Direct developing predatory gastropods (*Nucella* spp.) retain vestiges of ancestral novelties in foregut development

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Abstract

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Predatory gastropods (Neogastropoda) feed with a proboscis (elongate snout) and complex foregut. The presence of developmental modules (semi-autonomous components) in foregut development may have facilitated emergence of predatory feeding. In species with indirect development (feeding larval stage) physical and temporal separation of developing foregut modules (dorsal=larval esophagus; ventral=juvenile feeding structures) allows larval feeding and rapid switch to carnivory. However, previous studies on neogastropods with direct development (no feeding larval stage) did not identify foregut developmental modules. Thus, I investigated foregut development in two predatory, direct developing neogastropods: *Nucella lamellosa* and *N. ostrina* (Muricoidea), using histological sectioning, 3D reconstructions, TEM, and SEM. In both species, I showed evidence for dissociable dorsal and ventral foregut developmental modules. In *N. lamellosa,* the two modules were physically separate, although they were not separate in *N. ostrina*. My results reconcile differences in previous descriptions of foregut development between neogastropods with indirect and direct development.

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1.0 Introduction

The Phylum Mollusca is second only to Arthropoda in terms of diversity of species and molluscs are found in nearly every aquatic ecosystem on earth. Most of this impressive diversity is found in the Class Gastropoda and about 60% of the extant gastropods are found in the clade Caenogastropoda (Ponder *et al.,* 2008). In parallel with an extensive invasion of diverse marine habitats, caenogastropods have acquired many novel feeding strategies to exploit a variety of nutritional sources (Ponder and Lindberg, 1997; Ponder *et al.,* 2008). The ancestral feeding condition for gastropods, which is still retained by some extant caenogastropod groups, is herbivorous grazing, whereas more derived feeding systems within caenogastropods include: scavenging, carnivorous grazing, predation on active prey, parasitism, and suspension feeding (Ponder and Lindberg, 1997). Of particular interest is the transition from herbivorous grazing to predatory feeding, which accompanied the emergence of a large clade of gastropods, the Neogastropoda, during the late Cretaceous (Ponder, 1973; Taylor *et al.*, 1980; Kantor, 1996). The Neogastropoda includes highly specialized predators such as groups that drill into the shells of their prey (*e.g. Nucella*) and others that inject neurotoxins into active prey (*e.g. Conus*).

My results extend previous research that suggested a novelty in foregut development facilitated the evolutionary transition from herbivorous grazing to predatory feeding in caenogastropods (Abro, 1969; Page, 2000, 2002, 2005, 2011). This developmental novelty occurred within the ancestral caenogastropod life history, which included a feeding (planktotrophic) larval stage, and involved the physical and temporal separation of the larval esophagus from the developing post-metamorphic foregut. This novel developmental trajectory provided the larva with a functional esophagus for feeding, but simultaneously allowed a highly modified post-metamorphic foregut to develop without interfering with larval feeding. Furthermore, this separation of the dorsal component (larval esophagus) from the ventral components (developing post-metamorphic foregut) is evidence for the presence of dissociable developmental modules (semi-autonomous components of a developing organism) in foregut development. However, studies on neogastropods that have switched from a life history with a free-

living planktotrophic larva to a life history with fully encapsulated (direct) development did not identify this novel pattern of foregut development or the presence of developmental modules (Ball *et al.,* 1997a, b; Ball, 2002). The disparity in pattern of foregut development among neogastropods suggests either separate origins of predatory feeding within the Neogastropoda or, more likely, that developmental changes have occurred as a result of the loss of a free-living, feeding larval stage. The second of these two possibilities is supported by the results of my study, because I identified evidence of dissociable developmental modules in the foregut development of two direct developing species of *Nucella*.

As background to this subject, I provide a description of the derived anatomical features of the adult foregut of several neogastropod lineages, an overview of the life histories of gastropods, and a summary of current knowledge about foregut development in herbivorous and predatory gastropods. I will also review hypotheses about how derived feeding structures of predatory gastropods evolved from the ancestral feeding structures of herbivorous grazers.

1.1 Proboscis and foregut of predatory gastropods

Emergence of a proboscis is correlated with the shift from herbivory to carnivory in many caenogastropods, particularly in the predatory neogastropods (Taylor *et al.,* 1980). Whereas herbivorous grazers have a short snout and use rhythmic movements of the radula to rasp algal films from hard substrates, predators use an extensible, muscular proboscis to reach food that is more difficult to access (compare Figure 1A and B to 1C and D). The proboscis shifted the mouth from a ventral position (ancestral grazer) to a terminal position on the head (Kantor, 1996) and allowed predators to gain access to novel food sources. The proboscis can be retracted into the head when not in use for feeding and at least four and possibly five proboscis types are recognized in caenogastropods on the basis of retraction pattern. These are called: acrembolic, pleurembolic, intraembolic, and polyembolic, and a fifth type called teinoembolic has been recognized by Golding *et al.* (2009). For example, the pleurembolic proboscis sits within a proboscis sac (rhynchocoel) when not in use and retractor muscles insert primarily in the mid-region of the pleurembolic proboscis so that it is only partially

inverted when it is retracted (Fretter, 1969; Salvini-Plawen, 1988, Golding *et al.*, 2009). The proboscis sac is sometimes referred to as a rhynchocoel; however, this term is a misnomer because the wall of the proboscis sac is not derived from mesoderm so the sac is not a coelom. Therefore, I will refer to this structure as the proboscis sac. The buccal mass of the pleurembolic proboscis is at a terminal position where it can be used, for instance, to drill into the shells of prey and then to access flesh from within the penetrated armor of the prey (Figure 1C; Kantor, 1996). The intraembolic proboscis, which is found among members of the Conoidea, is different because the buccal mass is located at the base of the proboscis (Figure 1D). The retractor muscles insert along the entire length of the proboscis and thus the proboscis is not inverted when it is retracted, instead the proboscis wall folds like an accordion (Golding *et al.*, 2009). Proboscis morphology is extremely diverse in terms of biomechanical operation, retractor muscles, and muscle layers that compose the proboscis wall (Golding *et al.,* 2009). On the basis of this, Golding *et al.* (2009) has suggested that several caenogastropod lineages acquired a proboscis independently during the transition to predatory feeding (Golding *et al.,* 2009).

With the emergence of these proboscis types, the foregut of predators also changed considerably from the ancestral condition. Nonetheless, as reviewed by Fretter and Graham (1994), the foreguts of predators share some common features with the ancestral foregut of herbivorous grazers. The ancestral gastropod foregut is approximated by studying the foregut of the two most basal lineages of gastropods: the patellogastropods (true limpets) and vetigastropods (*e.g.* abalone, keyhole limpets, top snails). This foregut structure is also retained in many extant herbivorous caenogastropods. In general, the mouth of herbivorous grazers opens into an oral tube that immediately expands as the buccal cavity, which receives the ducts of the acinous salivary glands and leads to the esophagus. The radular sac is a diverticulum extending from the ventro-posterior region of the buccal cavity that secretes a ribbon of radular teeth *i.e.* the radula. The radula emerges from the radular sac and extends over the floor of the buccal cavity toward the mouth. Members of the neogastropod family Conidae are exceptional because the radular teeth are in the form of individual hollow harpoons that are attached to the buccal mass by only a slender basal ligament (Kohn *et al.,* 1972; Kantor and Taylor, 1991). Odontophoral cartilages support the radula and a complex set

of muscles produce the rhythmic movements of the radula. The floor of the buccal cavity, radular sac, odontophoral cartilages, and musculature are collectively called the buccal mass. In the ancestral herbivorous gastropods (Patellogastropoda, Vetigastropoda, and some Caenogastropoda), the mid-dorsal portion of the buccal cavity and anterior esophagus is in the form of a ciliated channel (dorsal food groove), demarcated by dorso-lateral folds (Figure 1B; Haszprunar, 1988; Salvini-Plawen, 1988). Many authors have identified a putative dorsal food groove in the foregut of predatory caenogastropods (*e.g.* Graham, 1941; Strong, 2003); however, a developmental study showed loss of the dorsal food groove at metamorphosis in the predatory neogastropod, *Nassarius mendicus* (Page, 2000, 2005). Thus, the presence of a dorsal food groove in more derived caenogastropods is questionable.

Despite similarities to herbivorous grazers, predators have several specialized features. The elongate proboscis of predators requires elongation of some portion of the post-metamorphic foregut. In species with an intraembolic proboscis, the additional length comes from an elongate oral tube (Figure 1D), whereas species with a pleurembolic proboscis have an elongate anterior esophagus (Figure 1C), which is muscular so as to propel food down the long proboscis by peristaltic contractions. Furthermore, predatory feeding with a pleurembolic proboscis requires a valve structure, the valve of Leiblein (Figure 1C), which may prevent regurgitation of ingested food during proboscis elongation (Graham, 1941; Kantor, 1996). Finally, various glandular structures arose to meet functional needs such as efficient prey capture and immobilization as well as digestion of animal tissues (Kantor, 1996). For example, the venom gland in *Conus* produces neurotoxic peptides that paralyze prey (Figure 1D; Olivera, 2006), secretions from the accessory salivary glands in *Nucella* have a possible paralytic function (Andrews, 1991), and the mid-esophageal gland (gland of Leiblein) in many neogastropods may serve a role in digestion (Figure 1C; Andrews and Thorogood, 2005). Predatory feeding certainly required numerous anatomical changes, which prompts a larger question about how these changes were generated during the development of the immediate ancestors of neogastropods. That is, what fostered the emergence of the complex, elongate foregut of predators?

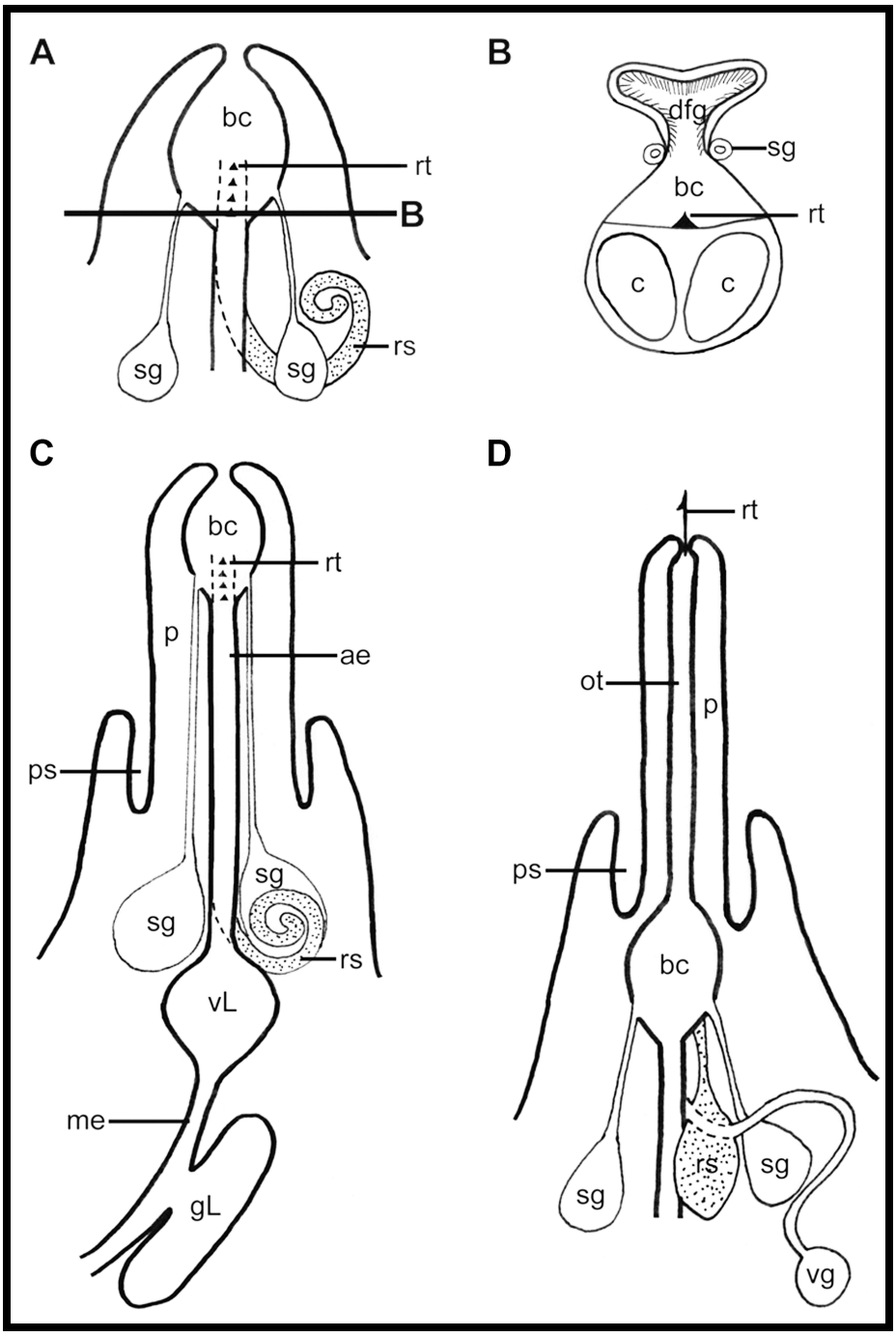

Figure 1. Comparative foregut anatomy in caenogastropods adapted from Page, 2000 and Page, 2011.

A. Dorsal view of a generalized herbivorous grazing caenogastropod; line B indicates the cross section in B. **B.** Cross section through the buccal cavity of a herbivorous grazing caenogasropod; notice the ciliated dorsal food groove, which runs the length of the buccal

cavity and anterior esophagus. **C.** Dorsal view of a pleurembolic proboscis type; notice the elongate anterior esophagus and the buccal cavity at tip of the extended proboscis. **D.** Dorsal view of an intraembolic proboscis type; notice the elongate oral tube and the buccal cavity at the base of the proboscis. Abbreviations: ae= anterior esophagus, bc= buccal cavity, c= cartilages, dfg= dorsal food groove, gL= gland of Leiblein, me= mid-esophagus, ot= oral tube, p= proboscis, ps= proboscis sac, rs= radular sac, rt= radular tooth, sg= salivary gland, vg= venom gland, vL= valve of Leiblein.

1.2 Life history evolution in caenogastropods

Gastropod life history patterns and life history transitions within the group must be considered in evolutionary and developmental studies. Gastropod life histories are highly variable and can be classified on the basis of presence or absence of a dispersive larva, presence or absence of metamorphosis, and nutritional source during development (Bonar, 1978).

In gastropods, an indirect life history includes a free-living, planktonic veliger larva that enables dispersal, whereas in a direct life history development takes place entirely within a benthic egg capsule and dispersal is therefore limited. The veliger larva is the characteristic larval type present in gastropods in general. Many gastropod species with fully encapsulated development pass through a stage that resembles a veliger larva within the egg capsule. The term larva refers to free-living, planktonic developmental stages, whereas the term embryo refers to encapsulated developmental stages. Because encapsulated veligers are not planktonic, they will be called veliger-like embryos. A metamorphic life history includes a veliger (free-living) or veliger- like embryo (encapsulated) that eventually undergoes metamorphosis into a juvenile. Metamorphosis involves loss of larval structures, such as the velar lobes, followed by the emergence of juvenile structures (Hadfield *et al.*, 2001), such as an elongate proboscis. Conversely, an ametamorphic life history does not pass through a veliger stage, but instead develops directly into a juvenile. Finally, if development passes through a veliger stage (free-living or encapsulated) the veliger or veliger-like embryo may be feeding or non-feeding. In the majority of caenogastropods, development includes a free-living veliger larva that captures and consumes microalgae; this is referred to as planktotrophic. Alternatively, development may include a veliger (free-living or encapsulated) that does not feed, but is provisioned by maternally provided yolk; this non-feeding veliger or veliger-like embryo is called lecithotrophic. Other unique life histories exist within gastropods. For instance, embryos of adelphophagic, direct developing species feed on nurse eggs (abortive sibling embryos) within the egg capsule. As illustrated in Figure 2, the four main gastropod life histories can be described as: A) indirect planktotrophic B) indirect lecithotrophic C) direct metamorphic D) direct ametamorphic.

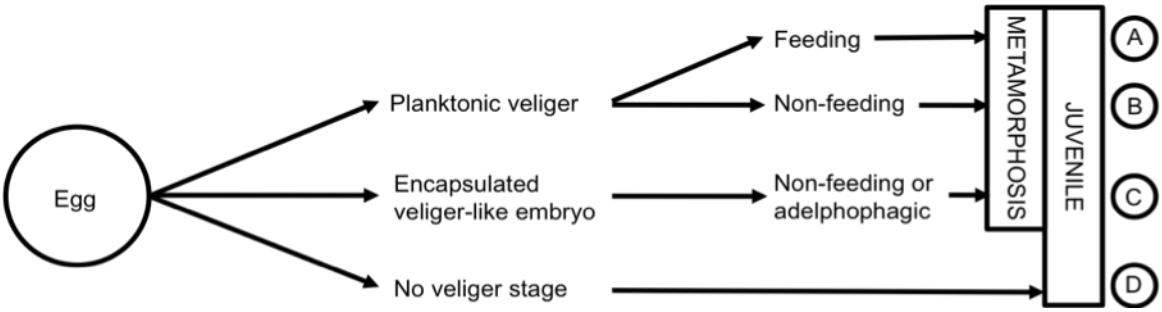

Figure 2. Summary of major life history patterns found in gastropods adapted from Bonar (1978).

A. Indirect planktotrophic. **B.** Indirect lecithotrophic. **C.** Direct metamorphic. **D.** Direct ametamorphic.

There is much variety in the morphology of gastropod veliger larvae between species and between developmental modes; nonetheless, a generalized veliger can be described as follows. The veliger has a calcified larval shell (protoconch), a foot, and velar lobes. The velar lobes usually consist of two flattened extensions of the head, which are used for swimming and sometimes feeding. Encapsulated veliger-like embryos, however, use the velar lobes to rotate within the egg capsule, which may aid in gas exchange (Hunter and Vogel, 1986) and uptake of capsular albumin (Rivest, 1992). At the edge of the velar lobes are two ciliary bands: the prototroch and the metatroch. The prototroch (or pre-oral ciliary band) consists of a band of long compound cilia, which produce swimming and feeding currents. The metatroch (or post-oral ciliary band) consists of a band of shorter cilia that beat towards the prototroch. Together, the prototroch and metatroch produce an opposed band feeding mechanism to capture food particles (Strathmann and Leise, 1979). Between these ciliary bands is the food groove, which gives rise to short cilia that transfer collected food to the mouth.

Current phylogenies suggest that the ancestral gastropod had an indirect lecithotrophic (non-feeding) veliger larva as is currently present in the basal clades: Patellogastropoda and Vetigastropoda (Figure 3**;** Haszprunar *et al.,* 1995; Lindberg and Guralnick, 2003; Aktipis *et al.,* 2008). Although members of these clades have a prototroch for swimming, they are unable to capture food and do not demonstrate use of the opposed band feeding mechanism (Hadfield *et al.,* 1997). However, the ancestor of the Neritimorpha, Caenogastropoda, and Heterobranchia is thought to have acquired a planktotrophic larva (Figure 3**;** Lindberg and Guralnick, 2003; Aktipis *et al.,* 2008). Indeed, the majority of extant species in these clades have a planktotrophic larva in their development (Thompson, 1976; Fretter and Graham, 1994; Kano, 2006). Additionally, the similarities in feeding and digestive systems in planktotrophic larvae from these three clades are consistent with the hypothesis that they share a common ancestor that acquired a feeding larval stage (Page and Ferguson, 2013). It should be noted, however, that alternative phylogenies do not place the Neritimorpha as sister group to the Caenogastropoda and Heterbranchia and thus there may have been two origins of planktotrophy within the gastropods (Haszprunar *et al.,* 1995; Ponder, 1991). Nevertheless, all phylogenies agree with the hypothesis that the ancestral condition in the

Caenogastropoda was planktotrophic development. However, numerous shifts in life history pattern have occurred in caenogastropods. For instance, secondary loss of a feeding larva has occurred independently numerous times in the caenogastropods (Hadfield and Iaea, 1989; Duda and Palumbi, 1999; Collin, 2004) and there is evidence that larval planktotrophy was reacquired after loss of a feeding larva in calyptraeid gastropods (Collin, 2004; Collin *et al.*, 2007).

