp, Fig. 2B, C) and inner (ilp, Fig. 2C) longitudinal layers arranged each in a single row and the intermediate layer composed of radially oriented fibers (rp, Fig. 2B, C). The radial fibers are often combined in larger bundles.

***Planorbarius corneus*.** The penis sheath and penis are very small relative to a large, muscular preputium. The muscle fibers in the penis sheath are oriented either in longitudinal or in circular direction, but there are no clear boundaries between muscle layers. The circular fibers tend to lie close to the outer (ocs, Fig. 3A, B) and to the luminal (ics, Fig. 3A, B) walls of the penis sheath and the longitudinal fibers (lps, Fig. 3A, B) are concentrated primarily in the interior, but muscle fibers of different orientation overlap with one another at the boundaries between these loosely demarcated layers. The inner circular muscles are much thinner than the outer and are arranged in about 3–8 rows (vs. about 8–12 rows in the outer layer) and the longitudinal layer is about 6–8 fiber thick.

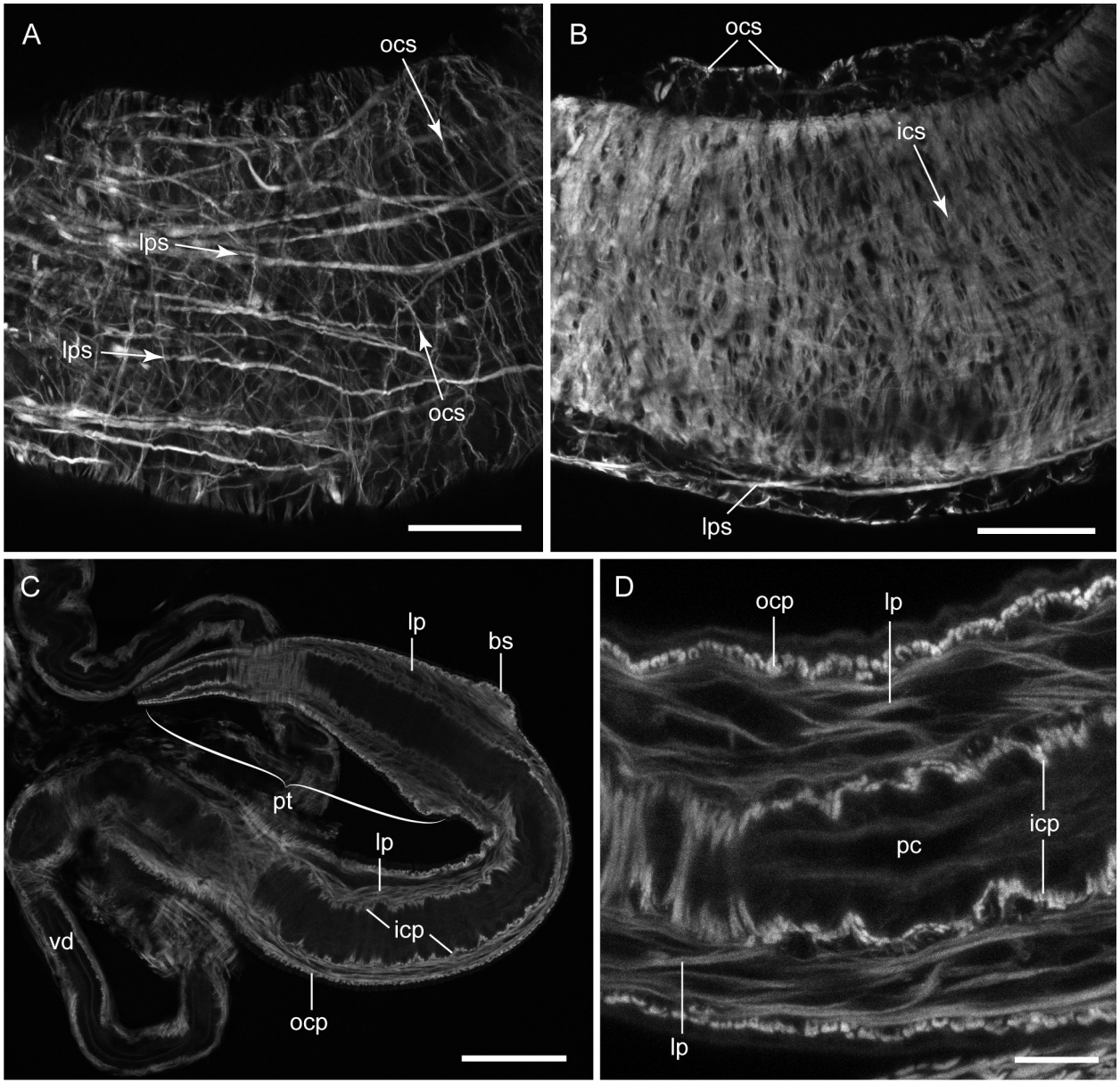

Fig. 1. Musculature of the penial complex of *Aplexa hypnorum*. A, B. Musculature in the wall of the penis sheath. C. Overall view of the vas deferens and penis. The penis sheath is removed. D. Longitudinal projection through the central axis of the penis. Abbreviations: bs – basal swelling of conical penis tip; icp – inner circular penial muscles; ics – inner circular muscles of penis sheath; lp – longitudinal penial muscles; lps – longitudinal muscles of penis sheath; ocp – outer circular penis muscles; ocs – outer circular muscles of penis sheath; pc – penial canal; pt – penial tip; vd – vas deference. Scale bars: A–C = 100 μm, D – 10 μm.

