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The ontogeny of the lower reproductive tract of the landsnail *Helix aspersa* (Gastropoda: Mollusca)

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Abstract We provide a size-based ontogenetic sequence of the development and differentiation of the lower reproductive tract of the heterobranch gastropod *Helix aspersa* (Müller 1774). Twelve development stages distributed among nine size classes were recognized based on readily visible changes in morphology and changes in tissue density. Geometric morphometrics was used to calculate the deformation between stages as represented by thin-plate spline bending energies. The developmental stages and sequence of developmental events are also compared to previously published scenarios for the evolution of stylommatophoran and other pulmonate reproductive tracts. These comparisons suggest that heterochronies, which include both acceleration and retardation, are operating in the morphological evolution of the pulmonate lower reproductive tract. This supports previous observations that largest number of developmental changes coincides with the transition to sexual maturity, which is also seen in the exponential curve of bending energies we observed in *Helix aspersa*. The belated organogenesis makes the ontogeny of the complex hermaphroditic reproductive system of pulmonates readily observable in size-friendly juveniles. This observation, coupled with the ease of raising individuals in the laboratory, recommends *Helix aspersa* as a potential model laboratory system for investigating molluscan evolutionary development.

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Introduction

The reproductive system of molluscs forms late in development (Raven 1966; Moor 1983); in several taxa the reproductive system does not develop until after larval settlement and metamorphosis [Solengastres (Thompson 1960), Polyplacophora, (Pearse 1977), Gastropoda (Moritz 1939)]. In many pulmonate and opisthobranch gastropods the formation of the reproductive tract is entirely a juvenile developmental event (Tardy 1970; Moor 1983; Visser 1988). This belated organogenesis makes the ontogeny of these complex structures readily observable in juveniles rather than larvae. Most heterobranch gastropods (traditionally divided into the "lower Heterobranchia," Opisthobranchia, and Pulmonata) have the added value of having a hermaphroditic reproductive system that is assembled from two distinct germ lines – mesoderm and ectoderm (Rouzaud 1885; Hoffmann 1922; Tardy 1970; Griffond and Bride 1985).

Molluscan reproductive systems are also important sources of characters for taxonomic groupings, character analysis, and phylogenetic analyses (Ghiselin 1965, 1987; Visser 1988; Gomez 2001). Fretter and Graham (1994) diagrammed 13 reproductive tract configurations in "prosobranch" gastropods (Patellogastropoda, Vetigastropoda, Neritimorpha, Caenogastropoda), while another 32 configurations have been proposed just for heterobranch gastropods (Beeman 1977; Berry 1977; Fretter and Graham 1994; Visser 1988; Gomez 2001). In terrestrial pulmonate snails, an easily observable reproductive system ontogeny with abundant morphological markers, combined with successful husbandry methods, provides an excellent opportunity to evaluate reproductive system character polarities, document

potential heterochronic changes in lineages, and investigate evolutionary trends in hermaphroditic reproductive systems. This includes whether ontogenetic differentiations of structures correspond to phylogenetic relationships within certain clades.

Shileyko (1973, 1978); Visser (1977), and Sirgel (1973) discussed some of these topics in the context of the evolution of the pulmonate terminal reproductive tract. However, Visser (1988) was one of the first to use comparative framework when he created ontogenetic sequences for "basommatophoran" (*Lymnaea*), "systellommatophoran" (*Gonaxis*), and stylommatophoran (*Elmlimux*) exemplars and then compared these ontogenies with the adult morphologies within each group.[1] Visser (1988: Fig. 10) also constructed evolutionary scenarios based on ontogenetic and taxonomic data and interpreted the different morphologies of these structures to heterochronic events in pulmonate development. He regarded the "Basommatophora" as having a neotenic origin from a common ancestor shared with "Systellommatophoran" and stylommatophoran taxa. He also considered the role of acceleration and retardation in the generation of the morphological variation seen in the terminal section of the stylommatophoran reproductive tract. However, testing of Visser's (1988) stylommatophoran scenario is limited by the lack of more detailed ontogenetic data. While taxonomically broad, the Visser (1988) ontogenetic data are unit-less and only follow the development of the bursa copulatrix and penis in fewer than four sequences.