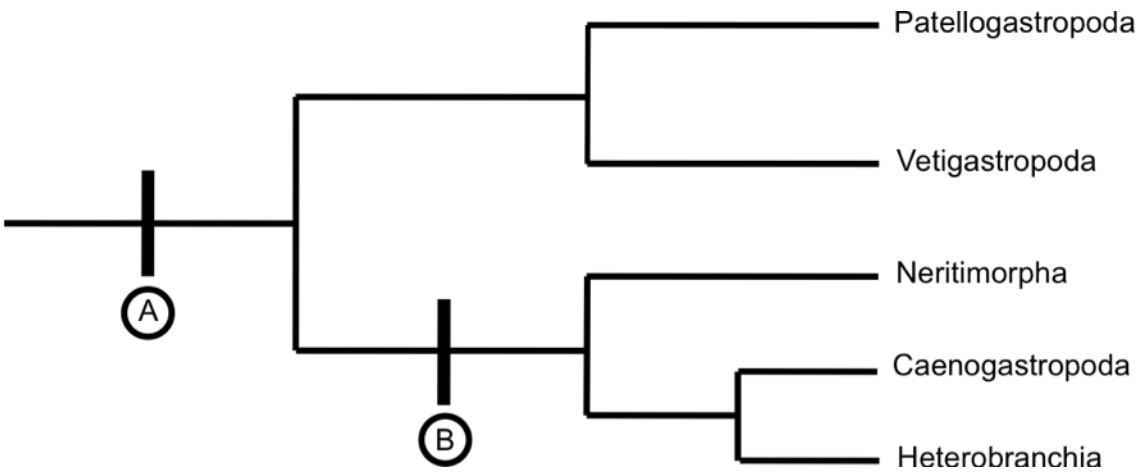

Figure 3. Current phylogeny of major gastropod clades as adapted from Lindberg and Guralnick (2003) and Aktipis *et al.* (2008).

A. Indirect lecithotrophic larva. **B.** Indirect planktotrophic larva.

1.3 Caenogastropod foregut development

Emergence of novel feeding strategies in post-metamorphic caenogastropods was complicated by the fact that it most likely occurred in the context of an indirect, planktotrophic life history. Unlike invertebrates that undergo radical changes at metamorphosis (*e.g.* echinoid echinoderms), caenogastropods undergo relatively modest metamorphic changes following settlement and definitive structures are largely formed by elaboration of larval structures during the larval stage (Page, 2002, 2005). Thus, in order for a novel feeding strategy to arise in the adult, two requirements must be met: a) the larva must be able to capture microalgae unimpeded during development and b) the definitive foregut must be ready for use by the juvenile shortly after metamorphosis (Fretter, 1969; Page, 2000). The larva requires a spacious, ciliated chamber for capture and consumption of microalgae, whereas the the juvenile feeding requirements may be markedly different. Therefore, specialized adult feeding structures may be realized through a novel developmental program that works around larval constraints (*i.e.* the structural limitations of the simple ciliated larval mouth and esophagus) or by loss of a functional larval stage (Page, 2000). Previous work has examined foregut development in both herbivorous and predatory caenogastropod species to understand how predatory feeding arose in the latter group (Table 1).

Table 1: Studies that investigated foregut development in a caenogastropod

Species and Superfamily	Development	Adult feeding habit	Proboscis type	*Foregut novelty	Reference
Lacuna vincta, Littorinoidea	Indirect planktotrophic	Herbivorous grazing with radula	n/a	No	Fretter, 1969; Page, 2000
Trichotropis cancellata, Capuloidea	Indirect planktotrophic	Ctenidial suspension feeder and kleptoparasite	Pseudo-proboscis	No	Parries and Page, 2003
Euspira lewisii, Naticoidea	Indirect planktotrophic	Predator: bivalves, gastropods, scaphopods	Acrembolic	**Yes	Page and Pedersen, 1998; Page, 2000
Marsenina stearnsii, Lamellaroidea	Indirect planktotrophic	Predator: colonial ascidians	Short pleurembolic	**Yes	Page, 2002
Nassarius incrassatus, Buccinoidea	Indirect planktotrophic	Carnivore: scavenger	Pleurembolic	Yes	Abro, 1969; Fretter, 1969
Nassarius mendicus, Buccinoidea	Indirect planktotrophic	Carnivore: scavenger	Pleurembolic	Yes	Page, 2000, 2005
Nucella lapillus, Muricoidea	Direct adelphophagic	Predator: bivalves, barnacles	Pleurembolic	No	Ball *et al*., 1997a, b; Ball, 2002
Conus anemone, Conoidea	Indirect lecithotrophic	Predator	Intraembolic	No	Ball, 2002
Conus lividus, Conoidea	Indirect planktotrophic	Predator	Intraembolic	Yes	Page, 2011

*Developmental novelty is defined here as the physical separation of the dorsal and ventral foregut developmental modules and destruction of the larval mouth and distal larval esophagus at metamorphosis followed by formation of a new mouth after metamorphosis

**The aperture of the larval mouth was not destroyed at metamorphosis, but the lips of the aperture were replaced

1.3.1 Foregut development in herbivorous caenogastropods

Lacuna vincta (Littorinoidea) is an example of a caenogastropod that has retained the ancestral feeding mode of herbivorous grazing. The adult foregut of this species develops by elaboration of the larval esophagus (Figure 4A; Page, 2000). The larval esophagus is a simple ciliated tube that transfers microalgae from the mouth to the stomach (Fretter, 1969; Page, 2000). A patch of non-ciliated cells embedded in the ventral wall of the distal larval esophagus forms an out-pocketing at approximately 20-30% of completed larval development (Page, 2000). The ventral out-pocketing is subsequently elaborated into definitive foregut structures, including the dorsoventrally flattened buccal cavity, the radular sac that secretes the radula, and the salivary glands (Page, 2000). Because the definitive foregut develops from a ventral trough beneath the larval esophagus, the ciliated larval esophagus remains as an unimpeded conduit for microalgae (Fretter, 1969; Page, 2000). At metamorphosis, the distal larval esophagus is reduced in size but it is nevertheless retained as the ciliated dorsal food groove that extends down the roof of the buccal cavity and anterior esophagus of the juvenile and adult stages (Page, 2000). Additionally, the radula extends onto the floor of the buccal cavity and supportive odontophoral cartilages and intrinsic musculature differentiate in association with the buccal cavity (Fretter, 1969; Page, 2000).

A similar developmental pattern was also identified in *Trichotropis cancellata* (Capuloidea), an herbivorous species with a highly specialized feeding mode. *T. cancellata* feeds by ctenidial suspension feeding and kletoparasitism using a pseudoproboscis to redirect streams of algae away from the radioles of feeding polychaetes (Pernet and Kohn, 1998; Parries and Page, 2003). Like *L. vincta,* the planktotrophic larvae of *T. cancellata* have a ciliated larval esophagus and a ventral out-pocketing of non-ciliated cells develops into the buccal cavity and radular sac. The developing buccal cavity and radular sac remain connected to the larval esophagus, but thickened lateral ridges constrict the connection and enable the larval esophagus to transmit algal cells. At metamorphic competence, the pseudoproboscis, a novelty of larval development in this species, forms as an enlarged swelling of the lower lip with a median strip of elongate cilia. Because the pseudoproboscis does not disrupt larval feeding, it develops nearly to completion in the larva. Similar to the metamorphic

conversion of the larval esophagus of *L. vincta*, the larval esophagus of *T. cancellata* transformed into the dorsal food groove of the buccal cavity and anterior esophagus at metamorphosis. Juveniles of *T. cancellata* were able to feed with their pseudoproboscis within several hours to several days after metamorphosis (Parries and Page, 2003). The functional requirements for feeding in both the larva and the juvenile/adult were clearly met in these examples.

1.3.2 Proboscis and foregut development in predatory caenogastropods

In the predatory caenogastropods, *Euspira lewisii* (Naticoidea) and *Marsenina stearnsii* (Lamellaroidea), foregut development began much like that of *L. vincta* with formation of a ventral out-pocketing beneath the larval esophagus. However, in these species the ventral out-pocketing bifurcated and formed anterior and posterior projections with only a narrow connection to the larval esophagus (Figure 4B; Page and Pedersen, 1998; Page, 2000, 2002). The anterior projection of the out-pocketing formed the definitive buccal cavity with jaws in the form of lateral serrated plates. The posterior projection of the out-pocketing formed the radular sac. At metamorphosis, the buccal cavity ruptured through the floor of the larval esophagus to expose the post-metamorphic jaws to the outside. This resulted in a transitory stage with two mouth apertures in *M. stearnsii* (Page, 2002). The portion of the larval esophagus distal to the connection to the ventral anlage was destroyed at metamorphosis allowing the definitive foregut to be slotted into place. Development of the jaws and the buccal mass during larval development allowed juveniles of both *M. stearnsii* and *E. lewisii* to adopt a predatory existence shortly after metamorphosis. By six days post-metamorphosis the ascidian prey of *M. stearnsii* (*Tridemnon opacum*) was observed in the guts of juveniles (Page, 2002). Three to five days after metamorphosis juveniles of *E. lewisii* began drilling holes in the shells of small bivalves (Pedersen and Page, 2000).

Studies on the neogastropods: *Nassarius incrassatus* and *Nassarius mendicus* (Buccinoidea) demonstrated a more profound spatial uncoupling between the larval esophagus and the ventral out-pocketing (or anlage) of the definitive foregut, which facilitated development of the complex foregut of predators during the larval stage. Abro (1969), as summarized by Fretter (1969), demonstrated that foregut development in *N.*

incrassatus began with a ventral out-pocketing beneath the larval esophagus, which elaborated into a "<" shape. This anlage of cells gave rise to the entire distal foregut of the adult including: the buccal cavity, radular sac, anterior esophagus, valve of Leiblein, and the proboscis itself. The anterior tip of the "<" developed into the buccal cavity, the ventral arm became the radular sac, and the dorsal arm became the anterior esophagus and valve of Leiblein. The definitive foregut remained attached to the larval esophagus by a small duct at the site of initial out-pocketing, which in late stage larvae was the valve of Leiblein. At metamorphosis, the larval mouth became the adult mouth, but the rest of the larval esophagus between this opening and the valve of Leiblein was occluded. The buccal cavity then opened to the exterior and the juvenile began feeding with a small proboscis (Abro, 1969; Fretter, 1969).

Subsequent studies on *Nassarius mendicus* confirmed much of what Abro (1969) observed for *N. incrassatus*. However, the later study suggested that Abro misinterpreted the fate of the mouth and the development of the proboscis. Similar to *N. incrassatus*, the ventral out-pocketing of *Nassarius mendicus* formed a tripartite (人) shape in lateral view that connected to the larval esophagus by a narrow duct at the valve of Leiblein (Figure 4C; Page, 2005). The anterior most portion of the out-pocketing formed the buccal cavity, the posterior section formed the radular sac, and the long neck connecting to the larval esophagus formed the elongate anterior esophagus and the valve of Leiblein. In both *N. incrassatus* and *N. mendicus* the elongate anterior esophagus and the valve of Leiblein can be interpreted as elaborations of the buccal cavity. Contrary to Abro's interpretation, Page (2005) showed via scanning electron microscopy that the larval mouth was sealed shut at metamorphosis and a transitory stage occurred at this time that lacked a mouth. The larval mouth did not form the definitive mouth, but rather a new mouth was formed as the definitive buccal cavity ruptured to the outside. The portion of the larval esophagus anterior to the valve of Leiblein was destroyed at metamorphosis and thus the dorsal food groove of ancestral gastropods is not retained in the post-metamorphic foregut of *N. mendicus*. The juvenile acquired an elongate proboscis and was capable of feeding by three days after metamorphosis (Page, 2005).

The neogastropod *Conus lividus* (Conoidea) has a highly derived post-metamorphic foregut and thus differences in foregut development are not surprising. In

this species, like other members of the Conoidea, the extra length of foregut that extends down the proboscis consists of a highly elongate oral tube, rather than anterior esophagus. During planktotrophic larval development of *C. lividus* a distinctive ventral trench of non-ciliated cells extend down most of the ciliated larval esphagus (Page, 2011). The anterior portion of this trench formed the post-metamorphic oral tube, whereas a diverticulum extending from the posterior region of the ventral trench formed the radular sac, which came to sit on the right side. At metamorphosis, destruction of the larval mouth and larval esophagus resulted in a stage that had no mouth. Despite differences in foregut development, *C. lividus* still demonstrated uncoupling of the larval esophagus and the developing definitive foregut and destruction of the larval mouth and distal larval esophagus at metamorphosis.

Pre-metamorphic development of the pleurembolic and intraembolic proboscis types followed a similar pattern in the species examined (*i.e. Nucella lapillus,* Ball *et al.*, 1997a, b; *Conus anemone,* Ball *et al.,* 2002; *Marsenina stearnsii*, Page, 2002; *Nassarius mendicus,* Page, 2000; Page, 2005; *Conus lividus,* Page, 2011). In general, the initial development of the proboscis involved formation of a short snout of thickened epidermis surrounding the larval mouth. The proboscis sac began as dorsal and ventral invaginations of the head region. Immediately following metamorphic loss of the velar lobes, the invaginations became confluent laterally to form a continuous sac surrounding the proboscis. After metamorphosis, the proboscis elongated and led to an anterior displacement of foregut structures.

1.3.3 Foregut development in predators with direct development

Descriptions of foregut development in species that have lost the planktotrophic larval stage do not mention the presence (or loss at metamorphosis) of the larval esophagus (*i.e.* the esophagus used for feeding in planktotrophic larvae). For example, the neogastropod, *Nucella lapillus* (Muricoidea), is a direct developing adelphophagic species that passes through a veliger-like stage during its fully encapsulated development (Stockmann-Bosbach, 1988). Ball *et al.* (1997a, b) and Ball (2002) examined proboscis and foregut development in late stage embryos of *N. lapillus* that had finished consuming nurse eggs (Figure 4D). The buccal cavity and radular sac developed beneath an

esophagus that was present in developmental stages. However, this esophagus was not identified as the homologue of the larval esophagus, but rather the esophagus and mouth present prior to metamorphosis were presumably carried through to the juvenile stages. Similarly, vestiges of the larval esophagus were not identified in the neogastropod *Conus anemone* (Conoidea), a species with a short lived non-feeding larva (Ball, 2002). The developmental pattern described for *N. lapillus* and *C. anemone* was incongruent with the developmental trajectory observed in predatory caenogastropods with planktotrophic development. This certainly warrants further investigation of foregut development in direct developing neogastropods.

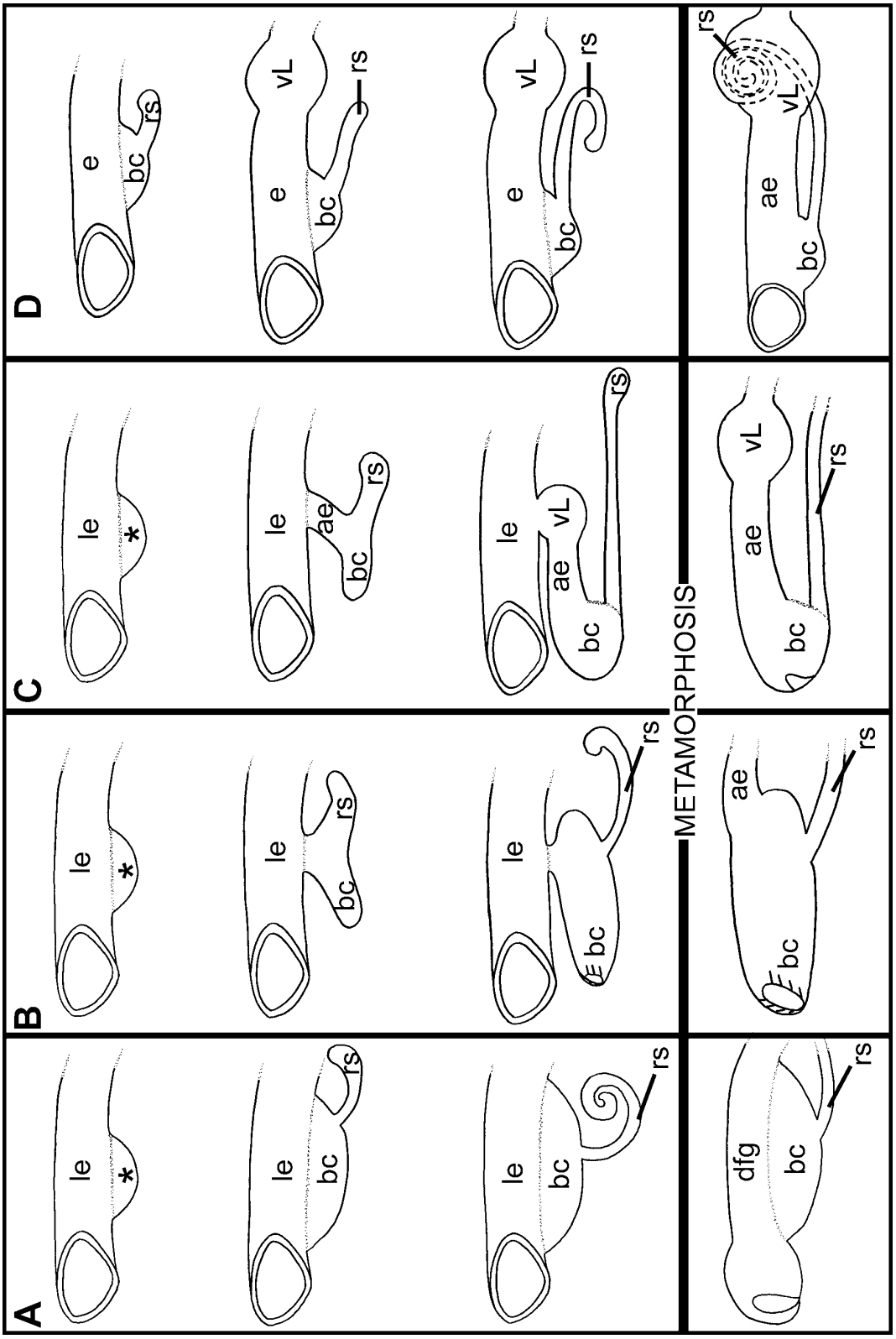

Figure 4. Comparative foregut development in caenogastropods adapted from Page, 2000 and Ball *et al.,* 1997a, b and Ball, 2002.

A. Foregut development in the herbivorous caenogastropod, *Lacuna vincta*; notice the retention of the larval esophagus as the dorsal food groove after metamorphosis; this species has indirect planktotrophic development. **B.** Foregut development in the predatory caenogastropod, *Euspira lewisii*; notice the destruction of the distal larval esophagus at metamorphosis, which allows the post-metamorphic buccal cavity with jaws to be used for feeding in the juvenile; this species has indirect planktotrophic development. **C.** Foregut development in the predatory neogastropod *Nassarius mendicus*; notice that the entire post-metamorphic anterior esophagus is derived from the ventral out-pocketing and that the distal larval esophagus anterior to the valve of Leiblein is destroyed at metamorphosis; this species has indirect planktotrophic development. **D.** Foregut development in the predatory neogastropod *Nucella lapillus*; notice that the mouth and esophagus present in development stages are retained through metamorphosis; this species has direct adelphophagic development. Abbreviations: ae= anterior esophagus, bc= buccal cavity, dfg= dorsal food groove, e= esophagus present in developmental stages of *N. lapillus*, le= larval esophagus, rs= radular sac, vL= valve of Leiblein.

1.4 Hypotheses about foregut evolution

Studies of foregut development in caenogastropods with a planktotrophic larval stage have demonstrated the presence of dorsal and ventral foregut developmental modules (semi-autonomous components of a developing organism), which have different ontogenetic fates (*e.g.* Werner, 1955; Fretter, 1969; Thiriot-Quiévreux, 1969, 1974; Page, 2000, 2002, 2005, 2011; Parries and Page, 2003). The dorsal module consists of the larval esophagus, which in some species is retained after metamorphosis as the dorsal food groove. The ventral module consists of post-metamorphic foregut components such as the buccal cavity, radular sac, and acinous salivary glands. In caenogastropod foregut evolution the dorsal and ventral modules have demonstrated both temporal and spatial dissociability, which Raff (1996) has identified as a hallmark feature of a developmental module. Two important novelties in foregut development, which involve uncoupling of the dorsal and ventral modules, likely had a strong influence on the success of the caenogastropods. The first involves temporal separation between the appearance of the dorsal and ventral modules during development and the second involves the physical separation of the dorsal and ventral modules during development.

Temporal separation resulting in early development of the dorsal module relative to the ventral module may have given rise to larval planktotrophy. Page (2011, 2012) proposed that the dorsal module (post-metamorphic dorsal food groove) was co-opted by the larval stage to serve as a ciliated larval esophagus when feeding larvae evolved within the Gastropoda. Essentially, a functional larval gut may have been borrowed from a portion of the adult gut of ancient gastropods, so as to permit larval feeding (Page and Ferguson, 2013). This homology is suggested by the development of the dorsal food groove in herbivorous species with a planktotrophic larva, *e.g. Lacuna vincta*. At metamorphosis, the larval esophagus of this species is directly transformed into the dorsal food groove of the adult stage (Page, 2000). A heterochronic shift (evolutionary change in developmental timing) may have resulted in early development of one component of the adult foregut, the dorsal food groove, relative to the development of the ventral components of the adult foregut. The early development of the dorsal food groove may have permitted this adult structure to be co-opted by the larva for feeding on microalgae, thus facilitating the emergence of larval planktotrophy.

Furthermore, a conserved developmental trajectory, which allowed for development of most of the components of the complex post-metamorphic foregut prior to actual metamorphosis, has been identified in: *Euspira lewisii, Marsenina stearnsii, Nassarius mendicus,* and *Conus lividus*. In caenogastropods with highly derived structures for post-metamorphic feeding, the ventral module not only develops later than the dorsal module, but it also develops in almost complete separation from the dorsal module. The physical separation of the dorsal developmental module (larval esophagus) and the ventral developmental module (post-metamorphic foregut) allowed for both larval feeding and simultaneous development of the complex foregut of predators. At metamorphosis the larval mouth and distal larval esophagus were destroyed, allowing the post-metamorphic foregut to be slotted into place and after metamorphosis a new mouth was formed. The spatial separation of the dorsal and ventral foregut developmental modules, destruction of larval structures at metamorphosis, and the formation of a new mouth after metamorphosis may have enabled predators to evolve unconventional designs for the definitive foregut, free from larval constraints.