A small cone-shaped penis (pe, Fig. 3A) has a subterminal ejaculatory opening so that the tip of the penis forms a small terminal papilla. The penial muscle arrangement is similar to that in the penis sheath: the circular muscle fibers form inner (icp, Fig. 3D) and outer (ocp, Fig. 3C, D) layers, while the longitudinal fibers (lp, Fig. 3C, D) lie in the interior, but muscle layers are indistinct and fibers of different orientation overlap with one another. Both circular layers are 2–3 fiber thick and the longitudinal layer comprises 3–4 rows of muscle fibers. In the penial papilla, the inner circular layer is absent.

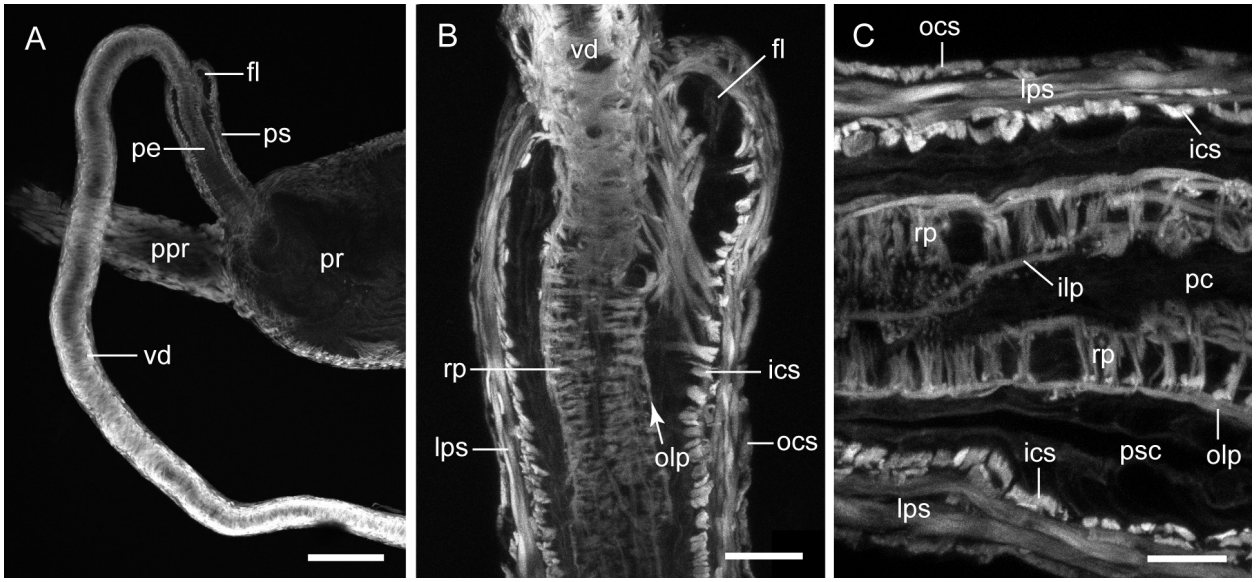

Fig. 2. Musculature of the penial complex of *Kolhymorbis bogatovi*. A. Overall view of the vas deferens and the penial complex. B. Longitudinal projection through the penial complex at the base of the penis sheath. C. Longitudinal projection through the central axis of the penis and penis sheath. Abbreviations: fl – flagellum; ics – inner circular muscles of penis sheath; ilp – inner longitudinal penial muscles; lps – longitudinal muscles of penis sheath; ocs – outer circular muscles of penis sheath; olp – outer longitudinal penial muscles; pc – penial canal; pe – penis; pr – preputium; ppr – preputium retractor muscle; ps – penis sheath; psc – penis sheath cavity; rp – radial penis muscles. Scale bars: A = 100 μm, B = C = 20 μm, C = 10 μm