To provide better resolution of the ontogeny of the stylommatophoran reproductive tract, we undertook a study of the European Brown Garden Snail, *Helix (Cornu) aspersa* (Müller 1774). *H. aspersa* is native to the Mediterranean region and Western Europe but has been introduced widely throughout the world, including California. The hermaphroditic reproductive tract of *H. aspersa* consists of an ovotestis and male and female organs and accessory glands that facilitate reciprocal fertilization and egg formation (Gomot and Griffond 1993). The different canals, glands, and structures differentiate only after hatching from a simple tubule, which is formed from a single ectodermal invagination on the anterior right side of the embryo (Tardy 1970; Griffond and Bride 1985). The site of invagination forms the genital pore, the middle and anterior portions of the tube form the bursa copulatrix, accessory glands, dart sac, penis, epiphallus, flagellum, and vas deferens, while the posterior terminus of the tube contacts the gonad and secondary structures (Fig. 1J) (Griffond and Bride 1985). The aims of this study were to: (1) determine the ontogenetic sequence of the lower reproductive tract in *H. aspersa*, (2) quantify the ontogenetic change occurring using geometric morphometrics, and (3) compare the development of the lower reproductive tract *H. aspersa* to published examples from other pulmonate taxa.

Materials and methods

Adult and juvenile snails from all size and age classes were collected at Point Isabel, Richmond, California (37° 54' 16" N, 122° 19' 13" W) and in Trestle Glen, Oakland, California (37° 48' 24" N, 122° 13' 48" W). The snails were housed in aerated buckets with garden soil and fed diets supplemented with calcium and other essential minerals and nutrients (R. Chase, personal communication). Some individuals were bred in captivity, producing a wide range of young, small snails. Eighty-seven snails between 6 mm−26 mm (shell diameter) were selected randomly for dissection. Some of these snails were collected in the wild, while others were offspring born in the laboratory. Snails were drowned for approximately 24 h and then preserved in either 10% formalin or 70% ethanol prior to dissection. Each shell was also measured, the number of whorls recorded, and the presence or absence of an apertural thickening indicating termination of growth and sexual maturity noted.

Snails were dissected by making a dorsal incision in the epidermis along the center of the body axis, beginning at the edge of the shell and extending anteriorly until reaching the eyestalks. The lower reproductive system was readily visible within the incision and not coiled within the shell. Structures were observed under a dissecting microscope at 10× magnification. The state and development of the lower portion of the reproductive tract were noted and drawn. While we did note variation in the size of organs among specimens of the same size class, in most cases the shapes of the organs showed less variation in their respective size classes. When shape variability was present we recorded the variation as sub-stages (see below). Thus, the morphologies illustrated here depict the characteristic states observed in each size class. Digital images of the dissections are presented in the Appendix. The comparison of drawings, notes, and photographs revealed, based on visible changes in morphology as well as tissue density, 12 stages of development distributed among 9 size classes. Stages that were found to have two character states within their size class (e.g., C, D, and F) were designated as sub–stages (e.g., C1, C2).

Geometric morphometrics

Geometric morphometrics was used to compare geometric landmarks plotted on pairs of successive developmental

[1] Recent phylogenetic work has revealed that both the "Basommatophora" and "Systellommatophora" as used by Visser (1988) and other earlier workers represent polyphyletic and paraphyletic groupings [Jörger et al. (2010)].