Although this theory offers an explanation of how novel post-metamorphic feeding structures may have evolved in caenogastropods, this pattern was not identified in species that have lost a planktotrophic larval stage. This incongruence may be the result of one of two possibilities: either the difference in foregut development reflects separate origins of predatory feeding within the neogastropods, or changes in foregut development have accompanied the shift in life history and thus vestiges of the dorsal module (larval esophagus) were not recognized by Ball *et al.*, (1997a, b) and Ball (2002). Although the monophyly of the Neogastropoda is currently under debate (*e.g.* Cunha *et al.*, 2009), gastropod life history patterns are not reliable indicators of ancestry. That is, the loss of a planktotrophic larva has occurred independently numerous times in many lineages of gastropods, such as within the genus *Conus* (Duda and Palumbi, 1999). Instead, differences in foregut development between species with and without a planktotrophic larva most likely reflect different selective pressures associated with the shift in life history pattern rather than a difference in how development was originally modified to facilitate the evolution of a predatory feeding system.

For example, significant changes to larval feeding structures, such as the velar lobes of gastropods, have been shown to accompany the loss of a feeding larva (*e.g.* Hadfield and Iaea, 1989; Hofstee and Pernet, 2011). Similarly, loss of a feeding larva may result in changes to the larval esophagus. Essentially, reduced selective pressure for larval feeding structures may have quelled any reminiscence of a functional larval esophagus. However, because the dorsal developmental module (larval esophagus) functions as the source of cells for the ventral developmental module (post-metamorphic foregut) in species with the ancestral life history (indirect development), vestiges of the larval esophagus should still be apparent in derived species with direct development. Thus, it is puzzling that no vestigial larval esophagus *i.e.* no dorsal module was identified in *N. lapillus* or *C. anemone*. Overall, changes to the pattern of foregut development may accompany the loss of a feeding larval stage, which could potentially mask an important developmental novelty that was present in an ancestor. Therefore, careful examination of foregut development in direct developing species is necessary in order help resolve this controversy.

1.5 Objective of present study

I have investigated foregut development in two direct developing neogastropods: *Nucella lamellosa* (Gmelin, 1791) and *Nucella ostrina* (Gould, 1852). *N. lamellosa* and *N. ostrina* are predatory species that drill into the shells of their prey, including barnacles and bivalves, using a chemomechanical mechanism. An accessory boring organ on their foot produces acidic secretions, which softens the calcareous shells of their prey (Clelland and Saleuddin, 2000). The radula, which is at the tip of a pleurembolic proboscis, rasps at the softened area. Repeated alternating applications of the accessory boring organ and the radula eventually results in a borehole. The extended proboscis can then be inserted through the borehole to feed on the soft tissues within. Both these species exhibit a complex post-metamorphic foregut specialized for predatory feeding including: an elongate, muscularized anterior esophagus, valve of Leiblein (a specialized portion of the esophagus), acinous and accessory salivary glands, and a highly developed gland of Leiblein (an absorptive and secretory glandular structure) in the mid-esophagus.

N. lamellosa and *N. ostrina* are congenerics of *N. lapillus*, which was the subject of a previous study on foregut development by Ball *et al.* (1997a, b). All three species have direct metamorphic life histories (Figure 2C) and thus pre-metamorphic development in these species takes place within benthic egg capsules and they all hatch as crawl-away juveniles. Additionally, all three species pass through a veliger-like stage during embryonic development and undergo metamorphosis, defined as loss of the velar lobes, prior to juvenile hatching. However, whereas *N. lamellosa* is non-feeding (lecithotrophic), *N. ostrina*, like *N. lapillus*, is adelphophagic and feeds on nurse eggs during early embryonic stages.

This study was conducted to determine if vestiges of the larval esophagus are present in species that have lost a planktotrophic larval stage. Furthermore, I looked for evidence of dorsal and ventral modules that were dissociable during foregut development. The sequence of events during foregut development was examined in these direct developing species to determine if foregut development followed a pattern that was reminiscent of the developmental trajectory previously observed in species with indirect planktotrophic development. Finally, differences in foregut development associated with a shift in life history were investigated by comparing a species with non-feeding developmental stages, *N. lamellosa,* and a species with feeding (adelphophagic) developmental stages, *N. ostrina*. Overall, this study set out to reconcile the profoundly different descriptions of foregut development given for predatory neogastropods with indirect planktotrophic development (*i.e. Nassarius mendicus*, Buccinoidea) and with direct development (*i.e. Nucella lapillus*, Muricoidea).

2.0 Materials and Methods

A more detailed analysis of foregut development was conducted for *Nucella lamellosa* than for *Nucella ostrina*. Developmental stages of *N. lamellosa* were reared in the laboratory from oviposition to juvenile hatching, whereas developmental stages of *N. ostrina* were obtained by opening field collected egg capsules and the chronological order of developmental stages was reconstructed. In both species, foregut development was investigated with histological sectioning at key developmental stages and sections were examined with a light microscope. However, surface-rendered 3D reconstructions and additional analysis of developing tissues by transmission electron microscopy were conducted for *N. lamellosa* only.

2.1 Collections, culture, and developmental timetable

Nucella lamellosa

Adults and egg capsules of *N. lamellosa* were collected from Telegraph Bay, Victoria, BC, Canada on January 10, 2013. Adults, together with their deposited egg capsules, were found in large breeding aggregations on the sides of rocks in the intertidal zone at low tide. Egg capsules were removed from the substrate by wedging a sharp knife under the attachment points of the egg capsules. Adults and egg capsules were slowly introduced to the re-circulating seawater system at the University of Victoria, where they were maintained at temperatures ranging from 11-12˚C.

Adults were placed in a large aquarium with flowing seawater. From January 10 to February 8, 2013 the adults deposited egg capsules on the sides of the glass aquarium. Periodically I removed clumps of egg capsules from the adult aquarium, however, I also left egg capsules on the side of the adult tank as a control to ensure that removal did not alter or negatively affect development. Although clumps of egg capsules were circled and date of oviposition marked with a wax pen, determining the exact timing of egg capsule deposition proved difficult because adults attached new egg capsules at random to older egg capsules. Egg capsules removed from the adult tank and egg capsules collected from Telegraph Bay (January 10, 2013) were maintained in large glass bowls with flowing seawater.

When juveniles began to hatch from egg capsules, the capsules were placed in small mesh containers. Hatched juveniles were then transferred from the mesh containers to sealed 1.41 L Glad Ware® freezer containers with four mesh-covered windows cut into the sidewalls. These were submerged in the seatable with flowing seawater. Small rocks with newly metamorphosed barnacles were added to these containers to provide food for the juveniles. The barnacles were collected from the shoreline of Mount Douglas Park, Victoria on April 13, 2013. Feeding began by April 15, 2013, as evidenced by one or more drill holes in the rostral shell plate, scutum, or tergum of the barnacles. The barnacles with drill holes were devoid of living tissues.

Embryos were frequently removed from their egg capsules to track the timing of development and the appearance of key features. I carefully removed embryos from their egg capsules using iridectomy scissors and fine forceps under a dissecting microscope. Holding onto the base of the egg capsule I cut off the top and made an incision down the length of the capsule. Subsequently, I cut off the base of the capsule and using two pairs of forceps, held the capsule open and gently agitated the embryos out of the capsule. Embryos were photographed using an Olympus DP72 Digital Camera attached to an Olympus stereomicroscope and Olympus DP2-BSW computer software. Developmental stages approximately one week apart could be discerned through careful examination of external anatomy.

Nucella ostrina

Egg capsules of *N. ostrina* were collected from East Sooke, near Victoria, BC, Canada on April 28, 2013. The egg capsules of *N. ostrina* were found in cracks and crevices of the rocky intertidal at an exposed site with moderate to heavy wave action. Egg capsules were maintained in the re-circulating seawater system at the University of Victoria in large glass bowls with flowing seawater. No adults of *N. ostrina* were collected.

Embryos of *N. ostrina* were liberated from egg capsules and categorized into developmental stages using methods described above for *N. lamellosa.* Unlike *N. lamellosa*, however, egg capsules were not maintained throughout development. The field collected egg capsules contained, by chance, embryos at various stages of

development. Many capsules were opened to acquire the full range of developmental stages.

2.2 Preparation of specimens for histological sectioning, transmission electron microscopy (TEM), and scanning electron microscopy (SEM)

Excapsulated developmental stages of *N. lamellosa* and *N. ostrina* were anaesthetized and fixed at weekly intervals, not including the earliest stages (*i.e.* pre-veliger-like stages). Embryos were liberated from their egg capsule and placed in an 8 mL glass vial for subsequent processing. Embryos were anaesthetized to prevent contraction of musculature. Anesthesia was achieved by slowly replacing regular seawater in the fixation vial with an artificial seawater solution containing high Mg^{2+} and low Ca^{2+} concentrations (Audesirk and Audesirk, 1980). Every 15 to 20 minutes, a portion of the seawater in the vial was removed and replaced with high Mg^{2+} /low Ca^{2+} seawater until the embryos were in full strength high Mg^{2+} /low Ca^{2+} seawater. Time for anesthesia ranged from 1.5 to 3 hours depending on the developmental stage. Advanced stages required longer periods of anesthesia, particularly when they were capable of contracting into their shell. During anesthesia, the temperature of the vial was maintained at 12˚C.

Once the embryos were sufficiently relaxed, a stronger anesthetic was used. Three drops of chlorotone were added to the vial and swirled vigorously to mix the solution. This was repeated 6 times at 1.5 minute intervals with the solution in the vial maintained just above 0˚C (vial surrounded by scant shards of ice). Subsequently, the anaesthetizing solution was removed and replaced with a glutaraldehyde primary fixative consisting of 2.5% glutaraldehyde, 0.2 M phosphate buffer (pH 7.6) and 0.14 M sodium chloride (Cloney and Florey, 1968). The fixative was then replaced with a second fill of 2.5% glutaraldehyde fixative. Specimens were left in the primary fixative for a minimum of 12 hours and a maximum of 7 days at 8˚C.

After primary fixation, the solution in the vial was replaced with a 1:1 solution of 2.5% glutaraldehyde fixative and 10% ethylenediaminotetraacetic acid, disodium salt (EDTA). The EDTA is a calcium chelating agent that decalcified the shells of the embryos. The decalcifying solution in the vial was replaced every 1 hour to 12 hours for

a total decalcification period of 3 hours to 2 days at room temperature. Advanced embryos with heavily calcified shells required the much longer decalcification periods.

Once decalcification was complete, specimens were rinsed three times for 15 minutes each in 2.5 % sodium bicarbonate ($NaHCO_3$) buffer (pH 7.2) at room temperature. Next, the embryos were post-fixed in a 2% solution of osmium tetroxide (OsO_4) at room temperature. The 2% OsO_4 was a 1:1 solution of 2.5% $NaHCO_3$ and 4% OsO_4. After an hour of post-fixation, the solution was removed and replaced twice with 2.5% $NaHCO_3$ to rinse off the post-fixative.

Dehydration of the specimens was accomplished with a graded acetone dilution series (30%, 50%, 70%, 90%, 95%, and 4 x 100%). The specimens were left in each dilution for 20 minutes. Over a period of 24 hours, Embed 812 resin (an Epon 812 substitute; Electron Microscopy Sciences) was infiltrated into the tissues of the specimens. Finally, specimens were embedded in the Embed 812 resin and put in an oven at 60˚C for 2 to 3 days to polymerize the resin.

2.2.1 Histological sectioning

I identified major stages of foregut development in *N. lamellosa* and *N. ostrina* by sectioning through the foregut of multiple stages in various orientations and viewing serial sections with the light microscope. Sections were cut at 1 μm thickness using glass knives on a Leica Ultracut UCT microtome. Sections were placed on glass slides and the tissues were stained with methylene blue and azure II (Richardson *et al.,* 1960). The slides were made permanent by applying Permount and a glass cover slip. Serial sections were photographed in their appropriate sequence using a Zeiss Axioskop compound light microscope with a Retiga 200T digital camera attached; the computer software used was QCapture Pro 5.1 (QImaging). Contrast and brightness of images were adjusted with Adobe Photoshop CS3.

2.2.2 Transmission electron microscopy (TEM)

In order to confirm the fate of the larval esophagus in *N. lamellosa* ultrathin sections were cut through the foregut of pre- and post-metamorphic stages. Sections destined for transmission electron microscopy (TEM) were cut at 80-90 nm thickness

using a Diatome diamond knife on a Leica Ultracut UCT microtome. Ultra-thin sections were cut through the larval and definitive foregut in a pre-metamorphic stage and through the definitive foregut in a post-metamorphic stage. Sections were collected on copper grids and stained with a 2% aqueous solution of uranyl acetate for 1 hour followed by a 0.2% solution of lead citrate for 7.5 minutes. Sections were examined with a Hitachi H-7000 transmission electron microscope and contrast and brightness of digital images were adjusted with Adobe Photoshop CS3.

2.2.3 Scanning electron microscopy (SEM)

To document the external appearance of the velar lobes and the juvenile proboscis in *N. lamellosa,* veliger-like embryos and feeding juveniles were prepared for scanning electron microscopy. These specimens were prepared according to the procedure for histological sectioning as described previously; however, there were three important differences in the procedure. First, all solutions added to the vial were filtered through a 0.20 μm syringe filter to keep debris off the specimens. The shells of specimens destined for SEM were not decalcified. Finally, dehydration was achieved with a graded ethanol dilution series rather than with acetone.

After dehydration, the specimens were transferred to mesh baskets and put in the critical point dryer. The critical point dryer flushed out the ethanol and replaced it with liquid CO_2. When the critical temperature and pressure was reached, the CO_2 sublimated from the surface of the specimens. This allowed for controlled drying of the specimens without surface damage. The dried specimens were then mounted on metal stubs using an eyelash tool and double-sided tape. SEM images were taken with a Hitachi S3500N scanning electron microscope and contrast and brightness of images was adjusted with Adobe Photoshop CS3.

2.3 Surface- rendered 3D reconstructions of the foregut

Surface-rendered 3D reconstructions of the foregut in pre- and post-metamorphic stages of *N. lamellosa* were produced using Reconstruct (v. 1.1.00) (Fiala, 2005). Images of serial sections through the foregut were ordered from anterior to posterior and imported into Reconstruct. Images of the histological sections were size-calibrated to

represent the appropriate thickness *i.e.* 1 μm. All sections were used to produce the 3D reconstruction except for rare sections that were lost. Sections were aligned and specific structures in the foregut were manually traced using a graphics tablet. Key components of the foregut, *e.g.* the larval esophagus and definitive esophagus, were traced in separate profiles and are shown in different colours. Stacks of each profile were reconstructed and together represented the 3D morphology of the foregut. The reconstructed profiles were surface rendered using a Boissant surfacing algorithim. Images of the 3D reconstructions at various orientations were captured and imported into Adobe Photoshop CS3 for surface smoothing and other minor editing.

3.0 Results

3.1 Life history and development of *Nucella lamellosa*

Development from oviposition to juvenile hatching required approximately 12 weeks at 11 to 12°C and key features were recorded at weekly intervals (Table 2). Each egg capsule contained 20-50 eggs, each approximately 500-600 μm in diameter. Early embryos were very yolky and bright yellow (Figure 5A). The embryos passed through a veliger-like stage within the egg capsule, which was characterized by a pair of ciliated velar lobes extending from the head area (Figure 5B-D). The encapsulated veliger-like embryos acquired dark pigmentation on the foot, visceral mass, and velar lobes (Figure 5D), which was visible through the egg capsules approximately three weeks prior to hatching. After approximately 11-12 weeks of development the encapsulated veliger-like embryos lost their ciliated velar lobes, an event that characterized metamorphosis (Figure 5E and F). The velar lobes were involuted into each side of the head, as shown in histological sections. Metamorphosis was a rapid process as egg capsules were found in which half of the individuals had velar lobes and the other half had lost them.

Veliger-like embryos of *N. lamellosa* had well developed velar lobes (Figure 6A). Large cells giving rise to long prototrochal cilia extended around the edges of the velar lobes and came together mid-ventrally to form the upper lip of the larval mouth. Posterior views of the velar lobes showed that a fully developed metatroch and food groove were also present (Figure 6B and 10A). *N. lamellosa* retained all three ciliary bands, which is similar to what is observed in planktotrophic species that use an opposed band feeding mechanism.

Shortly after metamorphosis, juveniles with an approximate shell length of 1 mm hatched from egg capsules and were ready to hunt for small barnacles (Figure 7A and B). Feeding was evidenced two days after juveniles had access to the barnacles, by one or more drill holes in the rostral shell plate, scutum, or tergum of the barnacles and an absence of soft tissues within (compare Figure 7C and D). Secretion of the teleoconch (post-metamorphic shell), which had a distinctive sculpture relative to the protoconch, was evident after juveniles began feeding on barnacles (Figure 7E and F). Like adults,

juveniles of *N. lamellosa* appeared to use a chemo-mechanical process to bore holes into the calcium carbonate shells of their prey. Scanning electron micrographs of the foot of juveniles that had been feeding on barnacles showed the opening of the accessory boring organ on the ventral surface of the propodium (Figure 8A). Secretions from this glandular structure serve to soften the shell of prey and the radula at the tip of the extended proboscis rasps away at the softened area. The short proboscis with the mouth at the tip (Figure 8B) is then inserted into the borehole to feed on the soft tissues of the prey.

Table 2. Embryogenesis in *Nucella lamellosa* maintained at 11-12˚C

Stage	Time to achieve stage (weeks)	Key features
1	0-2	Cleavage to gastrulation
2	2-3	Early embryo; formation of ciliated cephalic vesicle and absorptive cells of the larval kidney complex; early formation of shell
3	3-4	Onset of veliger-like morphology; rudimentary velar lobes, foot, and shell; mouth visible
4	4-5	Eyespots; metapodium (small pointed foot)
5	5-6	Rounding of visceral mass
6	6-7	Propodium (formation of crawling surface of the foot); right absorptive cell at a more dorsal position; shell covers visceral mass; tentacle rudiments
7	7-8	Black pigmentation on velar lobes, foot, and visceral mass; capable of contracting into shell; siphonal notch on left side of shell; oval-shaped velar lobes; reduced absorptive cells of the larval kidney complex
8	8-9	Onset of 2nd shell whorl; shell aperture flattened dorso-ventrally; pigmentation visible through egg capsule; ctendium visible through shell
9	9-10	Velar lobes crumpled and reduced in size
10	10-11	Notch at front of propodium; shell flared out at dorsal margin
11	11-12	Both pre-metamorphic (11a) and post-metamorphic (11b) stages present in the same capsule; velar lobes were absent in half of the individuals in the capsule
12	12	Velar lobes absent in all individuals; long tentacles
Juvenile	13 (1 week post-hatch)	Hatched from egg capsule; capable of feeding on newly metamorphosed barnacle prey

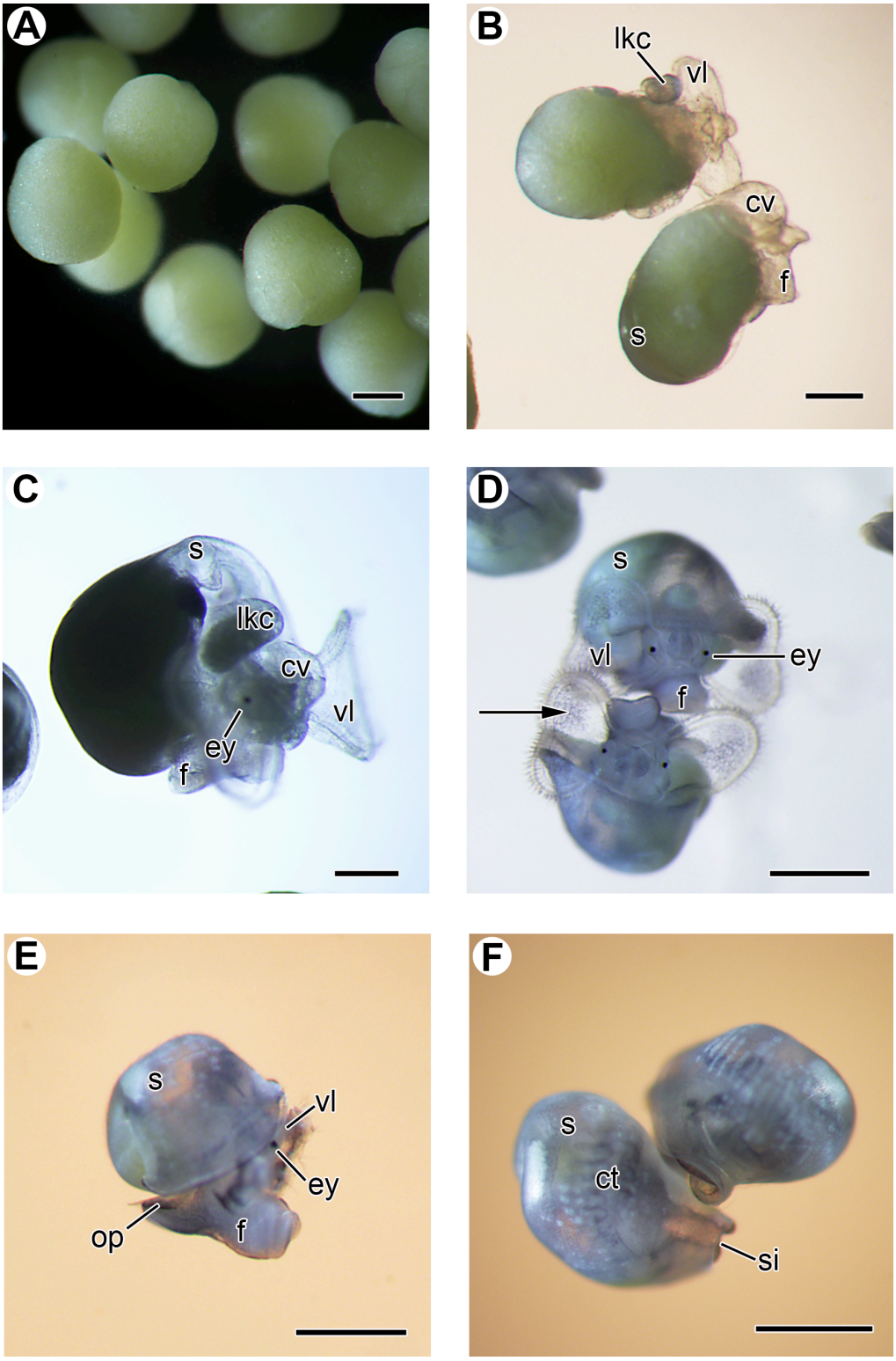

Figure 5. External features of encapsulated embryos of *Nucella lamellosa*.