DISCUSSION

The present study is a continuation of a series of studies (Soldatenko and Petrov 2012, 2016, 2019) that used phalloidin staining in combination with confocal microscopy to examine the male copulatory musculature in several representatives of the Hygrophila. Soldatenko and Petrov (2019) have shown that the musculature of the penial complex (penis sheath and penis) provides a set of clearly defined characters for several hygrophilan groups that previously lacked morphological synapomorphies. The three species included in the present study were selected as members of different hygrophilan taxa to verify phylogenetic hypotheses proposed based on mapping the characters of the male copulatory musculature onto a molecular phylogenetic tree (Soldatenko and Petrov 2019) by comparing these species with those examined earlier.

***Aplexa hypnorum*.** The family Physidae is currently subdivided into two subfamilies: Physinae and Aplexinae (Starobogatov 1967; Taylor 2003) and the only representative of the family for which musculature of the penial complex has so far been studied is *P. acuta* (Soldatenko and Petrov 2019), a member of the Physinae. *Aplexa hypnorum*, a representative of Physidae included in the present study, belongs to the other subfamily, Aplexinae, and the comparison of muscle arrangement in the penial complex of these two species may therefore clarify the degree of variation in male copulatory musculature across the two subfamilies. Penial musculature of *A. hypnorum* has essentially the same arrangement as that of *P. acuta*: in both there are outer and inner circular muscle layers and an intermediate layer of longitudinal muscles. In *Aplexa* the intermediate layer is much wider than in *P. acuta* and this helps better understand the arrangement of longitudinal muscles in this layer. The opposite endings of these muscle fibers attach to the inner and outer walls of the penis and can alternatively be regarded as obliquely oriented radial muscles. This complicates the interpretation of the plesiomorphic arrangement of penial muscles in the Hygrophila. It has previously been suggested (Soldatenko and Petrov 2019) that longitudinal fibers in the intermediate layer were antecedent to the radial fibers, but their arrangement in *A. hypnorum* blurs the distinction between these two types of muscle and gives some credence to the view that radial muscles may in fact represent a plesiomorphic condition for the Hygrophila.