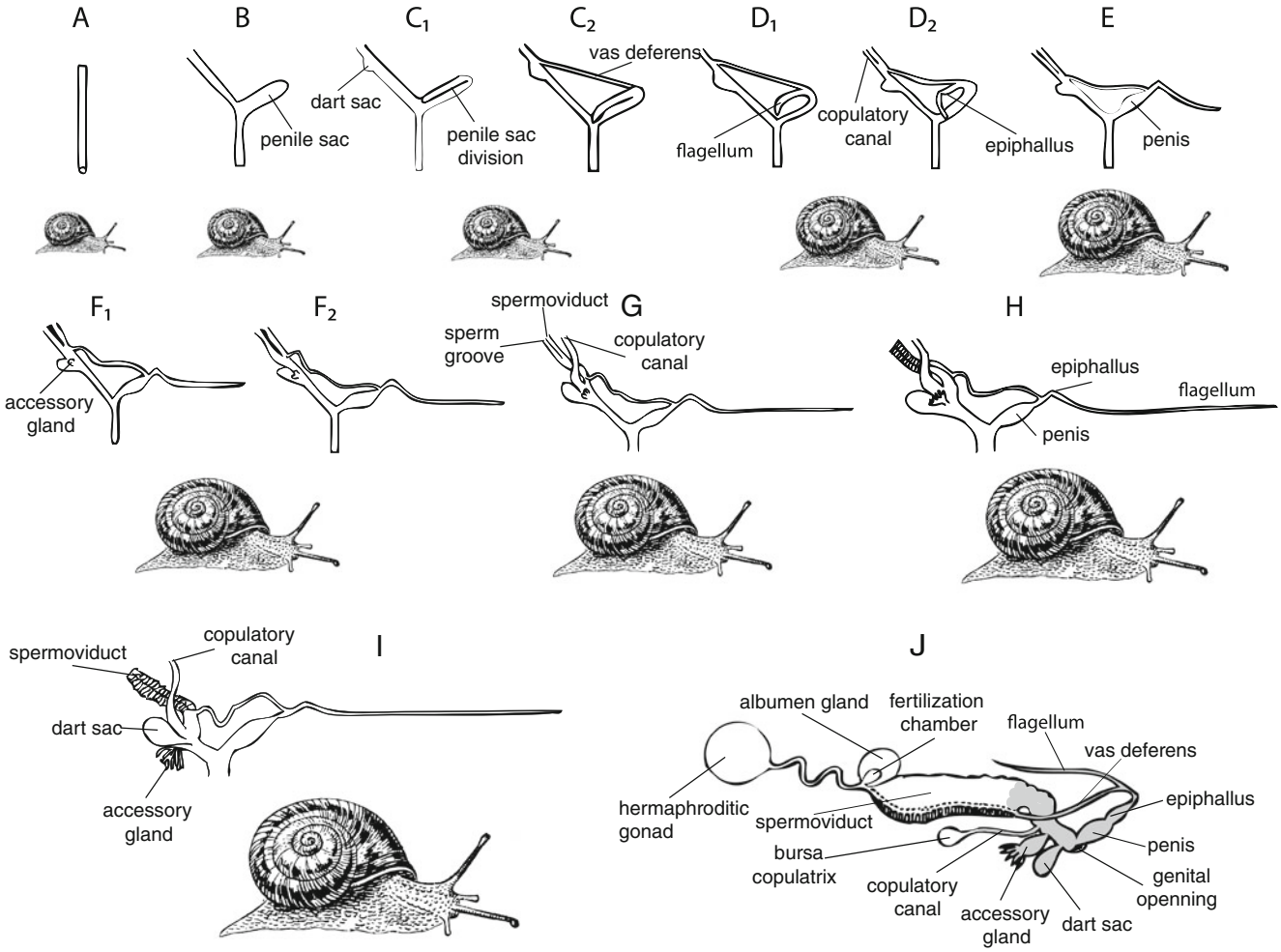

Fig. 1 Ontogeny of the lower reproductive tract of *Helix (Cornu) aspersa*. Illustration J is redrawn from Duncan (1960). Snail images are scaled to show the relative mean size of an individual at this stage of development (snail at stage A ≈ 6.75 mm)

stages. For stages divided into two sub-stages, the comparisons were made between the previous stage and the first sub-stage (e.g., B to C_1), and between the second sub-stage and the next stage (e.g., C_2 to D). Geometric morphometric software (http://life.bio.sunysb.edu/morph/) was used to quantify the degree of morphological deformation between developmental stages. In those cases where the structure would appear later in development, landmarks were placed relative to the position where the epithelial thickening would originate and the subsequent bud would appear. Therefore, these landmarks were not homologous between drawings, but they did maintain geometric homology throughout all comparisons. The program TpsDig2 (version 2.16) was used to digitize landmarks, and TpsSplin (version 1.20) was used to generate thin-plate spline comparisons between developmental stages and calculate the bending energies between successive developmental stages. Thin-plate spine analysis quantifies changes in landmark positions between objects and represents the magnitude of change between the objects in the bending energy variable (see Bookstein 1991; Zelditch et al. 2004).

Results

Sequence of development

Twelve stages (including sub-stages, designated A-I) were identified in the ontogeny of the lower reproductive tract of *H. aspersa*. These stages are documented in Fig. 1 and Table 1. The extent of the lower reproductive tract described here is shaded in Fig. 1J.

At stage A (Fig. 1A), the lower reproductive system is single tubule, which is derived from an invagination of ectodermal tissue (Tardy 1970; Griffond and Bride 1985; Visser 1988). Stage A was found in snails that averaged less than 7.00 mm in shell diameter. Stage B

Table 1 Material examined and geometric morphometric data

Stage	*n*	Mean shell diameter ± SD (mm)	Median shell diameter (mm)	Pairwise comparison	Bending energy (be)
A	5	≤6.88±0.629			
B	5	8.70±0.837	8	A-B	431.629
C_1 , C_2	10	9.50±1.155	9	B-C_1	0.915
D_1, D_2	16	12.63±1.348	11.75	C_2-D_1	1.942
E	6	15.03±1.381	13.25	D_2-E	45.995
F_1, F_2	16	16.28±1.798	15	*E*-F_1	6.258
G	15	17.47±1.246	17	F_2-G	28.089
H	6	19.45±1.129	18	G-H	45.759
I	8	≥23.69±1.580	21.5	H-I	114.974