A. Stage 1 embryos; scale bar= 200 μm. **B.** Stage 4 veliger-like embryos; scale bar= 200 μm. **C.** Stage 6 veliger-like embryo; scale bar= 200 μm. **D.** Stage 8 veliger-like embryos;

arrow indicates black pigmentation on the velar lobes; scale bar= 500 μm. **E.** Stage 11a pre-metamorphic veliger-like embryo with crumpled velar lobes (compare to D); scale bar= 500 μm. **F.** Stage 11b post-metamorphic embryos; scale bar= 500 μm. Abbreviations: ct= ctenidium, cv= cephalic vesicle, ey= eyespot, f= foot, lkc= larval kidney complex, op= operculum, s= shell, si= siphon, vl= velar lobe.

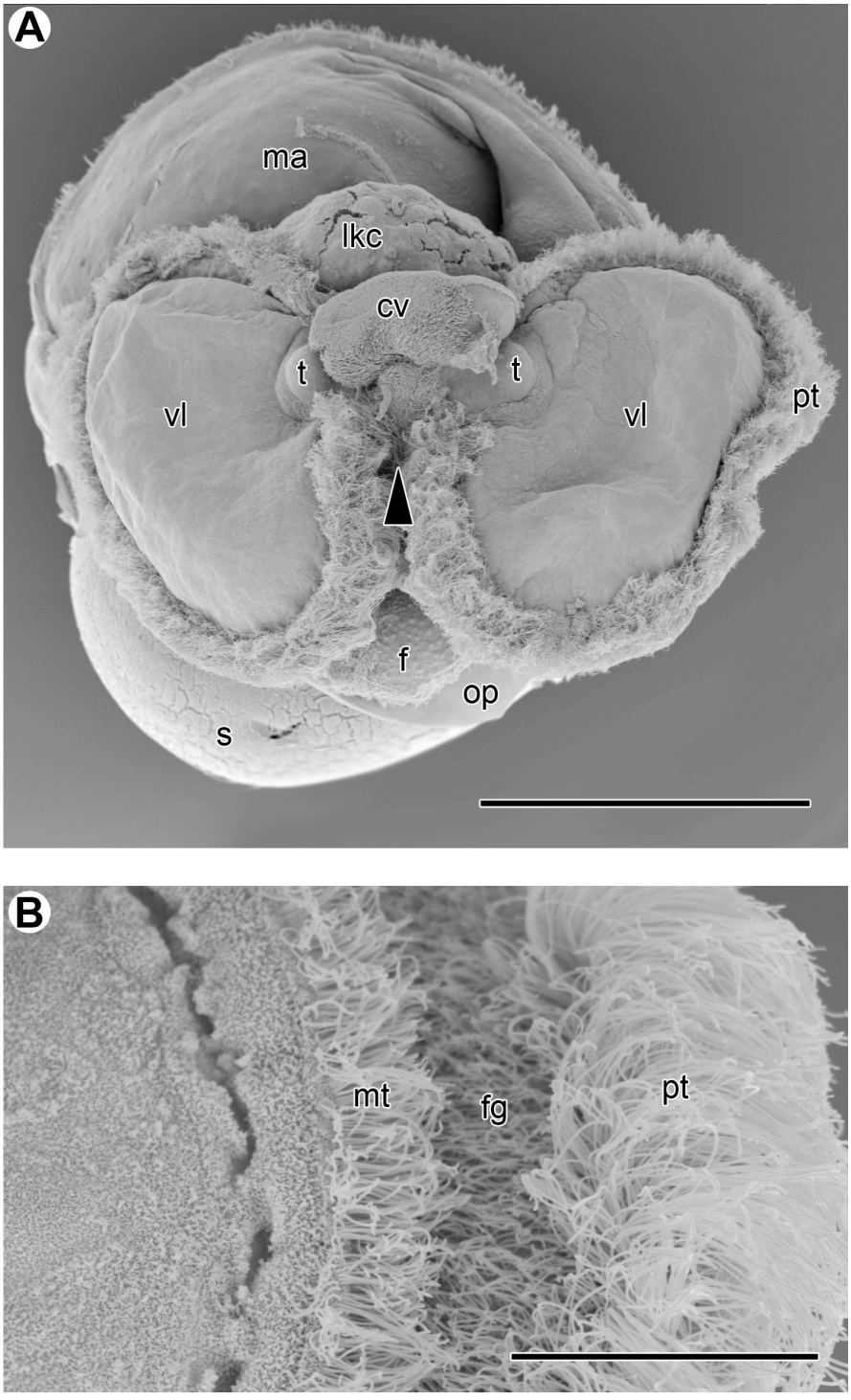

Figure 6. Scanning electron micrographs of stage 6 veliger-like embryos of *Nucella lamellosa*.

A. Anterior view showing well-developed velar lobes; location of the larval mouth is demarcated by an arrowhead; scale bar= 300 μm. **B.** Back edge of velar lobe showing

prototroch, metatroch, and food groove; scale bar= 30 μm. Abbreviations: cv= cephalic vesicle, f= foot, fg= food groove, lkc= larval kidney complex, ma= mantle, mt= metatroch, op= operculum, pt= prototroch, s= shell, t= tentacle, vl= velar lobe.

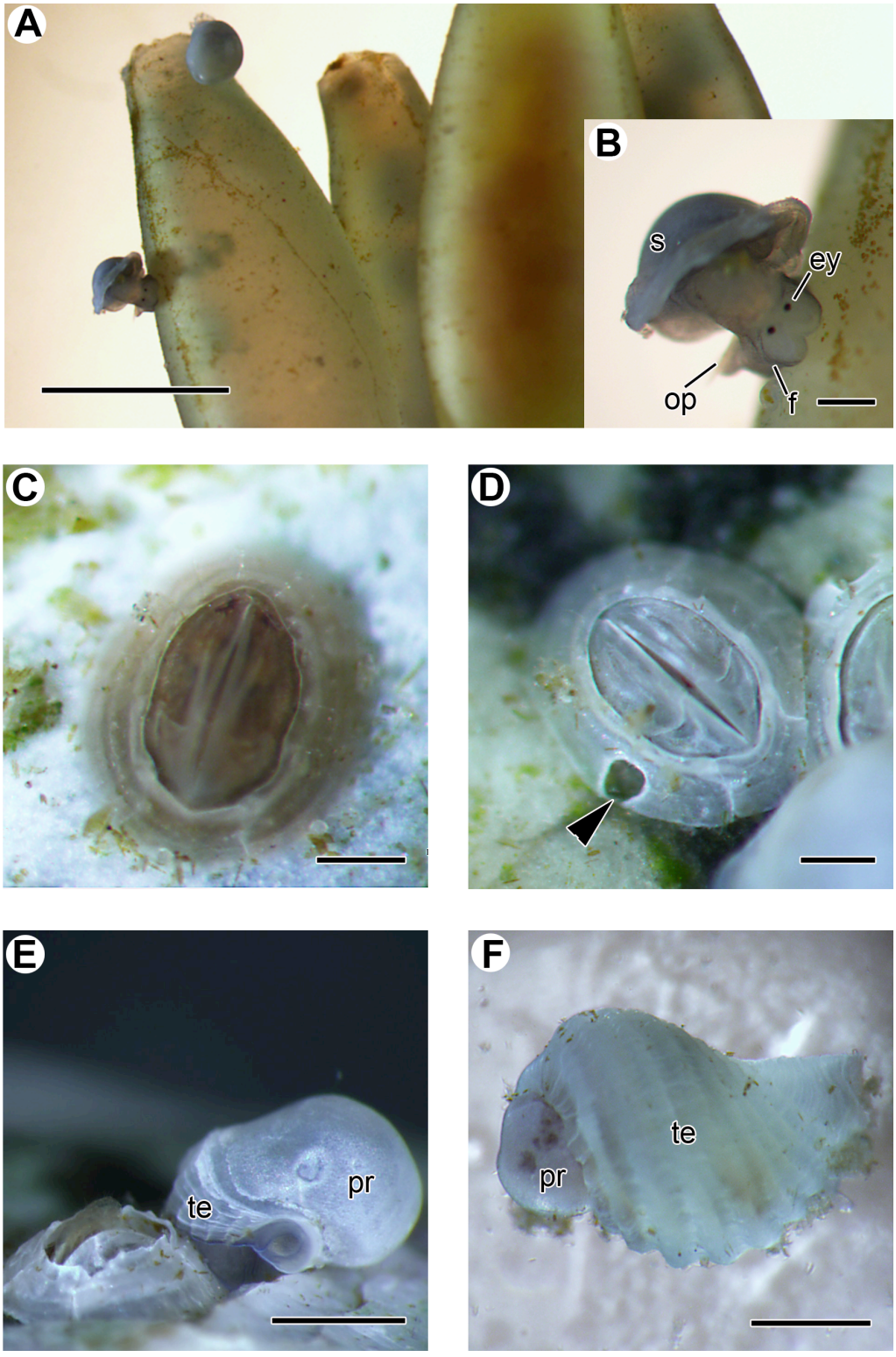

Figure 7. Juveniles and juvenile feeding in *Nucella lamellosa*.

A. Juveniles recently hatched from egg capsules; scale bar= 2 mm. **B.** Enlargement of juvenile in A; scale bar= 200 μm. **C.** Newly metamorphosed, living barnacle; scale bar=

200 μm. **D.** Dead barnacle, devoid of internal tissues and with a tiny drill hole (arrowhead); scale bar= 200 μm. **E.** Feeding juvenile; scale bar= 500 μm. **F.** Large juvenile with teleoconch; scale bar= 500 μm. Abbreviations: ey= eyespot, f=foot, op= operculum, pr=protoconch, s=shell, te=teleoconch.

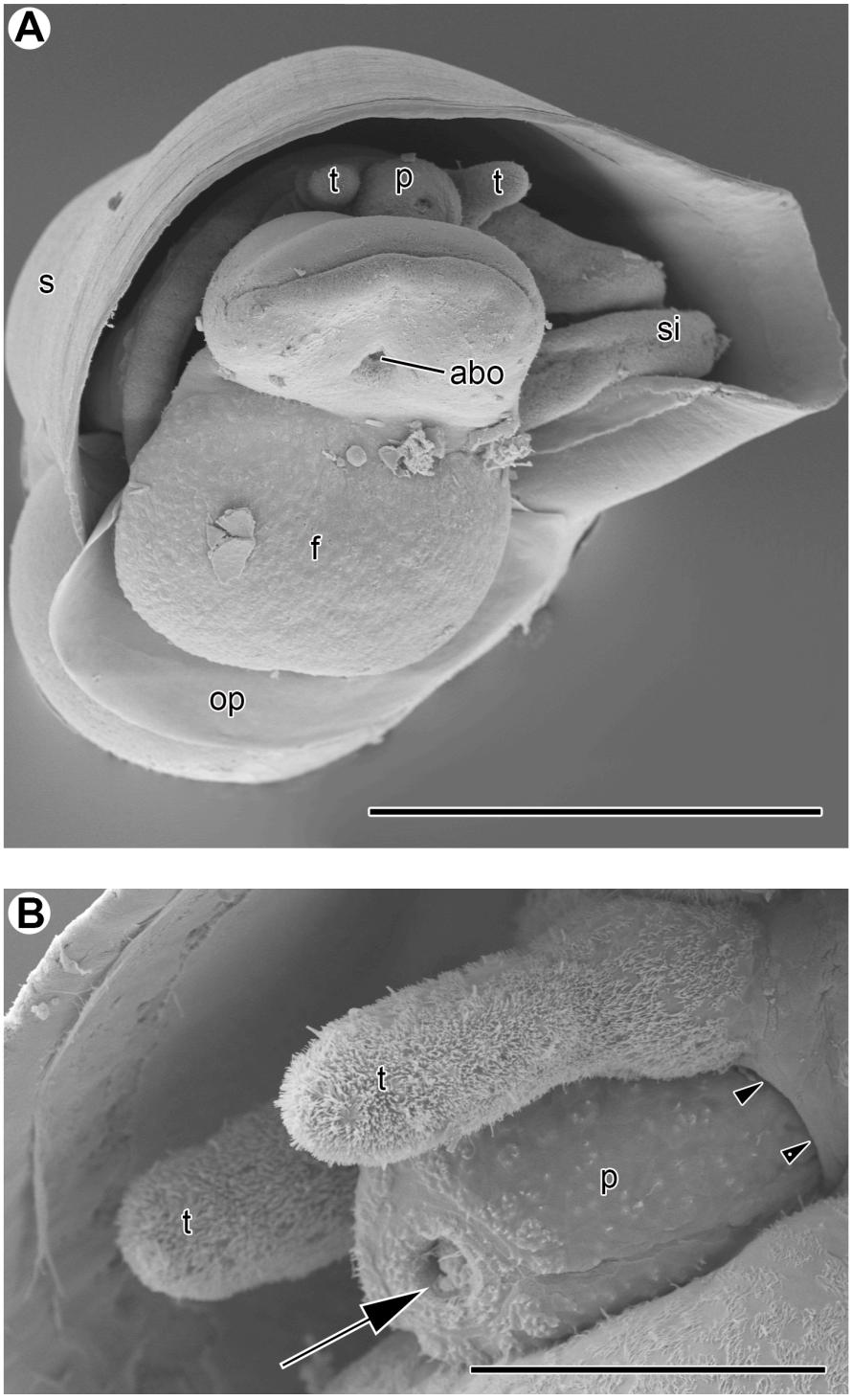

Figure 8. Scanning electron micrographs of feeding juveniles of *Nucella lamellosa.* **A.** Ventral view of a feeding juvenile showing the proboscis and the opening of the accessory boring organ on the foot; scale bar= 500 μm. **B.** Enlargement of the proboscis showing the mouth (arrow) and edges of the proboscis sac (small arrowheads); scale bar=

100 μm. Abbreviations: abo= accessory boring organ, f= foot, op= operculum, p= proboscis, s= shell, si= siphon, t= tentacle.

3.2 Life history and development of *Nucella ostrina*

Encapsulated embryos of *Nucella ostrina* fed on nurse eggs (abortive siblings) during early development and hatched from egg capsules as crawl-away juveniles. By examining embryos released from field-collected egg capsules of *N. ostrina*, I distinguished six distinct developmental stages (Table 3). Initially, embryos of *N. ostrina* were approximately the same size as nurse eggs *i.e.* approximately 200 μm diameter (Figure 9A). Each egg capsule contained hundreds of nurse eggs, but only approximately 20 viable embryos. By the time an early veliger-like stage was reached, embryos surrounded a central mass of nurse eggs and were completely engorged with yolk from ingested nurse eggs (Figure 9B). Eventually all nurse eggs were consumed and the veliger-like embryos completed encapsulated development using the energy stores from the earlier ingestion stages. Throughout subsequent development the yellow yolk from ingested nurse eggs was visible in the guts of viable embryos (Figure 9C). In one of the egg capsules that I examined, it appeared that nurse eggs had been unevenly shared among viable embryos, resulting in veliger-like embryos of the same developmental stage but with highly varied sizes; little to no yolk was present in the guts of the tiny embryos from these egg capsules (Figure 9D). The velar lobes of *N. ostrina*, similar to *N. lamellosa*, retained all three ciliary bands including a metatroch and food groove (Figure 10). However, relative to *N. lamellosa*, the metatroch was reduced in *N. ostrina* (compare Figure 10A to B). Veliger-like embryos underwent a behavioral change before metamorphosis in that embryos began to contract their velar lobes and remained within their shells. At metamorphosis the embryos were capable of crawling and began to reabsorb their velar lobes into the sides of their head (Figure 11A and B). After loss of their velar lobes, juveniles with an approximate shell length of 1-1.5 mm hatched from the egg capsules (Figure 11C and D). Yolk was still visible in the guts of hatching juveniles. Thus, juveniles had energy reserves for their subsequent hunt for prey. In the egg capsules where juvenile hatching was observed, approximately 10-20 juveniles were present.

Table 3. Developmental stages identified in *Nucella ostrina* from field collected egg capsules

Stage	Key features
1	Early cleavage; embryos are the same size as nurse eggs
2	Early ingestion stage; rudimentary velar lobes, foot, and shell; absorptive cells of the larval kidney complex; mouth and larval esophagus engorged with nurse eggs
3	Late ingestion stage; propodium (formation of crawling surface); heart beat
4	All nurse eggs consumed; round, extended velar lobes; siphonal notch in the shell; cephalic vesicle; capable of spinning; tentacle rudiments; larval kidney complex highly reduced
5	Large foot; tentacles; most remain retracted into shell; velar lobes appear contracted
6	Both pre-metamorphic (6a) and post-metamorphic (6b) stages present in the same capsule; velar lobes were absent in half of the individuals in the capsule
Juvenile	Hatched from egg capsule

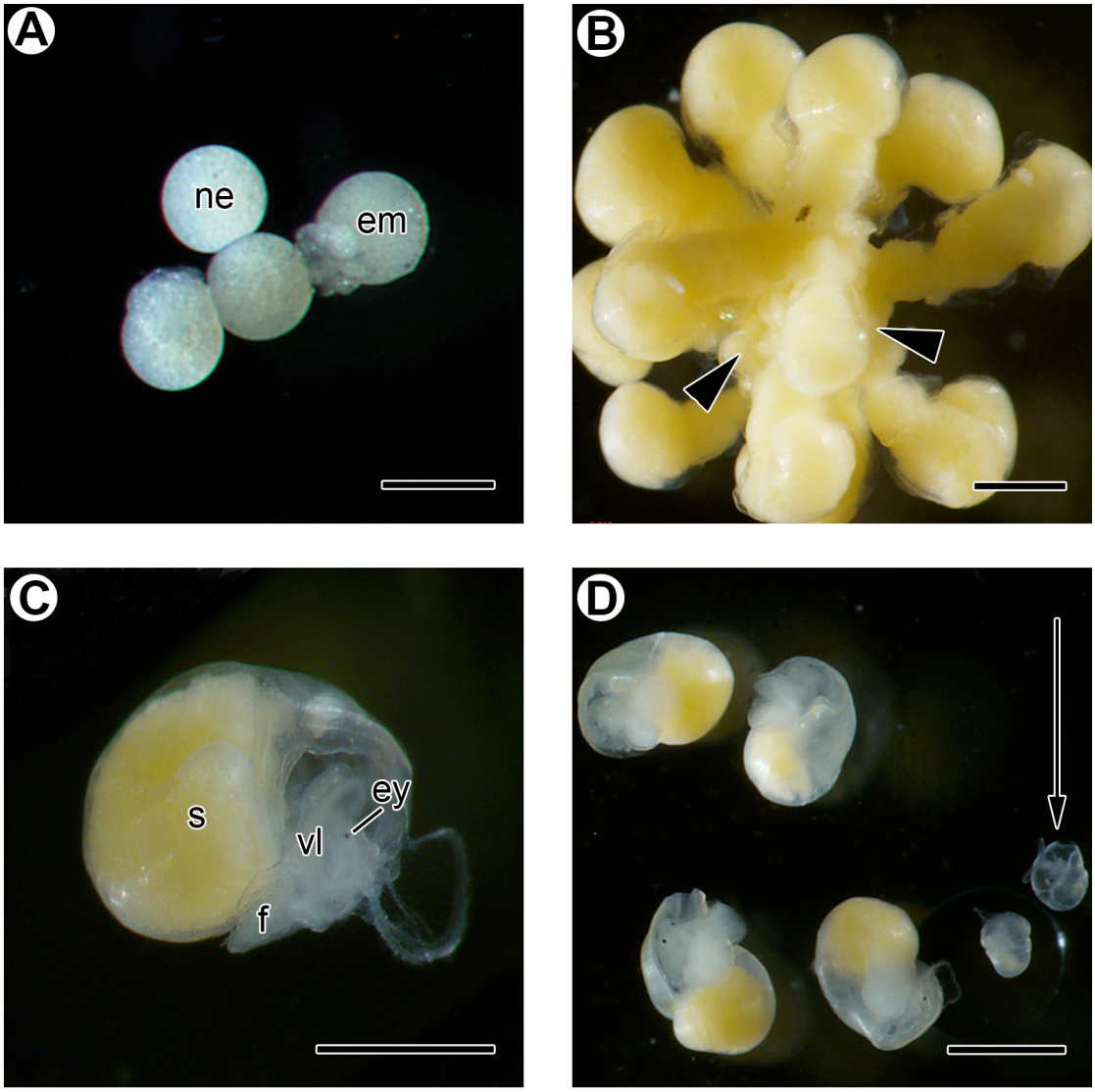

Figure 9. External features of encapsulated embryos of *Nucella ostrina*.
A. Stage 1 embryos and nurse eggs; embryos are undergoing early cleavage beside abortive nurse eggs; scale bar= 200 μm. **B.** Stage 2 veliger-like embryos; early ingestion stage; veliger-like embryos surround a central mass of nurse eggs (indicated by arrowheads) and are completely engorged with yolk; scale bar= 500 μm. **C.** Stage 4 veliger-like embryo; post-ingestion stage; scale bar= 500 μm. **D.** Stage 5 veliger-like embryos; note size differences due to unequal sharing of nurse eggs among embryos within this egg capsule; one of the small veliger-like embryos is indicated with an arrow; scale bar= 1 mm. Abbreviations: em= embryo, ey= eyespot, f= foot, ne= nurse egg, s= shell, vl= velar lobe.