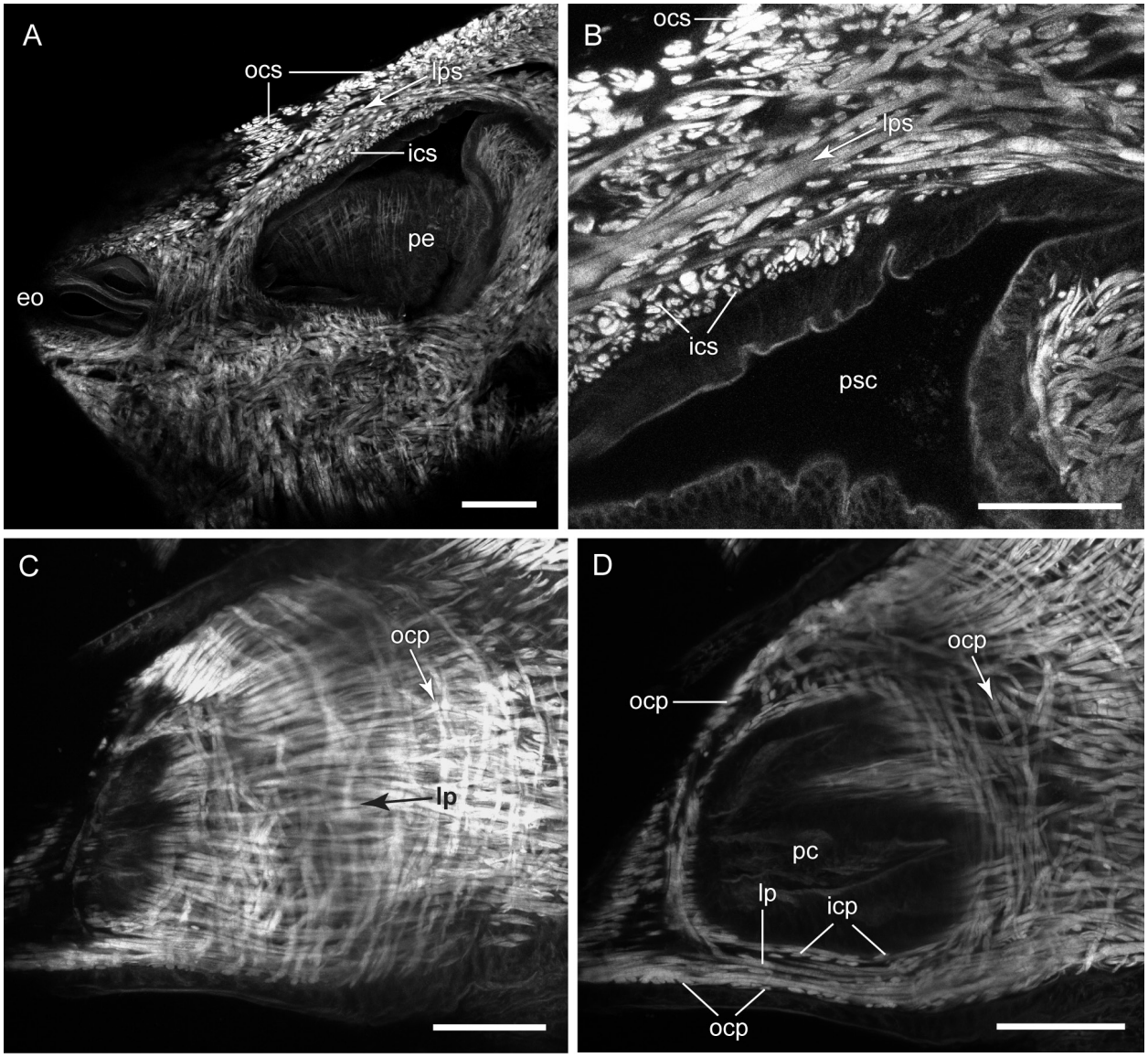

Fig. 3. Musculature of the penial complex of *Planorbarius corneus*. A. Overall view of the penis and penis sheath. B. Longitudinal projection through the wall of the penis sheath. C. Tangential projection through the wall of the penis. D. Slightly oblique longitudinal projection through the central axis of the penis. Abbreviations: eo – ejaculatory opening; icp – inner circular penial muscles; ics – inner circular muscles of penis sheath; lps – longitudinal muscles of penis sheath; ocp – outer circular penis muscles; ocs – outer circular muscles of penis sheath; pc – penial canal; pe – penis; psc – penis sheath cavity. Scale bars: A = 100 μm, B–D = 50 μm

The tip of the penis in *A. hypnorum* is set off from the penial shaft and is enlarged at its base probably serving to secure the penis in the partner's vagina. This is apparently achieved by contraction of longitudinal muscles at the base of the penis tip, which creates a bulge pressing against the walls of the vagina. The muscle arrangement in the penis tip of *A. hypnorum* remains essentially the same as in the penis shaft. Although an annular swelling at the penis tip is not universally present in the Physidae, *P. acuta* also has this type of penis tip, but in this species it lacks inner circular muscle fibers, which is almost certainly explained by a secondary loss.

The organization of the penis sheath musculature in *A. hypnorum* differs from that in *P. acuta*. In *Aplexa*, the penis sheath has three muscle layers (inner and

outer circular and intermediate longitudinal), while in *P. acuta* there are only two layers (outer longitudinal and inner circular). It has been previously hypothesized based on character mapping onto a molecular phylogenetic tree (Soldatenko and Petrov 2019) that the two-layered musculature of the penis sheath is plesiomorphic both for the Physidae and the Hygrophila in general. The presence of a three-layered penis sheath musculature in *A. hypnorum* raises the possibility that this condition can in fact be plesiomorphic (at least for the Physidae). If this assumption is correct, it can be argued that the outer circular layer in the penis sheath may have been lost in *P. acuta* (and probably in other Physinae), which agrees with molecular data that place *Aplexa* as the most basal genus of the Physidae (Wethington and Lydeard 2007).

***Kolhymorbis bogatovi*.** In *K. bogatovi*, the sequence of muscle fibers in both penis sheath and penis is the same as in the other two segmentinins (*Segmentina oelandica* (Westerlund, 1885) and *Hippeutis complanatus* (Linnaeus, 1758)) and in the eight species of Planorbini, in which the male copulatory musculature has been studied (Soldatenko and Petrov 2016, 2019). In all cases, the penis sheath musculature is three-layered and has the outer circular and intermediate longitudinal muscle layers. The inner layer shows some variation; in all species of Segmentinini including *K. bogatovi* it consists of circular muscle fibers, but in almost all Planorbini it is formed by crisscrossing diagonal fibers (Soldatenko and Petrov 2019). The only exception is *Gyraulus albus* (O.F. Müller, 1774), which, like segmentinins, has a circular inner layer (Soldatenko and Petrov 2019). This distinction between Segmentinini and Planorbini, however, should not be interpreted as having much phylogenetic importance, because circular muscle fibers can easily assume diagonal orientation if this is required by biomechanical factors. The penial musculature of Planorbini and Segmentinini is always composed of outer and inner circular fibers, with radial fibers crossing the space between these layers. The arrangement with the outer and inner muscle layers composed of circular fibers is unique in the Hygrophila and the presence of the same muscle arrangement in *K. bogatovi* adds further support for the argument that this character provides a strong synapomorphy for the clade comprising the tribes Planorbini and Segmentinini.