(≈8.70 mm) was characterized by the appearance of a thin distal outgrowth of tissue on the side of the initial tube (Fig. 1B). This outgrowth, referred to here as the penile bud, gives rise to the epiphallus, flagellum, and penis; the vas deferens is also likely derived, in part, from this outgrowth as well. In stage C (≈9.50 mm) the penile bud divided medially, giving rise to a folded blind tube. A second, smaller outgrowth of tissue representing the beginning of the dart sac appeared further up the main tube (Fig. 1C_1). In successive stages, the dart sac continued to enlarge. Some snails in this size class were also characterized by the appearance of the vas deferens, connecting from above the dart sac to the distal end of the folded blind tube (Fig. 1C_2). A partially formed vas deferens was not observed.

In stage D (≈12.5 mm), the penile bud completed its division and separated from the proximal end of penile sac, forming a blind tube folded back on itself (Fig. 1D). In some specimens (stage D_2), the penis became visible, as the proximal end of the penile bud widens and thickens. Also, just above the early dart sac the upper portion of the initial tube divided into two tubes, the copulatory canal and putative spermoviduct; the new spermoviduct also contained the vas deferens as it separated from the copulatory canal (Fig. 1D_2). The two canals became completely separated in stage E (≈15.0 mm), and the epiphallus became visible as a bend in the tissue tube between the penis and the flagellum (Fig. 1E). From this stage onwards, the flagellum is illustrated in Fig. 1 as extending to the right, away from the main tube of the reproductive system, so that its gradual and continuous elongation is evident (Fig. 1E-I).

In stage F (≈16.0 mm), the accessory gland appeared as a pair of round outgrowths slightly anterior to the maturing dart sac, one on each side of the tract (only the dorsal one is pictured in Fig. 1). Later in this stage (F_2), each of the outgrowths divided down the center, producing a pair of small buds on each side of the tube; the base of the copulatory canal also widened slightly in this stage (Fig. 1F_2). In stage G (≈17.5 mm), the accessory gland divided into units of three, and the spermoviduct and copulatory canal tubes enlarged slightly and crossed over each other; the sperm groove of the spermoviduct also began to differentiate (Fig. 1G).

Stages H and I (> 23.5 mm) represent stages at which full maturation of the reproductive system occurs. Stage H was characterized by further division of the accessory glands, and the exterior surface of the spermoviduct changed with the differentiation of a central region with tissue folds on either side (Fig. 1H). In stage I the tissue of the spermoviduct was completely differentiated, with highly textured creases and folds (Fig. 1I). The accessory glands each divided into clusters of approximately 12–15 long, thin, finger-like extensions, and the flagellum elongated and became coiled, extending up into the viscera enclosed by the shell. The dart sac, penis, genital atrium, spermoviduct, vas deferens, and copulatory canal all enlarged, and their tissues were noticeably thickened. The entire lower part of the reproductive tract was now packed tightly within a thin layer of mesentery tissue, housed posteriorly within the penultimate whorl of the shell.

Geometric morphometrics

Log transformed, non-affine bending energies for pairs of successive developmental stages are plotted in Fig. 2. The largest bending energy occurred between stages A-B. However, we consider this an artifact. Because the

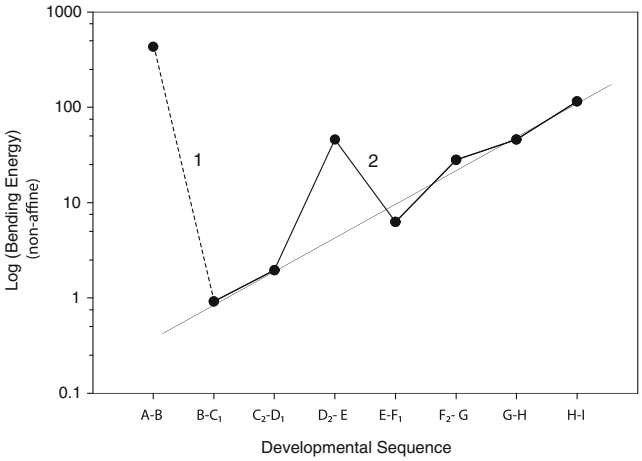

Fig. 2 The thin-plate spline bending energy of pairwise comparisons of adjacent developmental stages. 1 = initial landmark deformations, 2 = straightening of flagellum; both of these events likely represent artificial morphological changes. Linear regression of log-transformed bending energies, without the problematic A-B and D_2-E values, gives an r^2 value of 0.9565