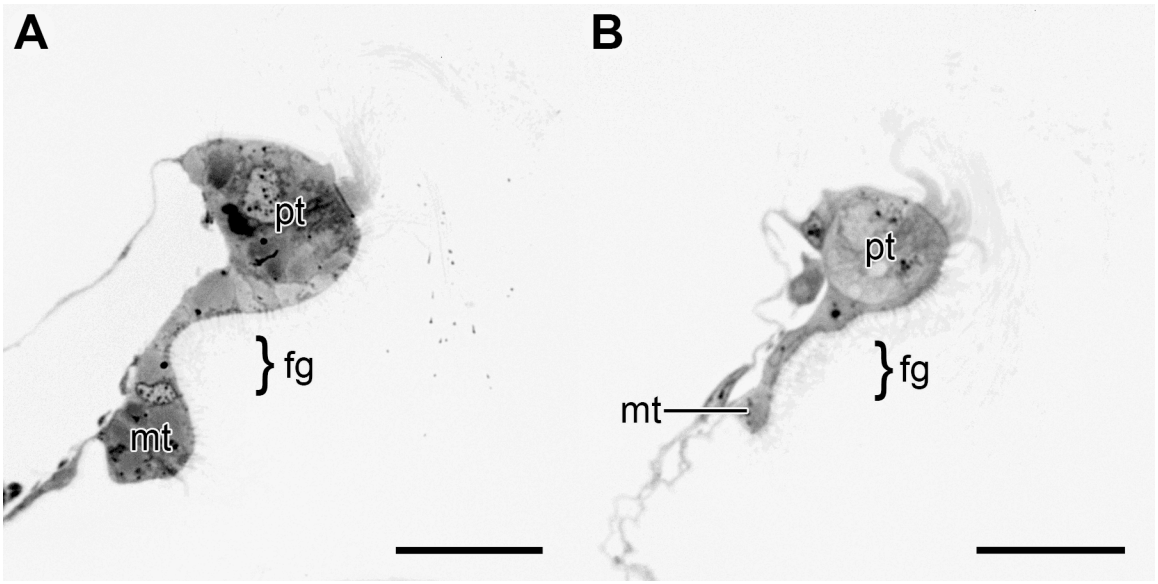

Figure 10. Cross sections through the velar lobes of *Nucella lamellosa* and *N. ostrina*. **A.** Velar lobe of *N. lamellosa* showing prototroch and metatroch with food groove in between; scale bar= 25 μm. **B.** Velar lobe of *N. ostrina* showing prototroch and metatroch with food groove in between; scale bar= 25 μm. Abbreviations: fg= food groove, mt= metatroch, pt= prototroch.

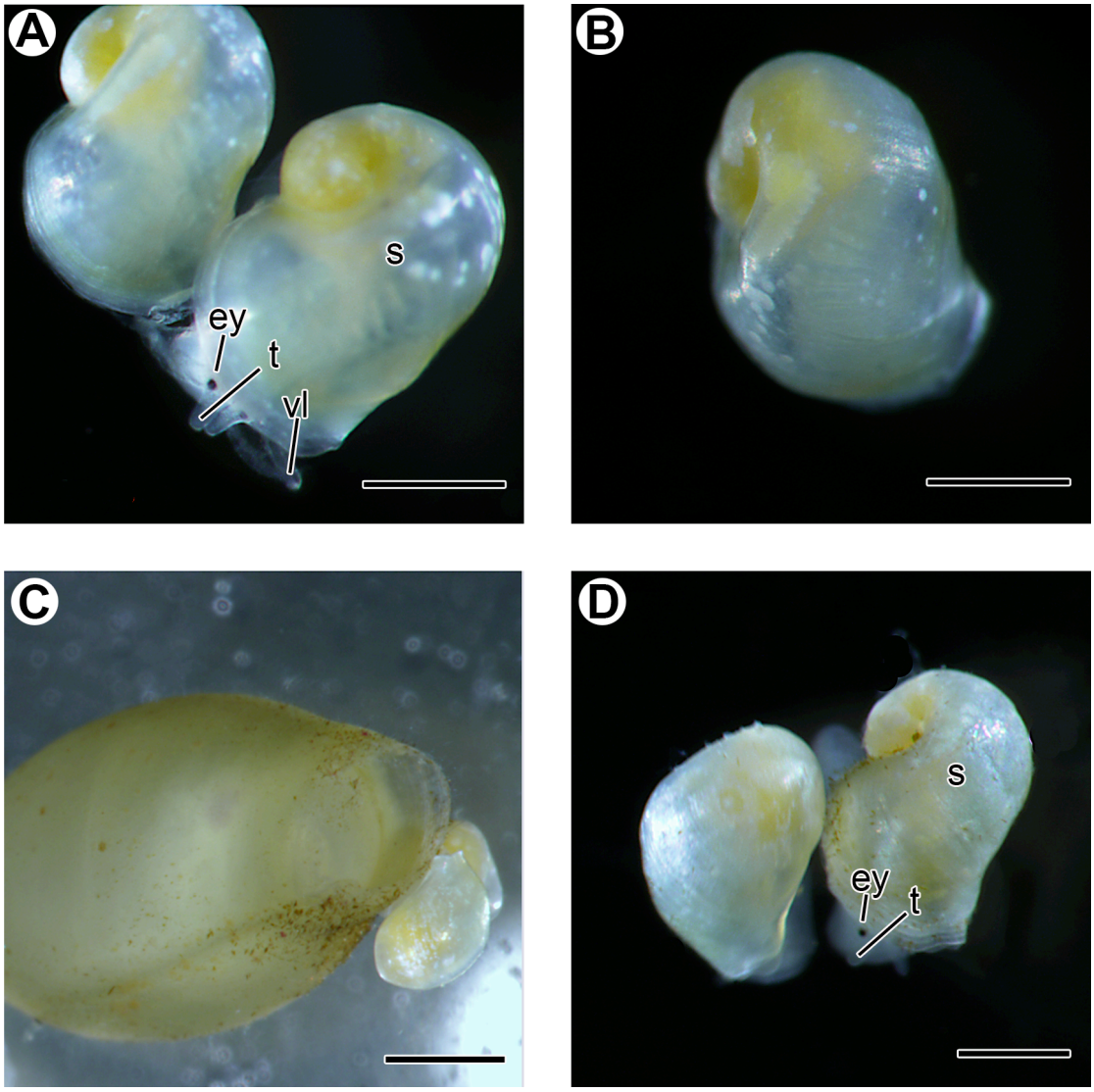

Figure 11. Metamorphosis and juvenile hatching in *Nucella ostrina*.

A. Pre-metamorphic stage 6a veliger-like embryo showing presence of velar lobes; scale bar= 500 μm. **B.** Post-metamorphic stage 6b specimen; scale bar= 500 μm. **C.** Juveniles recently hatched from egg capsule; scale bar= 1 mm. **D.** Crawling juveniles immediately after emergence from egg capsule; scale bar= 500 μm. Abbreviations: ey= eyespot, s=shell, t=tentacle, vl=velar lobe.

3.3 Proboscis and foregut development in *Nucella lamellosa*

Important events in the development of the foregut in *N. lamellosa* were ascertained with histological sectioning and light microscopy. Six developmental stages were examined in detail: stage 4**,** stage 6, stage 8, stage 11a, stage 11b, and a feeding juvenile one week after hatching from the egg capsule (Table 2, Figures 5-8). Sections of stage 4 veliger-like embryos showed the future definitive foregut as an out-pocketing of non-ciliated cells along the ventral wall of a ciliated tube (homologue of the larval esophagus) extending inward from the mouth. Sections of stage 6 veliger-like embryos showed that the ventral out-pocketing had become regionally differentiated into the major foregut components (buccal cavity, radular sac, and anterior esophagus). Sections of stage 8 veliger-like embryos showed further differentiation of the ventral components of the definitive foregut as well as the emergence of additional foregut components. Sections of stage 11a and 11b pre- and post-metamorphic stages showed the changes that occurred to the foregut at metamorphosis. Finally, sections of a feeding juvenile showed the foregut rearrangements that occurred as a result of proboscis elongation. These events are described in detail below.

3.3.1 Stages 4 and 6: regional differentiation of the ventral out-pocketing

By stage 4 the homologue of the larval esophagus was present in the form of a short ciliated tube extending from the mouth to the developing stomach. Because *N. lamellosa* is a non-feeding, direct developing species, this tube was most likely a vestigial structure. An out-pocketing along the ventral wall of the larval esophagus was present just posterior to the opening of the larval mouth (Figure 12A and B); this out-pocketing was the anlage of the definitive foregut. The cells of the ventral anlage were distinct from the cells of the larval esophagus because whereas the cells of the larval esophagus were ciliated, the cells of the ventral anlage were non-ciliated and stained slightly more densely with Richardson's stain. The out-pocketing was only connected to the larval esophagus by a narrow mid-sagittal strip (Figure 12A) but bulged out laterally (Figure 12B).

By stage 6 the ventral out-pocketing had become regionally differentiated into rudiments of the future buccal cavity, radular sac, and anterior esophagus of the juvenile

stage (Figure 12C). As seen in sagittal sections, the ventral out-pocketing had acquired anterior and posterior projections. The anterior projection was the future buccal cavity, whereas the posterior projection would later give rise to the anterior esophagus and the valve of Leiblein. At the junction between these two projections, the future radular sac had begun to differentiate as a diverticulum. Whereas the future buccal cavity projected anteriorly as a blind ending channel that was separate from the overlying larval esophagus, the posterior projection of the future anterior esophagus remained connected to the larval esophagus along a thin mid-sagittal strip (Figure 12D). Particles were found in the larval esophagus and developing buccal cavity of some of the specimens sectioned (Figure 12D). These particles were most likely ingested pieces of the fragile larval kidney complex that broke off during the mechanical disruption caused by opening of egg capsules.

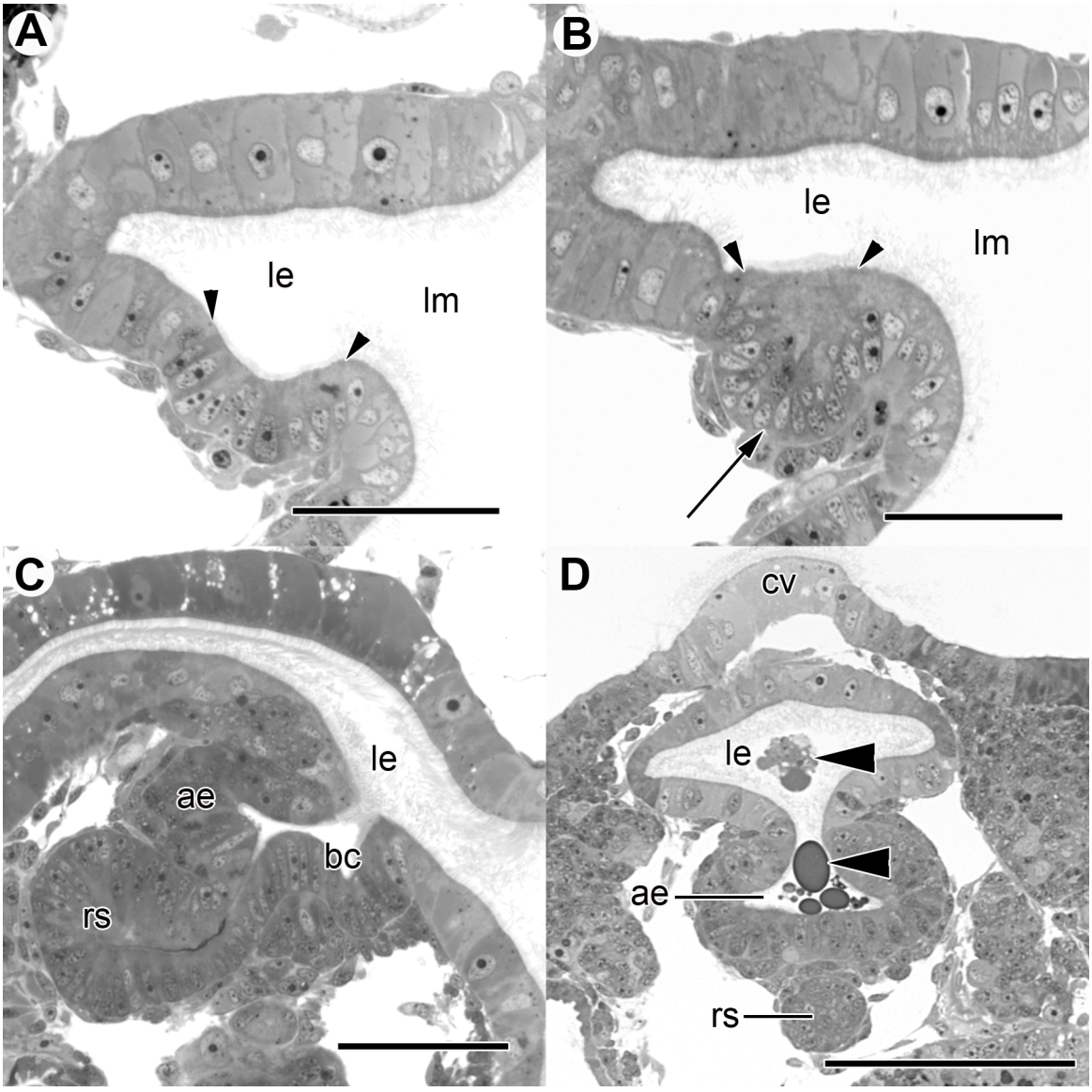

Figure 12. Sections showing the ventral out-pocketing in stage 4 veliger-like embryos and the major foregut components in stage 6 veliger-like embryos of *Nucella lamellosa*. **A.** Mid-sagittal section showing the ventral out-pocketing in a stage 4 veliger-like embryo; arrowheads indicate the border of the ciliated cells of the larval esophagus and the non-ciliated cells of the ventral out-pocketing; scale bar= 50 μm. **B.** Parasagittal section just right of the midline showing the larval esophagus and the lateral bulge of the ventral out-pocketing (arrow) in a stage 4 veliger-like embryo; arrowheads indicate the border of the ciliated cells of the larval esophagus and the non-ciliated cells of the ventral out-pocketing; scale bar= 50 μm. **C.** Sagittal section through the larval esophagus and the ventral out-pocketing showing differentiation of the major ventral components of the

definitive foregut in a stage 6 veliger-like embryo; scale bar= 50 μm **D.** Cross section through the larval esophagus and the developing anterior esophagus and radular sac of the definitive foregut in a stage 6 veliger-like embryo; arrowheads indicate fragments of the larval kidney complex; scale bar= 100 μm. Abbreviations: ae= anterior esophagus, bc= buccal cavity, cv= cephalic vesicle, le= larval esophagus, lm= larval mouth, rs= radular sac.

3.3.2 Stage 8: Additional differentiation of the major foregut components

By stage 8 additional structures of the definitive foregut had emerged. At the anterior end, the accessory salivary glands formed from the area that later became the lower lip of the definitive mouth, at the distal end of the future proboscis. A single duct extended posteriorly and divided into two glandular regions (Figure 13A and B). Early development of the proboscis consisted of epithelial thickenings dorsal and ventral to the larval mouth. The proboscis sac had begun to form as paired lateral invaginations, which in later stages took a more dorsal position (Figure 13B; compare to Figure 16D and F). Furthermore, the ventral trough had differentiated into many components of the definitive foregut. The distal larval esophagus and the definitive anterior esophagus/ valve of Leiblein remained continuous along the entire length of both foreguts. However, the anterior-most end of the anteriorly projecting definitive buccal cavity was separate from the larval esophagus (Figure 13B). The ducts of the acinous salivary glands extended from the posterior end of the buccal cavity and continued posteriorly nestled on either side of the definitive anterior esophagus (Figure 13C-E). Posterior from the definitive buccal cavity, the definitive anterior esophagus (connected to the larval esophagus), together with the acinous salivary glands, separated from the radular sac (compare Figure 13C and D). The odontoblasts of the radular sac had begun to secrete radular teeth, a pair of cartilaginous rods had formed beneath the radula, and the muscular of the odontophore had begun to differentiate (Figure 13D and E). At this stage the nerve ring (*i.e.* cerebral, pedal, and pleural ganglia and their connectives) had formed and the larval/definitive anterior esophagus, acinous salivary glands, and radular sac extended through this nerve ring (Figure 13D and E). The valve of Leiblein began to differentiate at the level of the nerve ring, but was still small (Figure 13E). The valve of Leiblein developed as an asymmetrical structure having a thicker right side compared to the left. This asymmetry was apparent by stage 8 and continued through to future stages. By the feeding juvenile stage, however, the asymmetry was lost. The mid-esophagus began posterior to the valve of Leiblein and remained largely undifferentiated, however, the ventral region consisted of a small trough of more densely staining cells (Figure 13F).

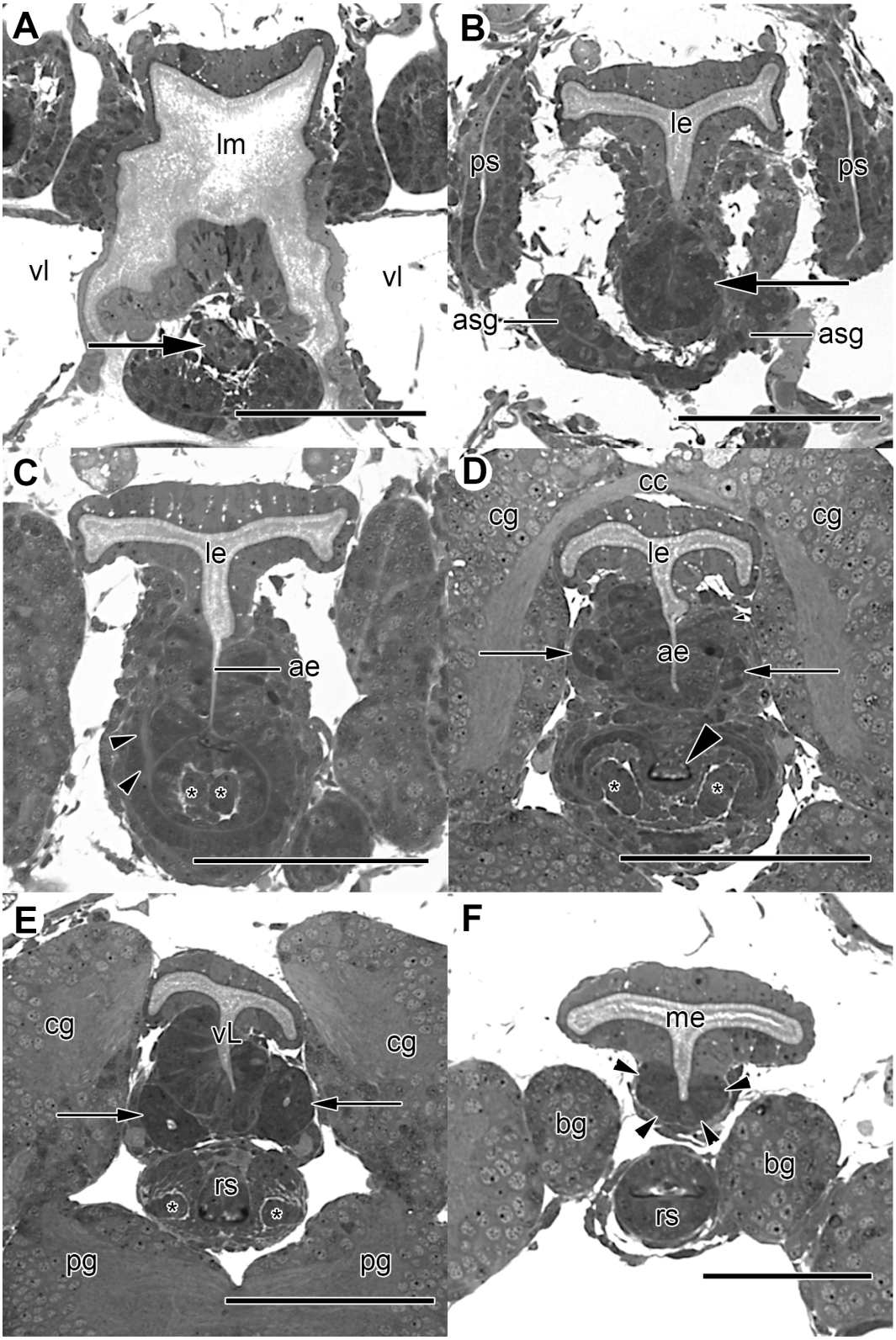

Figure 13. Cross sections through the foregut of a stage 8 veliger-like embryo of *Nucella lamellosa* ordered from anterior to posterior.