***Planorbarius corneus*.** The phylogenetic position of *P. corneus* within the Planorbidae remains contentious. Molecular phylogenetic studies place *Planorbarius* variously either as the sister group to the clade uniting the tribes Segmentinini and Planorbini (Soldatenko and Petrov 2019), in an unresolved polytomy with Planorbini + Segmentinini and the tribe Helisomatini (Albrecht et al. 2007) or as the sister group to Helisomatini (Saadi et al. 2020). Comparison of the male copulatory musculature of *P. corneus* with that of representatives of Segmentinini + Planorbini and Helisomatini can therefore be useful to decide, which of the phylogenetic hypotheses appears more likely. Although the penial musculature of *P. corneus* is unusual in that its muscle layers partially overlap with one another, it adheres to the muscle arrangement presumably plesiomorphic for the Hygrophila with the outer and inner circular and intermediate longitudinal muscles. The penial musculature in the clade comprising Segmentinini and Planorbini is fundamentally different and has a reversed arrangement of muscle layers (the inner and outer muscle layers are longitudinal), which indicates that *P. corneus* belongs to a lineage that diverged before the last common ancestor of Planorbini and Segmentinini.

The penis sheath musculature of *P. corneus* and Segmentinini + Planorbini has the same sequence of muscle layers (outer and inner circular and intermediate longitudinal), while in the more basal taxa the penis sheath musculature is in most cases only two-layered (outer longitudinal and inner circular). The three-layered penis sheath musculature may constitute a synapomorphy of the clade comprising *P. corneus* and Segmentinini + Planorbini, but this character seems less reliable than the arrangement of penial musculature, as was shown above in the case of Physidae.

In Helisomatini, the musculature of the penial complex has been studied in *B. glabrata* and *P. duryi* (Soldatenko and Petrov 2019). Since the musculature of the large, massive penis of *P. duryi* has been significantly modified as a result of the unusual mating behavior of this species (Soldatenko and Petrov 2012, 2019), it is difficult to compare it with that of *P. corneus*. The penis of *B. glabrata,* which is probably more typical in morphology for Helisomatini, has circular muscle fibers in the outer and inner layers (a plesiomorphic character also present in *P. corneus*), but the interior of the penis in this species contains two separate layers of longitudinal muscles and the radial muscle fibers. The penis of *P. corneus*, in contrast, has only one intermediate layer of longitudinal muscles.

Since the arrangement of penial muscles observed in *B. glabrata* is also present in the Ancylidae, which is a more basal group, it is likely that in the lineage leading to *P. corneus* the musculature in the interior of the penis has been simplified and the radial muscles have been lost. This simplification can be explained by a highly modified mating behavior of *P. corneus*, in which the copulation is guided by a very large preputium, the vas deferens and specialized preputial and atrial organs and the penis plays only a subsidiary role (Soldatenko and Petrov 2013).

CONCLUSION

The results of this study support and strengthen the conclusion of an earlier study (Soldatenko and Petrov 2019) that the musculature of the penial complex in the Hygrophila may provide a reliable source of phylogenetically informative characters. Although there is some variation in muscle arrangement within families or tribes that could probably be explained by reduction of muscle layers (as in the Physidae) or by biomechanical constraints (as in Planorbini), the organization of the male copulatory musculature is generally uniform within major hygrophilan groups, but displays variation between these taxa. The most reliable synapomorphy (circular muscle fibers in the inner and outer penial muscle layers) defines the clade comprising the tribes Planorbini and Segmentinini, a group that has no formal name in the currently accepted classification of the Hygrophila (Bouchet et al. 2017). The muscle sequence in the penial complex of *A. hypnorum* is essentially the same as in another physid, *P. acuta*, but this arrangement is probably plesiomorphic for the Hygrophila in general and cannot be taken as evidence of phylogenetic relationship. The sequence of muscles in the penis complex of *P. corneus* suggests that this species diverged prior to the common ancestor of Planorbini + Segmentinini, but the degree of its relationship with Helisomatini cannot be established, probably because the musculature of the penis and penis sheath in *Planorbarius* has been significantly modified as a result of its unusual mating biology.

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