initial morphology was a simple tube, it was necessary to estimate the positions of geometric landmarks for the structures that would subsequently appear along the tube (see Appendix Fig. 4 for example of landmark placement between successive developmental stages). The formation of the penile sac deformed most of these future landmarks, causing the large bending energy (1 in Fig. 2). In subsequent stages, morphological change was relegated to specific regions (e.g., penile sac differentiation or dart sac/spermoviduct/accessory glands differentiation), and the bending energy values appeared to more accurately reflect the morphological change seen between stages. However, the bending energy value for D_2 to E (2 in Fig. 2) was also likely inflated because of the artificial straightening of the flagellum beginning with Fig. 1E. With the exception of these two values, there was an exponential increase in bending energies, with the highest bending energy values associated with the onset of sexual maturity. The exponential increase in bending energies in *H. aspersa* with the transition to sexual maturity (Fig. 2) is similar to the finding of Runham and Laryea (1968) who summarize the "reproductive development" of five "organs" of the stylommatophoran slug *Agriolimax reticulatus* and concluded that developmental events increased with the transition to sexual maturity.

Discussion

The ontogeny of the reproductive tract of *Helix* has been the subject of study since Ihering (1875). During the first 100 years, one of the central questions associated with the ontogeny of the pulmonate reproductive system centered on the origin of reproductive tract. Some workers reported that it was derived from mesodermal tissue (Ihering 1875), others from an ectodermal invagination (Rouzaud 1885), and others (e.g., Rabl 1875) suggested that the lower tract was ectodermal, while the upper components (gonad, albumen gland,

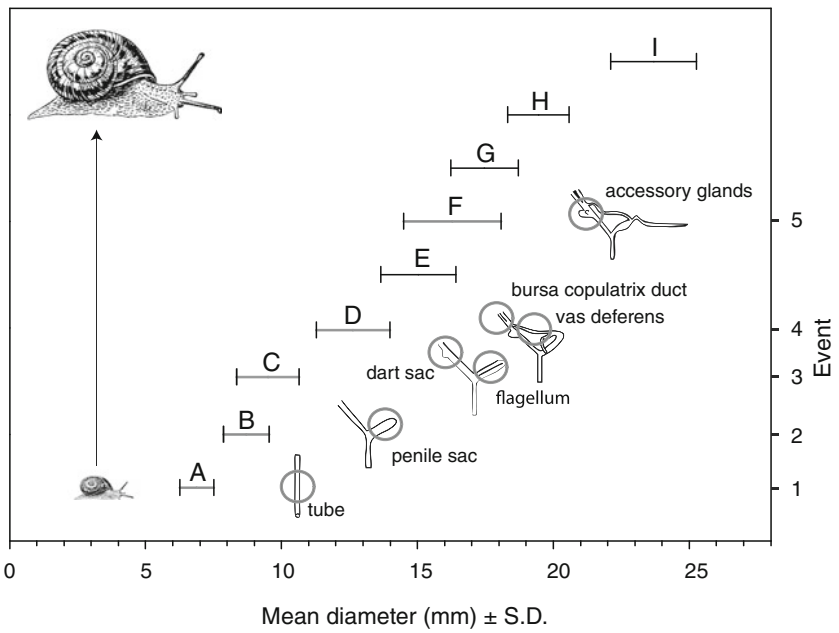

Fig. 3 Sequence event coding of the ontogeny of the lower reproductive tract of *Helix (Cornu) aspersa*. Snail images show relative size range (6.75 to 23 mm) of samples, and the circled reproductive tract structure highlights the associated developmental events that occur within the corresponding size classes. Event sequences are compared to other pulmonate taxa in Table 2

fertilization chamber) were derived from mesoderm [see reviews by Fraser (1946) and Martoja (1964)]. More recent studies have supported upper mesodermal and lower ectodermal derivations (Tardy 1970; Brisson and Regondaud 1971; Visser 1977, 1988; Enée and Griffond 1983; Griffond and Bride 1985). However, all these studies consistently reported that the reproductive tract originates from a simple epithelial tube that gives rise to the adult complex of tubes, glands, channels, and sacs (Runham 1982).