All scale bars= 100 μm. **A.** Anterior head region showing the spacious larval mouth; arrow indicates the common duct of the accessory salivary glands. **B.** Anterior projection of the definitive buccal cavity (arrow) beneath the larval esophagus; the common duct of the accessory salivary glands divided and terminated into two glandular regions. **C.** Continuity of the internal lumens of the larval esophagus, definitive anterior esophagus, and definitive buccal mass; arrowheads indicate the duct of the right acinous salivary gland extending off the posterior end of the buccal cavity; the asterisks indicate the odontophoral cartilages that support the radula. **D.** The larval esophagus and definitive esophagus have extended apart from the radular sac; the ducts of the acinous salivary glands are nestled beside the definitive anterior esophagus (indicated by the arrows); the arrowhead indicates the radula within the radular sac and the asterisks indicate the odontophoral cartilages that support the radula. **E.** Section at the level of the cerebropedal nerve ring showing the future valve of Leiblein; notice that the valve of Leiblein, acinous salivary glands (arrows), and radular sac extend through the nerve ring; the asterisks indicate the odontophoral cartilages that support the radula. **F.** Mid-esophagus with ventral trench of more darkly staining cells (indicated by the small arrowheads). Abbreviations: ae= anterior esophagus, asg= accessory salivary gland, bg= buccal ganglia, cc= cerebral commissure, cg= cerebral ganglion, le= larval esophagus, lm= larval mouth, me= mid-esophagus, pg= pedal ganglia, ps= proboscis sac, rs= radular sac, vl= velar lobe, vL= valve of Leiblein.

3.3.3 Stage 11: Metamorphic remodeling of the foregut

Stage 11 marked an important event in the life history of *N. lamellosa*; at this stage *N. lamellosa* underwent metamorphosis in preparation for its free-living juvenile existence. The events of metamorphosis were presumably rapid, as egg capsules containing both individuals with velar lobes and individuals that had lost their velar lobes were present. Significant changes to the foregut also took place at this stage, particularly the region of the foregut anterior to the valve of Leiblein (Figure 14 and 15).

Prior to metamorphosis, stage 11a veliger-like embryos still had a larval mouth and a larval esophagus (Figure 14A and C; Figure 15A and C; Figure 16A, C, and E). Beneath the larval esophagus the definitive foregut at stage 11a had differentiated to an advanced stage and consisted of the buccal cavity, radular sac, anterior esophagus, two pairs of salivary glands (accessory and acinous), and the valve of Leiblein (Figure 14A, C, and E; Figure 15C). At metamorphosis, stage 11b individuals sealed the larval mouth shut and the definitive mouth formed later in development, which resulted in a stage with no mouth (Figure 15B and D; Figure 16B). Furthermore, the larval esophagus was destroyed and the velar lobes were absorbed into the sides of the head (Figure 16D and F). By stage 11a the dorsal and ventral epithelial thickenings of the developing proboscis had formed a short snout. At metamorphosis the two epithelial thickenings met as the larval mouth was sealed shut (compare Figure 15A to B and 15C to D; compare Figure 16A to B). Additionally, by stage 11 the two lateral invaginations of the proboscis sac had deepened and taken a more dorsal position and a shallow ventral invagination of the future proboscis sac had formed (Figure 16F).

Whereas the larval esophagus and definitive anterior esophagus were connected along their entire length in stage 8 individuals, by stage 11a they had separated into two distinct tubes anterior to the valve of Leiblein (Figure 17A). However, the connection between the larval esophagus and definitive anterior esophagus was maintained posterior to the start of the valve of Leiblein (Figure 17C and E). At metamorphosis, the larval esophagus anterior to the valve of Leiblein was destroyed (Figure 17B) and the dorsal portion of the valve of Leiblein, which was continuous with the larval esophagus in earlier stages, was reduced but still maintained (Compare Figure 17C to D and 17E to F).

Finally, by stage 11a the foregut began to twist starting at the valve of Leiblein (Figure 17E and F).

The foregut continued to twist posterior to the valve of Leiblein (Figure 18A-F) eventually placing the developing gland of Leiblein at a right rather than ventral position (Figure 18E and F). By stage 11a the ventral trench in the mid-esophagus, particularly the glandular tissue of the gland of Leiblein, increased in size substantially (compare Figure 13F to 18A-F). At metamorphosis there were no major changes to the mid-esophagus other than a slight reduction in the dorsal portion, which was continuous with the larval esophagus in previous stages (compare Figure 18A to B and 18E to F).

Whereas the velar lobes were absorbed into the sides of the head at metamorphosis, the degenerating tissues of the larval esophagus were consumed after they dissociated. In specimens that were undergoing metamorphosis at the time of fixation, the valve of Leiblein, mid-esophagus, posterior esophagus, and stomach were completely engorged with dissociated cells (Figure 19A and B).

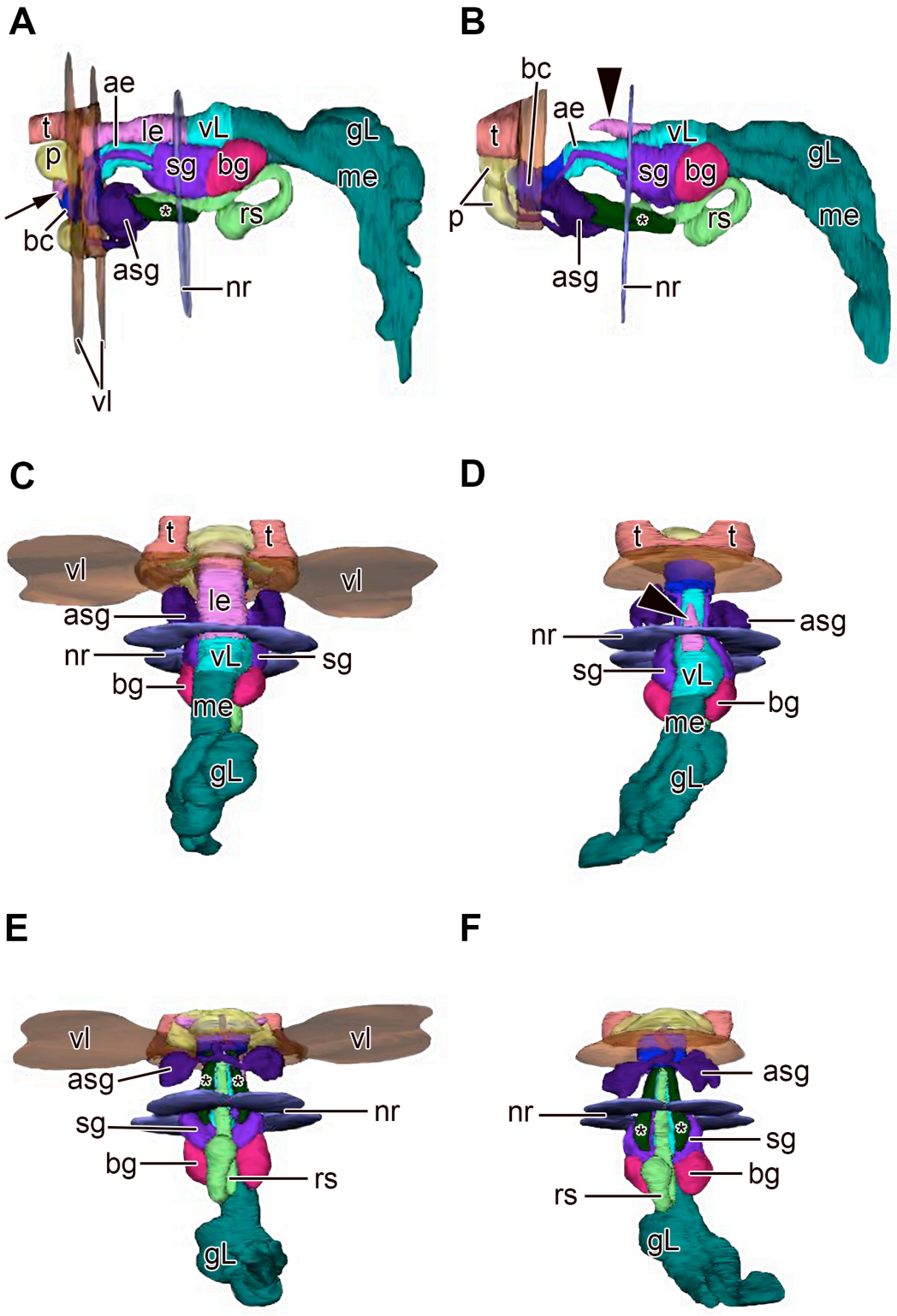

Figure 14. Surface-rendered 3D reconstructions showing the components of the foregut before and after metamorphosis in *Nucella lamellosa*.

Notice that major changes at metamorphosis include loss of the velar lobes and loss of the larval mouth and distal larval esophagus. **A.** Left lateral view of a stage 11a specimen prior to metamorphosis; arrow indicates the location of the larval mouth; asterisk indicates the odontophoral cartilages. **B.** Left lateral view of a stage 11b specimen after metamorphosis; arrowhead indicates the remnant of the distal larval esophagus; asterisk indicates the odontophoral cartilages. **C.** Dorsal view of a stage 11a specimen. **D.** Dorsal view of a stage 11b specimen; arrowhead indicates the remnant of the larval esophagus. **E.** Ventral view of a stage 11a specimen; asterisks indicate the odontophoral cartilages. **F.** Ventral view of a stage 11b specimen; asterisks indicate the odontophoral cartilages. Abbreviations: ae= anterior esophagus, asg= accessory salivary gland, bc= buccal cavity, bg= buccal ganglion, gL= gland of Leiblein, le= larval esophagus, me= midesophagus, nr= nerve ring, p= proboscis, rs= radular sac, sg= acinous salivary gland, t= tentacle, vl= velar lobe, vL= valve of Leiblein.

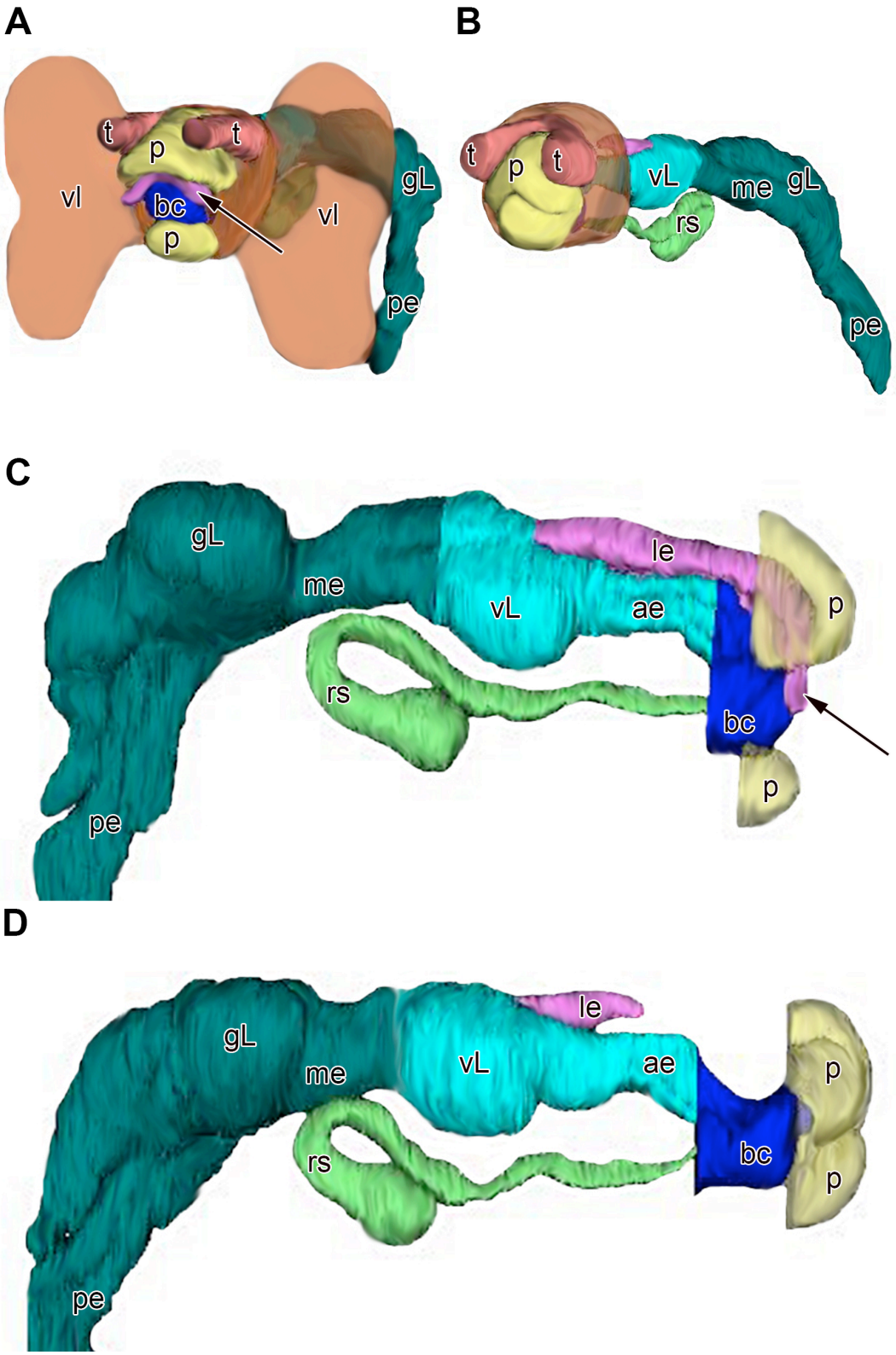

Figure 15. Surface-rendered 3D reconstructions showing the proboscis and foregut before and after metamorphosis in *Nucella lamellosa*.

A. Frontal view of a stage 11a specimen showing the thickened epithelium of the proboscis (yellow) located dorsal and ventral to the larval mouth prior to metamorphosis; the buccal cavity projects anteriorly beneath the larval esophagus, but has not yet ruptured to the outside.; the arrow indicates the larval mouth (pink). **B.** Frontal view of a stage 11b specimen showing the fusion of the larval mouth and the closure of the dorsal and ventral components of the proboscis after metamorphosis; this stage has no mouth. **C.** Right lateral view of a stage 11a specimen showing the major components of the foregut prior to metamorphosis; notice that the definitive foregut structures anterior to the valve of Leiblein develop as a ">" beneath the larval esophagus; arrow indicates the larval mouth. **D.** Right lateral view of a stage 11b specimen showing the major components of the foregut after metamorphosis; notice the destruction of the larval mouth and distal larval esophagus; also notice that the buccal cavity has not yet ruptured a new mouth. Abbreviations: ae= anterior esophagus, bc= buccal cavity, gL= gland of Leiblein, le= larval esophagus, me= midesophagus, p= proboscis, pe= posterior esophagus, rs= radular sac, vl= velar lobe, vL= valve of Leiblein, t= tentacles.

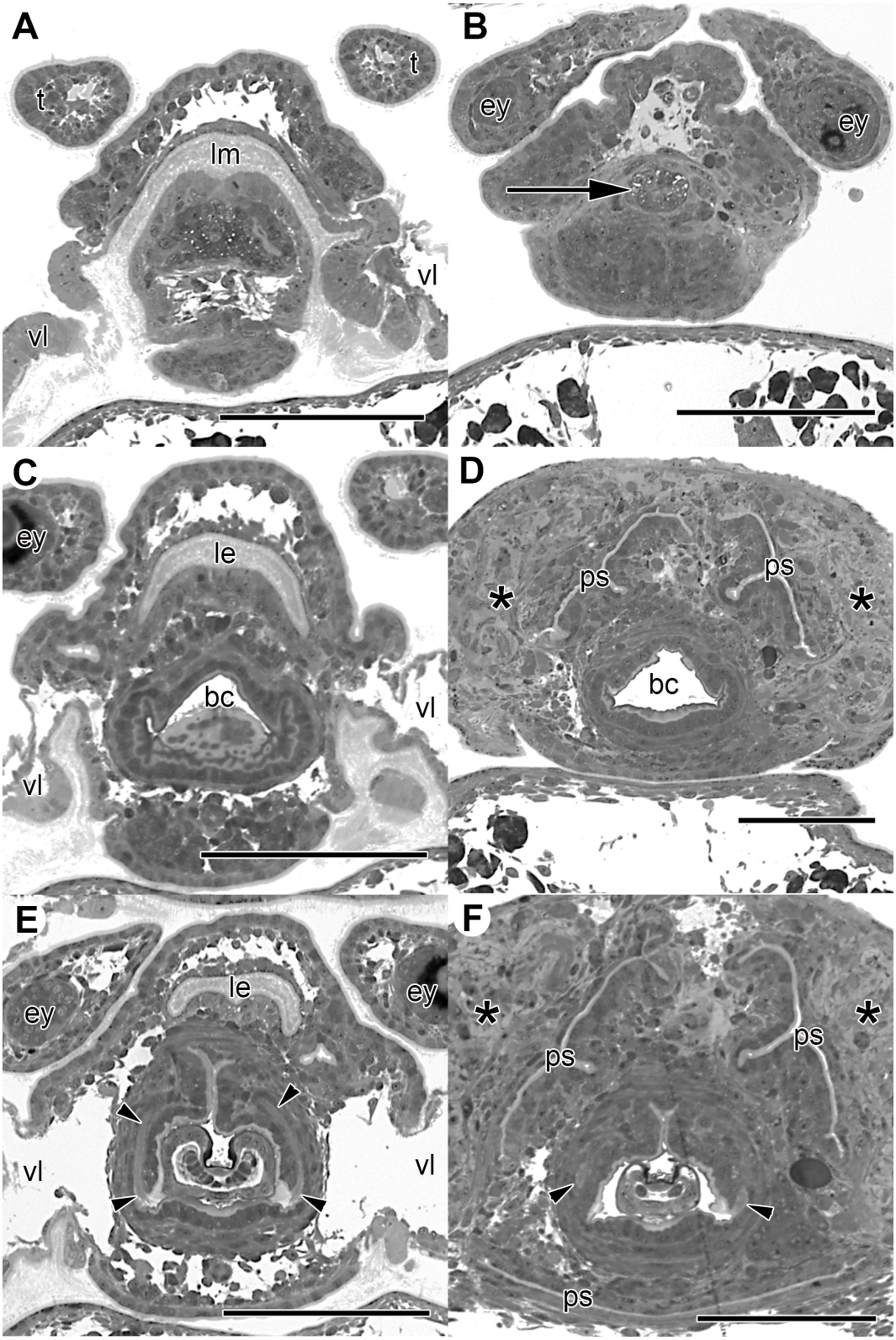

Figure 16. Cross sections through the anterior-most region of the head before and after metamorphosis in *Nucella lamellosa*.

Notice the destruction of the larval mouth and distal larval esophagus at metamorphosis; all scale bars= 100 μm. **A.** Stage 11a; snout showing the larval mouth and the thickened epithelium of the developing proboscis, which was interrupted by the velar lobes prior to metamorphosis. **B.** Stage 11b; snout showing the absence of a mouth and the thickened epithelium of the developing proboscis after metamorphosis; arrow indicates the cells of the oral tube, which eventually rupture a mouth in the juvenile. **C.** Stage 11a; larval esophagus and definitive buccal cavity are clearly two separate tubes. **D.** Stage 11b; absence of the larval esophagus with the definitive buccal cavity still present; large masses of reabsorbed velar lobes denoted by asterisks. **E.** Stage 11a; larval esophagus dorsal to the buccal mass; ducts of the acinous salivary glands (arrowheads) extending off the buccal cavity at the region where the radula comes onto the floor of the buccal cavity. **F.** Stage 11b; absence of a larval esophagus; ducts of the acinous salivary glands (arrowheads) extend off the buccal cavity; masses of reabsorbed velar lobes indicated by asterisks. Abbreviations: bc= buccal cavity, ey= eyespot, le= larval esophagus, lm= larval mouth, ps= proboscis sac, t=tentacles, vl= velar lobes.

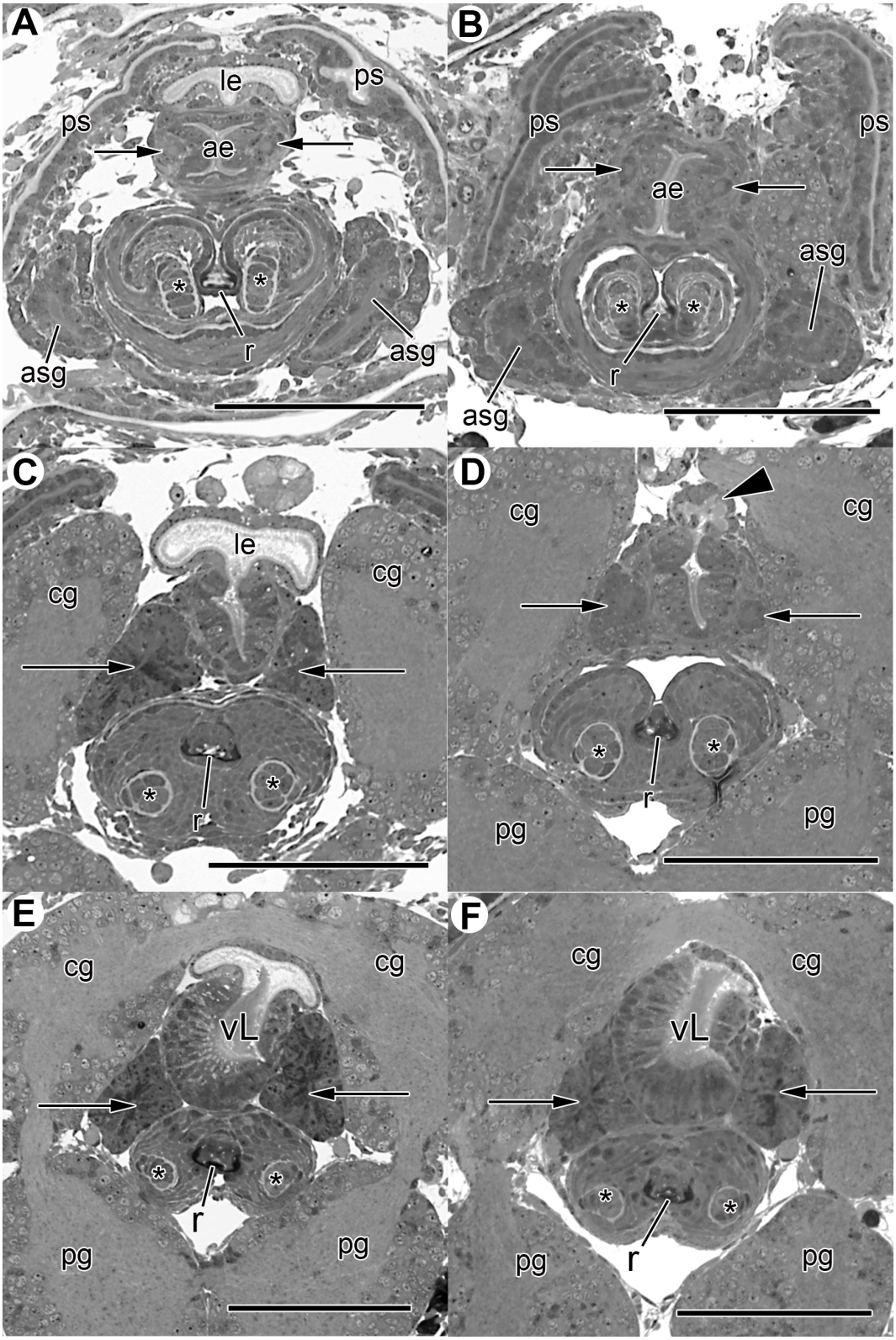

Figure 17. Cross sections through the anterior esophagus before and after metamorphosis in *Nucella lamellosa*.

Notice the destruction of the distal larval esophagus at metamorphosis; all scale bars= 100 μm. **A.** Stage 11a; the larval esophagus and definitive anterior esophagus are separate tubes prior to metamorphosis; the arrows indicate the ducts of the acinous salivary glands; the asterisks indicate the odontophoral cartilages that support the radula. **B.** Stage 11b; absence of a larval esophagus dorsal to the definitive anterior esophagus after metamorphosis; the arrows indicate the ducts of the acinous salivary glands; the asterisks indicate the odontophoral cartilages that support the radula. **C.** Stage 11a; larval esophagus and definitive anterior esophagus merge at the start of the valve of Leiblein; the arrows indicate the acinous salivary glands; the asterisks indicate the odontophoral cartilages that support the radula. **D.** Stage 11b; reduced remnant of the larval esophagus (arrowhead) remains at the start of the valve of Leiblein; the arrows indicate the acinous salivary glands; the asterisks indicate the odontophoral cartilages that support the radula. **E.** Stage 11a; valve of Leiblein, acinous salivary glands (arrows), and radular sac extend through the nerve ring; the asterisks indicate the odontophoral cartilages that support the radula. **F.** Stage 11b; valve of Leiblein, acinous salivary glands (arrrows), and radular sac extend through the nerve ring; dorsal portion of valve of Leiblein is reduced compared to E; the asterisks indicate the odontophoral cartilages that support the radula.
Abbreviations: ae= anterior esophagus, asg= accessory salivary gland, cg= cerebral ganglia, le= larval esophagus, pg= pedal ganglia, ps= proboscis sac, r= radula, vL= valve of Leiblein.

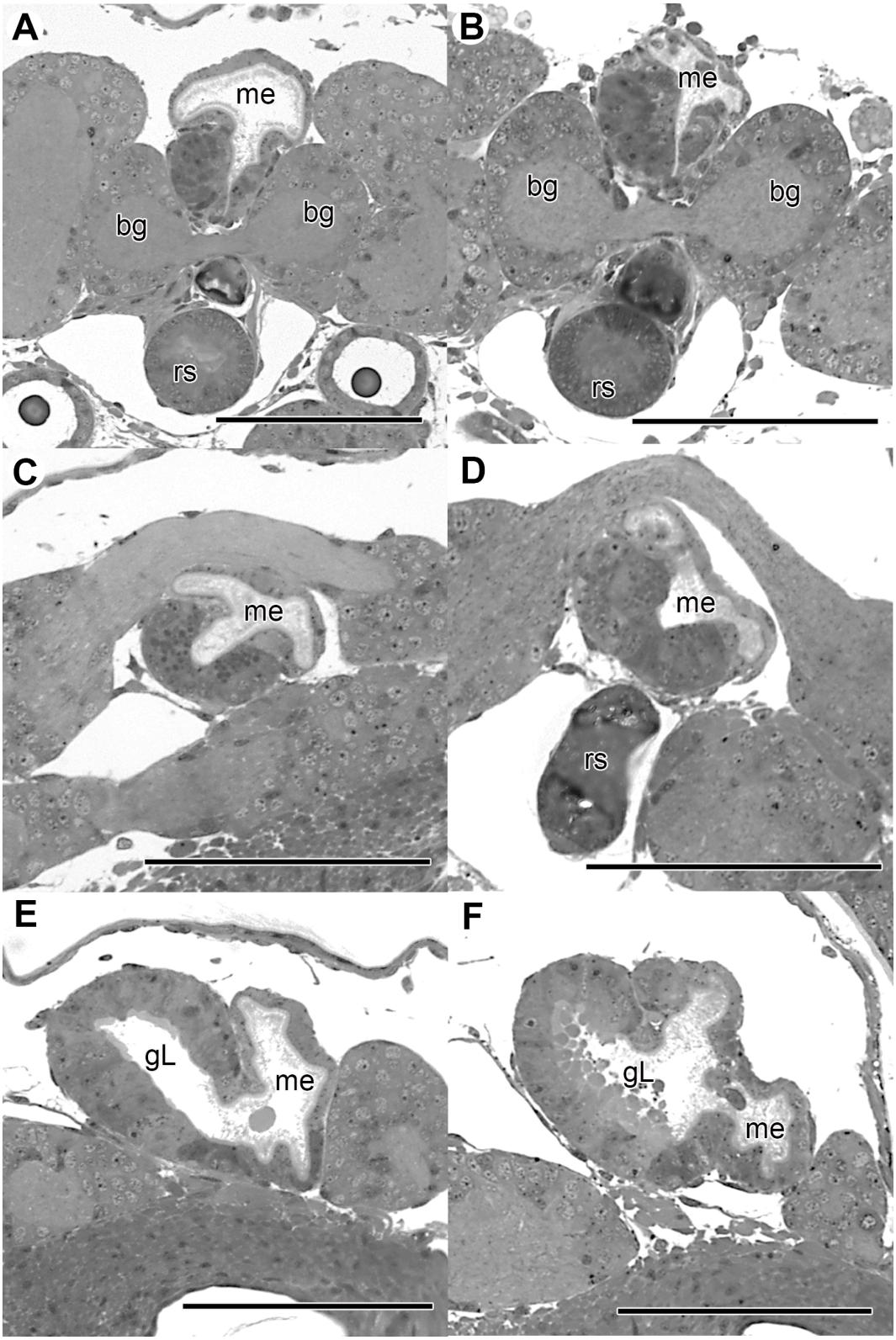

Figure 18. Cross sections through the mid-esophagus before and after metamorphosis in *Nucella lamellosa*.

All scale bars= 100 μm. **A.** Stage 11a, pre-metamorphosis; start of the mid-esophagus at the terminus of the valve of Leiblein, which is marked by the buccal commissure connecting the buccal ganglia. **B.** Stage 11b; start of the mid-esophagus showing a reduction in the dorsal portion of the mid-esophagus after metamorphosis. **C.** Stage 11a; mid-esophagus with ventral trench of darkly staining cells. **D.** Stage 11b; mid-esophagus with ventral trench of darkly staining cells. **E.** Stage 11a; developing gland of Leiblein extending off the right side of the mid-esophagus. **F.** Stage 11b; gland of Leiblein. Abbreviations: bg= buccal ganglia, gL= gland of Leiblein, me= mid-esophagus, rs= radular sac.

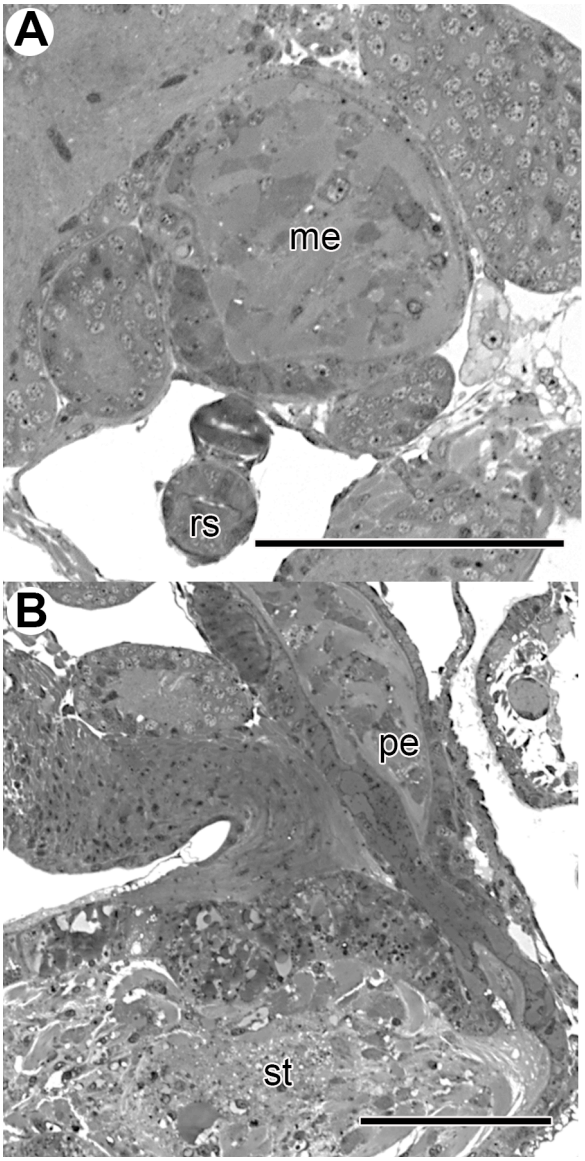

Figure 19. Metamorphosing stage 11 specimen of *Nucella lamellosa* in the process of ingesting the degenerating tissues of the larval esophagus.

All scale bars= 100 μm. **A.** Degenerating tissues fill the mid-esophagus. **B.** Degenerating tissues fill the posterior esophagus and stomach. Abbreviations: me= mid-esophagus, pe= posterior esophagus, rs= radular sac, st= stomach.

3.3.4 Juvenile: Rearrangement of the foregut with proboscis elongation

Elongation of the proboscis occurred after metamorphosis and hatching from the egg capsule. The two dorsal invaginations and the ventral invagination of the proboscis sac joined to form a continuous sheath around the proboscis (Figure 20A-D). At the tip of the proboscis a new mouth formed that led directly into the definitive buccal cavity (Figure 20A and B). Posterior, the radula projected onto the floor of the definitive buccal cavity. The anterior esophagus extended off the dorsal side of the buccal cavity and continued down the length of the proboscis with the radular sac extending in parallel in a ventral position (Figure 20C and D). The duct of the accessory salivary glands was difficult to trace in the juvenile, however, secretory material began to accumulate within the glandular regions (Figure 20D). The elongation of the proboscis pulled the anterior esophagus, acinous salivary glands, and the buccal mass anteriorly out of the cerebro-pedal nerve ring and the radula coiled on the right, dorsal side of the nerve ring (Figure 21A). Furthermore, the valve of Leiblein was pulled out of the nerve ring and sat dorsal to the cerebral commissure with the anterior portion of the midesophagus extending through the narrow passage of the nerve ring (Figure 21B). Posterior to the nerve ring the gland of Leiblein had grown substantially and the tissue of the gland had almost completely peeled away from the main tube of the mid-esophagus; the gland was therefore connected to the midesophagus by only a narrow duct (Figure 21C). The gland of Leiblein occupied most of the posterior cephalic hemocoel and the posterior esophagus (portion of esophagus posterior to the duct of the gland of Leiblein) extended to the stomach (Figure 21C and D).

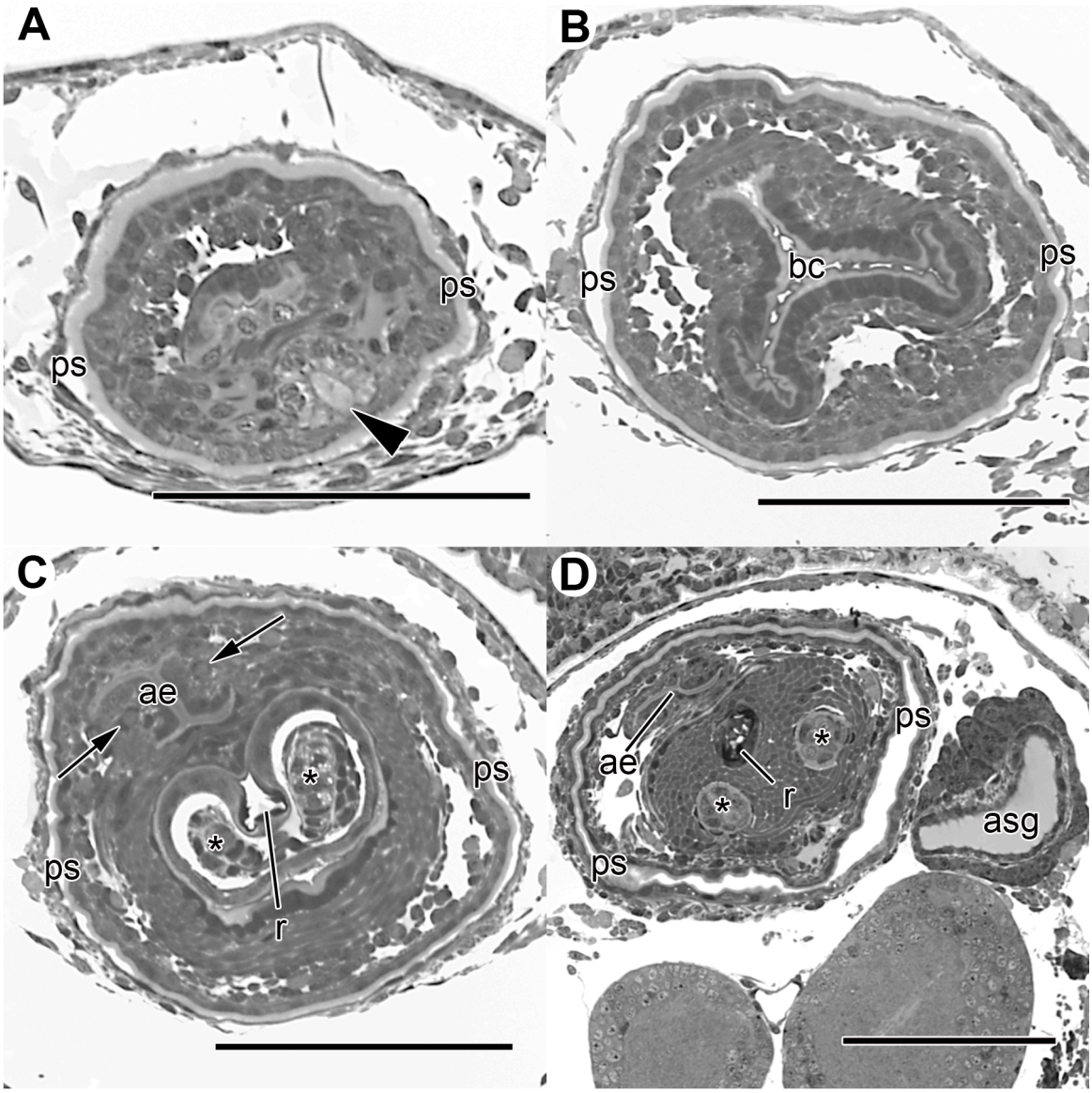

Figure 20. Cross sections through a retracted proboscis within its proboscis sac from a feeding juvenile of *Nucella lamellosa*.

All scale bars= 100 μm **A.** Anterior end of the proboscis including the mouth (arrowhead). **B.** Buccal cavity at the tip of the proboscis. **C.** Anterior esophagus with the buccal mass (radula, cartilage, and musculature) located ventrally; arrows indicate the ducts of the acinous salivary glands; asterisks indicate the odontophoral cartilages. **D.** Section near the base of the proboscis; secretory substance can be observed in the left accessory salivary gland. Abbreviations: ae= anterior esophagus, asg= accessory salivary gland, bc= buccal cavity, ps= proboscis sac, r= radula.