Many of the aforementioned and other studies have focused on the ontogeny of the upper mesodermal-derived tract rather than the lower ectodermal-derived tract (e.g., Runham 1978; Griffond and Bride 1981; Enée and Griffond 1983; Cuezzo 1990). Part of this focus has occurred because of the presence of hermaphroditism in the heterobranchs and the opportunity to link the ontogeny of the ovitestes with the evolution of hermaphroditism within Gastropoda. Many structures of the reproductive tract used in pulmonate systematics come from the lower portion of the reproductive tract, and understanding the development of these structures provides ontogenetic data to test character state polarities (Kluge and Strauss 1985; Schulmeister and Wheeler 2004). Duncan (1960) was one of the first to compare reproductive systems in the pulmonates. He suggested that the increasing separation of male and female ducts and the appearance of accessory structures (accessory glands, dart sac) indicated a trend of increasing complexity in the evolution of pulmonate reproductive systems. Visser (1977), on the other hand, proposed that the basommatophoran and stylommatophoran pulmonates were derived independently from male and female ancestors, respectively, thereby making hermaphroditism convergent and a secondary acquisition in both groups.

Visser (1988) again revisited the question of the evolution of the pulmonate reproductive system, but this time focused on the lower components of the pulmonate reproductive tract. Visser identified three modes of development in the Stylommatophora. In the first mode (e.g., *Gonaxis*) initial cellular differentiation involved the development of the spermoviduct, penis, and genital atrium along the body wall and below the mantle edge where the pneumostome is located. This event was followed by splitting of the vas deferens from the penis and finally the separation of the seminal groove from the spermoviduct, forming the spermathecal diverticulum. This mode involves the posterior placement of the genital opening and the reproductive system. The second mode (e.g., *Elisolimax*) was characterized by the initial anlage opening into the mantle cavity, followed by the formation a second posterior opening of the putative spermoviduct in a more posterior position, followed by an anterior migration of lateral body wall cells and forward displacement of the genital aperture and elongation of the pallial duct. The last stages in this mode were marked by the separation of the penis and genital atrium from the terminal duct. This separation produces the anterior placement of the genital opening. In the third mode (*Ariopelta)* development involved the simultaneous but transient retention of posterior female and anterior male genital openings, followed by a consolidation toward an anteriorly placed, combined genital opening, resulting in elongation of the pallial duct and creation of the genital atrium through in-rolling of the body wall at the composite genital aperture. The lower reproductive system completed development when the vas deferens separated from the penis.

Visser equated these modes, and those in other taxa, to heterochronic changes in the development of the ancestral pulmonate reproductive system. For example, he regarded the "Basommatophora" and "Systellommatophora" as neotenic because of their retention of embryonic ditremy (two genital openings) into the adult and pointed out that the

Table 2 Developmental sequence of the formation of lower reproductive tract structures in *Helix (Cornu) aspersa* compared to previously published sequences for major pulmonate taxa. Developmental stages were illustrated by Visser (1988) from the following sources: "Basommatophora" (Fraser 1946), "Systellommatophora" (Hoffmann 1925), and Stylommatophora (Visser 1977)

Taxon	Lower reproductive tract structures						
	Tube	Bursa copulatrix	Penile sac	Vas deferens	Flagellum	Dart sac	Accessory glands
"Basommatophora" *Lymnaea stagnalis appressa*	1	2	2	3/4	n/a	n/a	n/a
"Systellommatophora" *Vaginulidae*	1	2	2	3	n/a	n/a	n/a
Stylommatophora *Gonaxis gwandaensis*	1	3	2	3	-	-	-
Helix (Cornu) aspersa (Fig. 3)	1	4	2	4	3	3	5

Stylommatophora also recapitulated the ditrematic condition in early development, but established a monotrematic system (single opening) as the snails matured. Although unspecific, Visser also suggested that other modifications to the lower tract could be the result of heterochronic events, such as acceleration and/or retardation.

The ontogenetic sequence of the lower reproductive tract of *H. aspersa* described here does not appear to align with any of the aforementioned stylommatophoran modes. Although it does have a single, anteriorly placed genital opening, as in the second and third modes, differences in the timing of other developmental events suggest a different developmental pathway, most likely with a different suite of heterochronies. To compare our findings with Visser's (1988) evolutionary scenarios, we seriated (based on shell diameter) five developmental events in the formation of the lower reproductive tract of *H. aspersa* (Fig. 3). These five events were formation of the: (1) initial tubule, (2) penile sac, (3) dart sac and flagellum, (4) bursa copulatrix duct and vas deferens, and (5) accessory glands (Fig. 3). These events were then placed in a tabular form (Table 2) to permit comparison with the developmental sequences provided by Visser (1988) for "Basommatophora," "Systellommatophora," and Stylommatophora taxa. This simple comparison shows variation in the timing of the formation of the bursa copulatrix and another possible shift in the timing of the formation of the vas deferens (Table 2). To further examine the variation in timing of the formation of the bursa copulatrix, we mapped Visser's (1988) taxa (Table 2) on the recent heterobranch phylogeny of Jörger et al. (2010). These taxa suggest that the formation of the bursa copulatrix is delayed in the Stylommatophora.

As pointed out by Schrödl et al. (2011); Visser's (1977, 1988) evolutionary scenarios assumed that pulmonates gastropods were derived directly from a "prosobranch" lineage, which has been subsequently falsified by all recent morphological and molecular analyses (e.g., Wade et al. 2001; Wägele et al. 2008; Jörger et al. 2010; Kocot et al. 2011). This assumed ancestry constrained Visser's (1988) interpretation of the evolution of the pulmonate reproductive tract. In the past, scenarios of the evolution of heterobranch genital systems were not placed in a robust phylogenetic framework. Instead, the sequence of developmental events was laid out in a unit-less narrative without reference to a time or size axis. In light of the availability of the aforementioned robust molecular and morphological phylogenies, targeted studies of key taxa should prove useful in providing new hypotheses of the evolution of the pulmonate reproductive tract. As Schrödl et al. (2011) has argued, morphological variability, homologies, and the evolution of the heterobranch genital systems warrants much further investigation, a recommendation seconded by our results as well.

Several of the characters presented in Fig. 3 also represent characters commonly used in stylommatophoran systematics (Pilsbry 1940), and the ontogenetic data may prove useful in establishing character polarity. For example, in one of the early parsimony analyses of stylommatophoran relationships, Roth (1996) examined the occurrence of the dart sac in the stylommatophoran clade Helminthoglyptidae. Roth found the absence of a dart sac to be homoplastic, having occurred at least six times, and that the accessory glands had originated independently at least five times. Such large numbers of character transformations among relatively closely related taxa might seem unusual; however the ontogenetic sequence of the lower reproductive tract of *H. aspersa* supports Roth's hypothesis. The dart sac and accessory glands are topologically independent of the other structures making up the lower tract. Although the dart sac primordium appears early (Fig. 1B), it is not closely associated with any subsequent structures — with the possible exception of accessory glands — which appear in juxtaposition to it much later in development (Fig. $1F_1$). Thus, multiple losses or originations are possible without necessarily affecting other structures in the lower tract [see also Gilbertson et al. (2013)]. If other structures were dependent on the appearance of the dart sac and accessory glands, multiple losses and gains would be more difficult or a downstream cascade of other morphological changes anticipated.

Lastly, the belated organogenesis makes the ontogeny of the complex hermaphroditic reproductive system of pulmonates readily observable in size-friendly juveniles, rather than larvae, as is often required in other molluscan groups. This observation coupled with the ease with which many pulmonates taxa can be raised in large numbers over multiple generations (Daguzan 1983; García et al. 2006) provides a potential model laboratory system to investigate the molecular components of development by conducting gene expression studies at various stages of development.

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Appendix

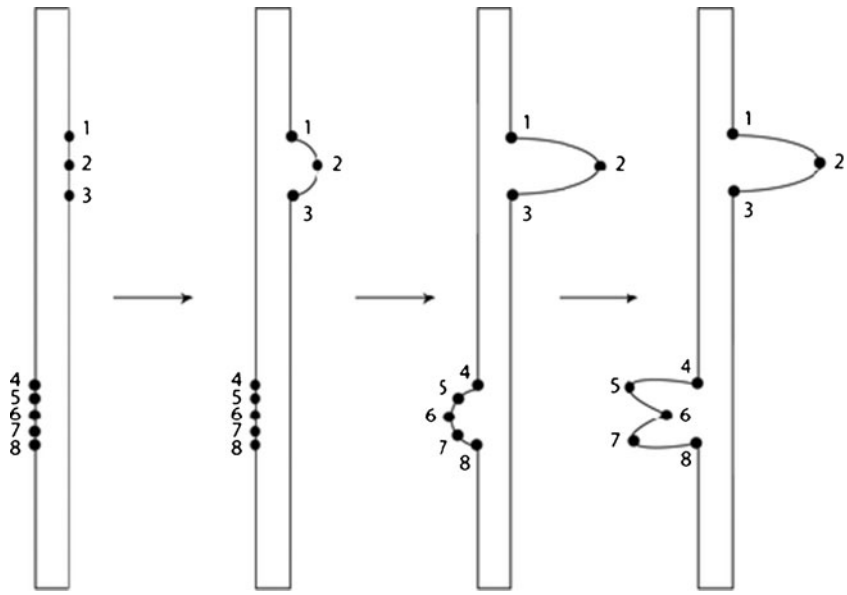

Fig. 4 Hypothetical developmental sequence of two protrusions appearing on a simple tube. The necessary landmarks to describe the basic shapes and relative positions of the final protrusions are projected back through the developmental sequence and placed in juxtaposition to capture subsequent development

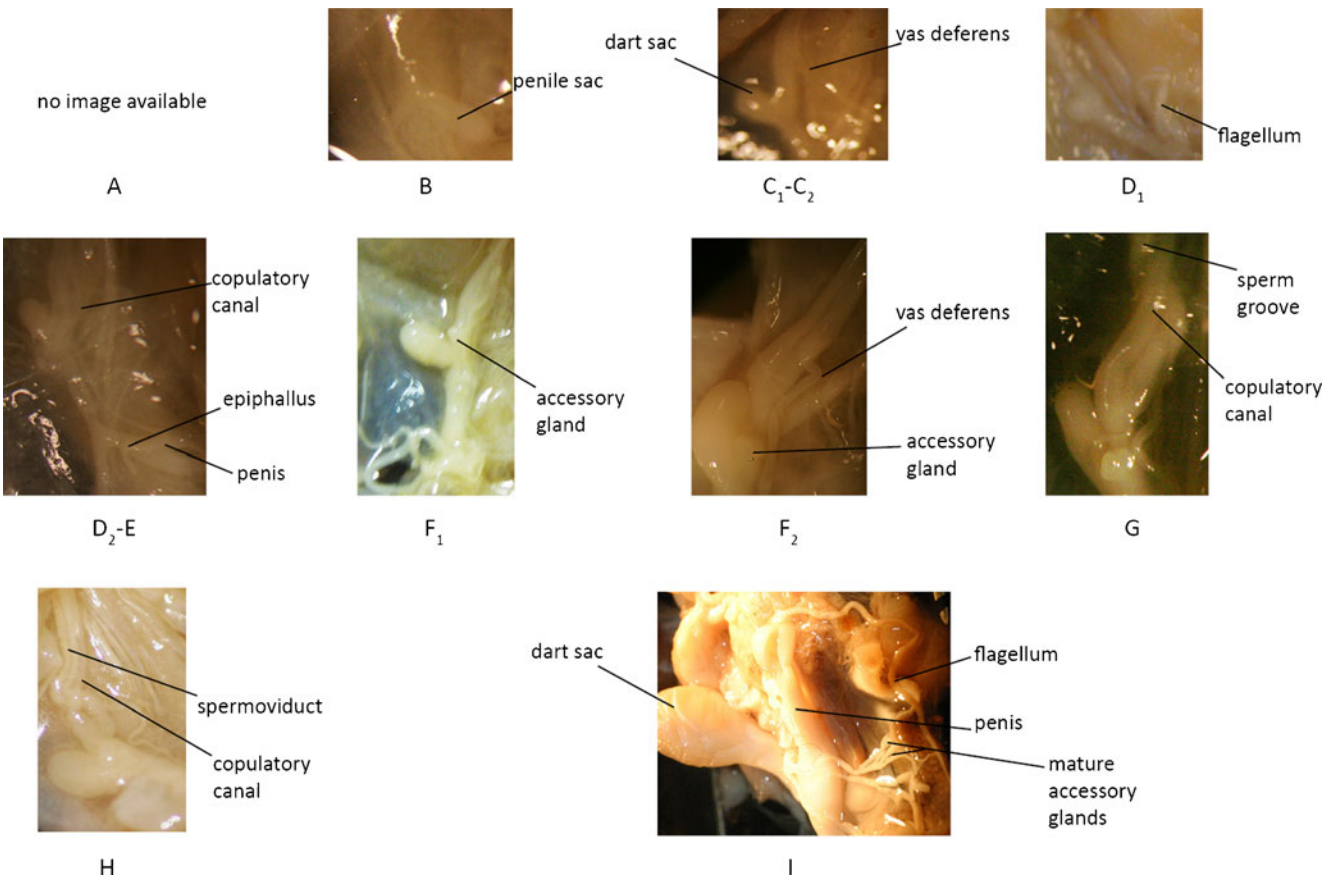

Plate 1 Photographs of the lower reproductive tract of *Helix aspersa* at each stage of development. Refer to Fig. 1 for corresponding illustrations

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