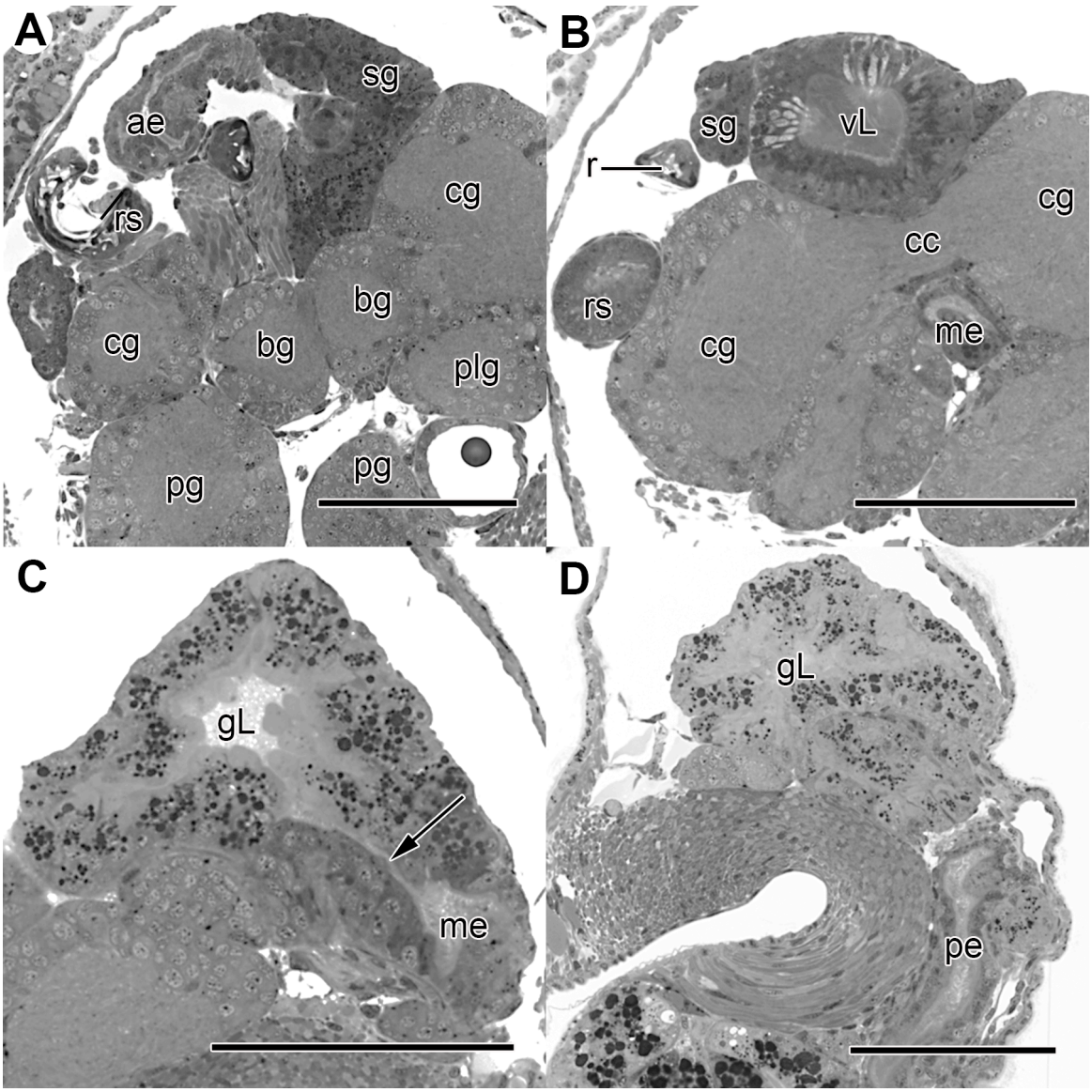

Figure 21. Cross sections through the foregut posterior to the proboscis of a feeding juvenile of *Nucella lamellosa.*

All scale bars= 100 μm. **A.** Anterior esophagus, acinous salivary glands, and radular sac are outside of the nerve ring; part of the coiled radula can be observed at a right dorsal position. **B.** The valve of Leiblein sits at a dorsal position above the cerebral commissure; only the mid-esophagus passes through the nerve ring. **C.** The gland of Leiblein is large and connects to the mid-esophagus by a narrow duct (arrow). **D.** The gland of Leiblein occupies most of the remaining space at the back of the head and the posterior esophagus continues to the stomach. Abbreviations: ae= anterior esophagus, bg= buccal ganglion, cc= cerebral commissure, cg= cerebral ganglion, gL= gland of Leiblein, me= mid-

esophagus, pe= posterior esophagus, pg= pedal ganglion, plg= pleural ganglion, r= radula, rs= radular sac, sg= acinous salivary gland, vL= valve of Leiblein.

3.4 Proboscis and foregut development in *Nucella ostrina*

I examined foregut development in six developmental stages of *Nucella ostrina* through histological sectioning. The stages examined were: stage 2, which occurred early in the phase of ingesting nurse eggs; stage 3, which occurred during the later phase of ingesting nurse eggs; stage 4, which occurred after all nurse eggs had been ingested; stage 6a, which immediately preceded metamorphic loss of the velar lobes; stage 6b, which immediately followed metamorphosis; and a recently hatched juvenile (Table 3, Figures 9 and 11). Foregut development in *N. ostrina* differed from that of *N. lamellosa* in that development of the definitive foregut was delayed relative to the development of external features. Furthermore, the larval esophagus was greatly expanded during early ingestion stages and the connection between the larval esophagus and definitive anterior esophagus remained continuous throughout development.

Differentiation of the definitive foregut was not observed in stages prior to stage 6 (although a stage 5 specimen was not sectioned). In stages 2, 3, and 4 only the larval esophagus with a ventral out-pocketing of non-ciliated, more densely staining cells had formed (Figure 22A-D). Furthermore, in stages 2 and 3, the larval esophagus was full of yolky particles from ingested nurse eggs and the expanded larval esophagus occupied nearly the entire cephalic hemocoel (Figure 22A and C). By stage 4 the nurse eggs had been fully consumed and the larval esophagus collapsed and its wall was highly folded (Figure 22D).

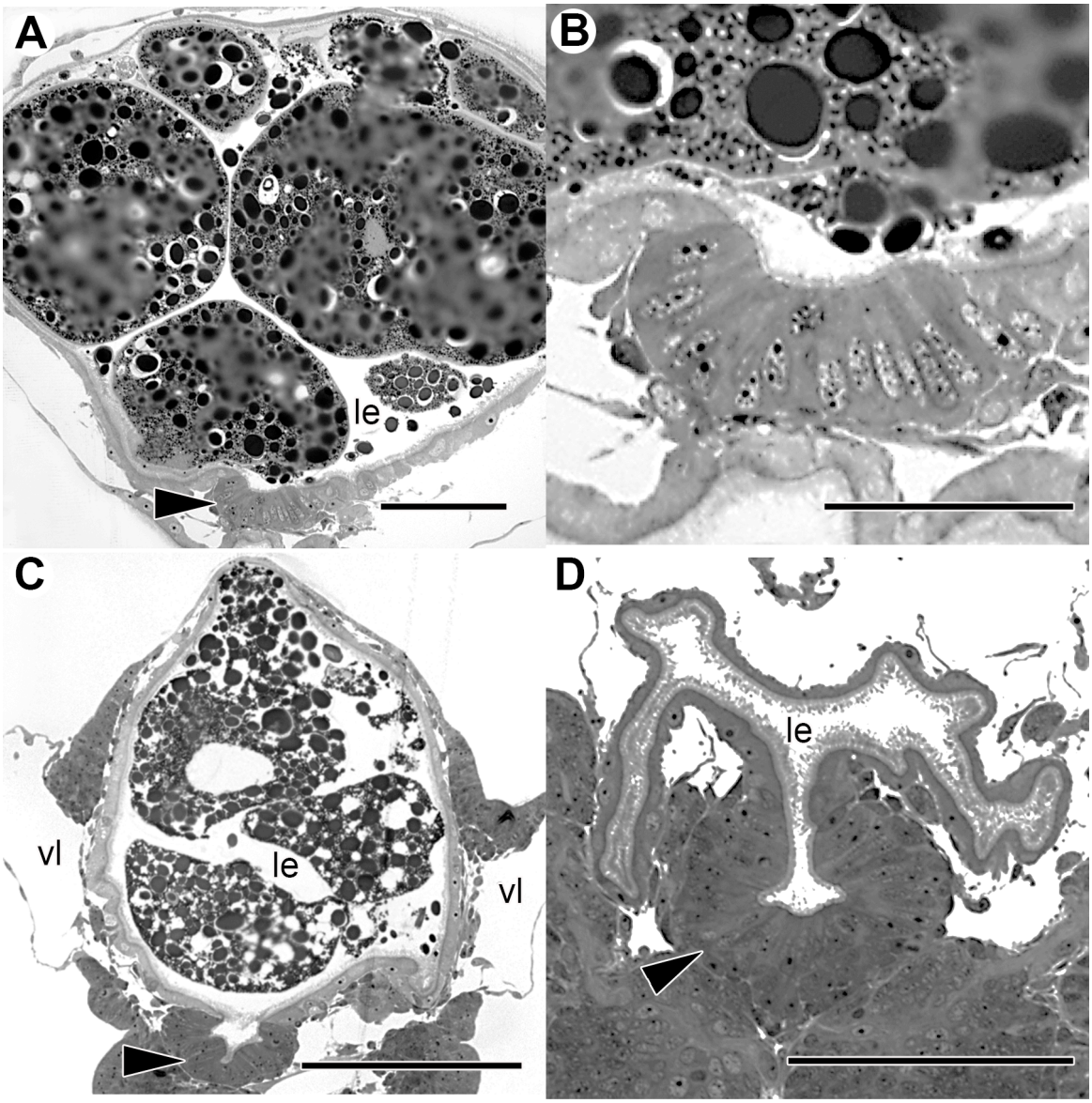

Figure 22. Cross sections through the foregut of *Nucella ostrina* showing formation of the ventral out-pocketing of non-ciliated, densely staining cells.

A. Stage 2 specimen with a greatly expanded larval esophagus that is full of yolk particles from ingested nurse eggs; arrowhead indicates the initial formation of the ventral out-pocketing that will become the definitive foregut; scale bar= 100 μm.

B. Enlargement of the ventral out-pocketing from A; scale bar= 50 μm. **C.** Stage 3; larval esophagus is still full of ingested nurse eggs; the ventral out-pocketing (arrowhead) has increased in size compared to stage 2; scale bar= 200 μm. **D.** Stage 4; nurse eggs have been fully consumed and the larval esophagus has collapsed as a result; arrowhead

indicates the ventral out-pocketing, which by this stage has developed into a long trench running down the ventral wall of the larval esophagus; scale bar= 100 μm. Abbreviations: le= larval esophagus, vl= velar lobe.

By stage 6a in *N. ostrina*, the major components of the definitive foregut had emerged and similar to stage 8 in *N. lamellosa*, the larval esophagus and definitive foregut remained continuous except at the anterior-most end. However, unlike embryos of *N. lamellosa*, embryos of *N. ostrina* underwent metamorphosis without dividing the larval esophagus and definitive anterior esophagus into two separate tubes. Additionally, the definitive anterior esophagus in pre-metamorphic stages of *N. ostrina* did not reach the level of differentiation that was observed in stage 11a (pre-metamorphosis) in *N. lamellosa*. Specifically, the pre-metamorphic anterior esophagus did not undergo the same shape changes that occurred prior to metamorphosis in *N. lamellosa*. Nonetheless, like *N. lamellosa*, destruction of the larval esophagus in *N. ostrina* occurred anterior to the valve of Leiblein. Pre-metamorphic stage 6a individuals had a larval mouth and larval esophagus, whereas in post-metamorphic stage 6b individuals the larval mouth and larval esophagus were destroyed (compare Figure 23A to B and 23C to D). The dorsal portion of the valve of Leiblein and the mid-esophagus was reduced at metamorphosis (compare Figure 23E to F and 24A to B). Additionally, the radular sac, valve of Leiblein, and acinous salivary glands extended through the nerve ring in both stage 6a and stage 6b specimens (Figure 23E and F). A twist in the mid-esophagus eventually placed the gland of Leiblein at a right position (Figure 24A-F). Finally, the proboscis sac in *N. ostrina* developed as one dorsal invagination, rather than two as was observed in *N. lamellosa* (Figure 23A and B).

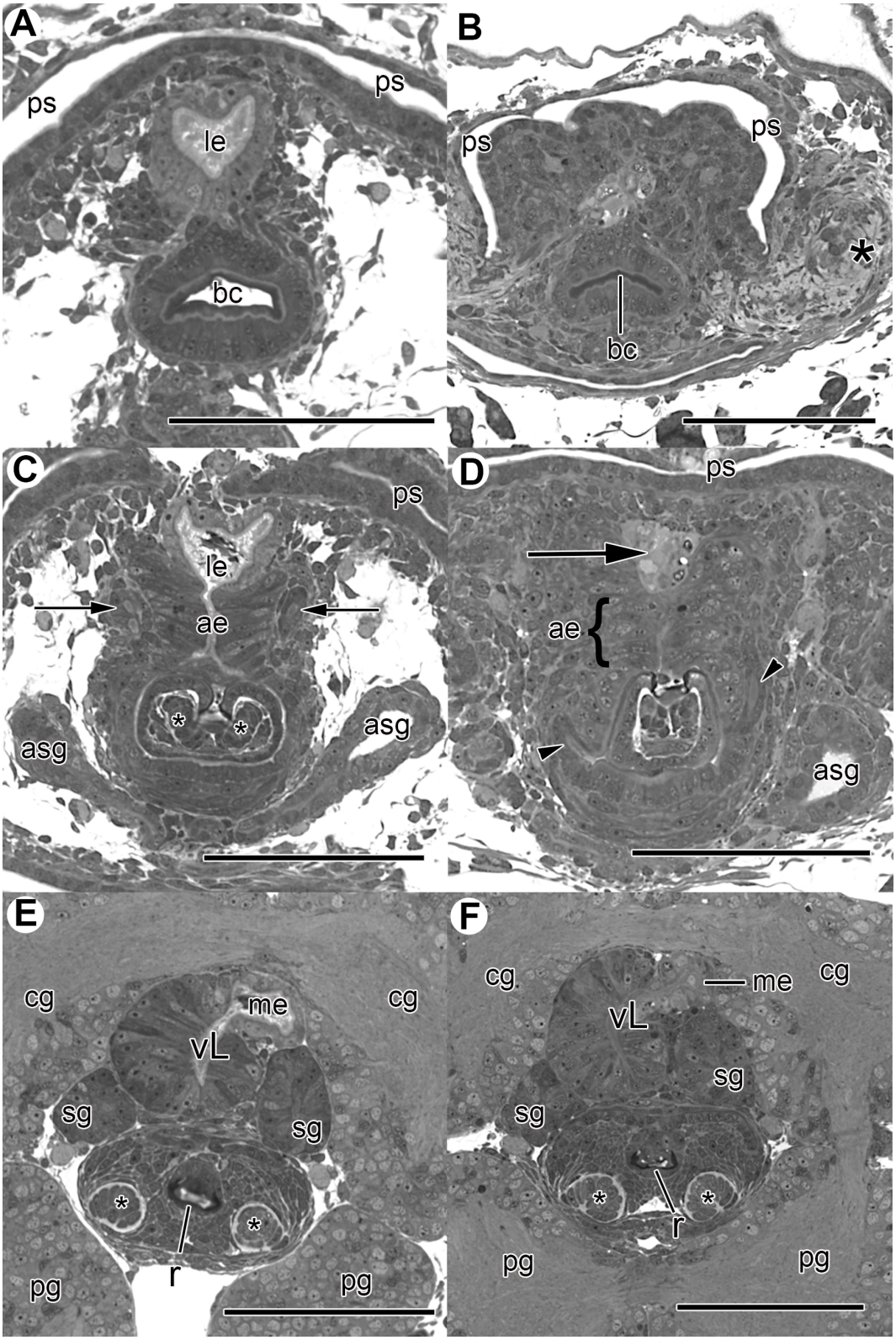

Figure 23. Cross sections through the anterior esophagus before and after metamorphosis in *Nucella ostrina.*

Notice the destruction of the larval mouth and the distal larval esophagus at metamorphosis. All scale bars= 100 μm. **A.** Stage 6a, pre-metamorphosis; larval esophagus with the definitive buccal cavity beneath; a deep dorsal invagination of the developing proboscis sac has formed. **B.** Stage 6b, post-metamorphosis; absence of the larval mouth and larval esophagus, only the anterior projection of the buccal cavity can be observed in this section; masses of the reabsorbed velar lobes are on either side of the head (asterisk indicates left mass). **C.** Stage 6a; continuity of the lumens of the larval esophagus, definitive anterior esophagus, and the buccal mass; the arrows indicate the location of the ducts of the acinous salivary glands. **D.** Stage 6b; dissociated cells of the larval esophagus fill the lumen (arrow); columnar cells of the anterior esophagus retained; the arrowheads indicate the ducts of the acinous salivary glands coming off the posterior end of the buccal cavity. **E.** Stage 6a; valve of Leiblein at the level of the nerve ring; the acinous salivary glands and radular sac pass through the nerve ring; the asterisks indicate the odontophoral cartilages that support the radula. **F.** Stage 6b; reduction of the dorsal portion of the valve of Leiblein; the asterisks indicate the odontophoral cartilages that support the radula. Abbreviations: ae= anterior esophagus, asg= accessory salivary gland, bc= buccal cavity, cg= cerebral ganglion, le= larval esophagus, me= mid-esophagus, pg= pedal ganglion, ps= proboscis sac, r= radula, sg= acinous salivary gland, vL= valve of Leiblein.

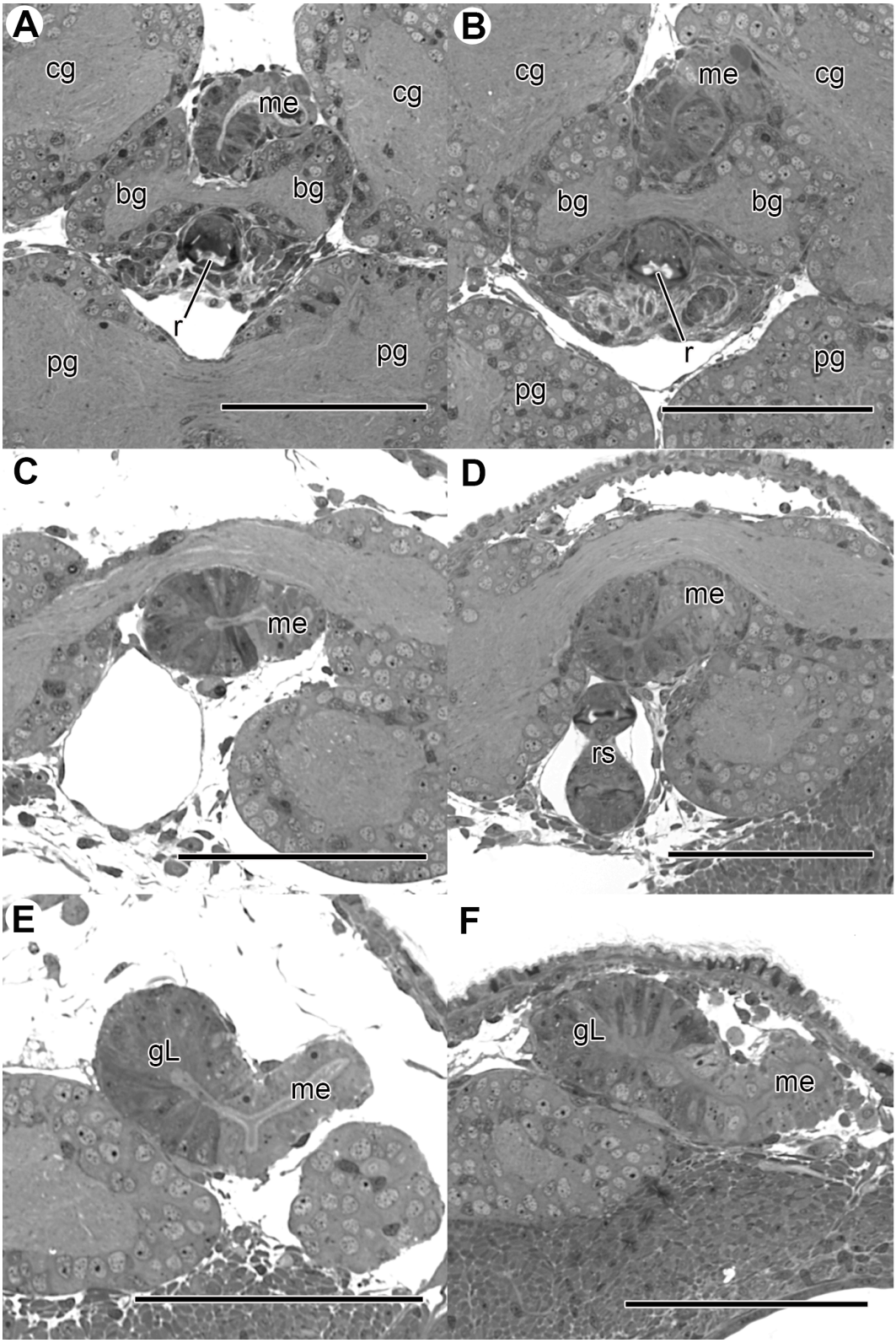

Figure 24. Cross sections through the mid-esophagus before and after metamorphosis in *Nucella ostrina.*

Notice that no major changes occur to the mid-esophagus at metamorphosis; all scale bars= 100 μm. **A.** Stage 6a, pre-metamorphosis; start of the mid-esophagus just posterior to the valve of Leiblein, which is at the level of the buccal commissure connecting the buccal ganglia. **B.** Stage 6b, post-metamorphosis; start of the mid-esophagus at the level of the buccal commissure. **C.** Stage 6a; the ventral glandular portion of the mid-esophagus has taken a right position. **D.** Stage 6b; the ventral glandular portion of the mid-esophagus has taken a right position. **E.** Stage 6a; gland of Leiblein at a right position. **F.** Stage 6b; gland of Leiblein at a right position. Abbreviations: bg= buccal ganglion, cg= cerebral ganglion, gL= gland of Leiblein, me= mid-esophagus, pg= pedal ganglion, r= radula, rs= radular sac.

After metamorphosis and juvenile hatching, proboscis elongation resulted in similar shifts in the foregut as were observed in *N. lamellosa.* A new mouth formed at the terminus of the proboscis, which led to the buccal cavity (Figure 25A and B). Further posteriorly, the buccal cavity gave rise to the definitive anterior esophagus and the radular sac, which ran the length of the proboscis (Figure 25C and D). By hatching, the anterior esophagus had fully differentiated and was in the form of a distinct tube (unlike stage 6 specimens). The valve of Leiblein, acinous salivary glands, and buccal mass were pulled anteriorly out of the nerve ring as the proboscis elongated. The valve of Leiblein took a position dorsal to the cerebral commissure and the radula coiled at a right dorsal position (Figure 26A). Only the mid-esophagus traversed the narrow passage through the nerve ring (Figure 26B). The gland of Leiblein increased in size and its connection to the mid-esophagus was reduced to a narrow duct; posterior to the duct, the posterior esophagus extended to the stomach (Figure 26C and D).

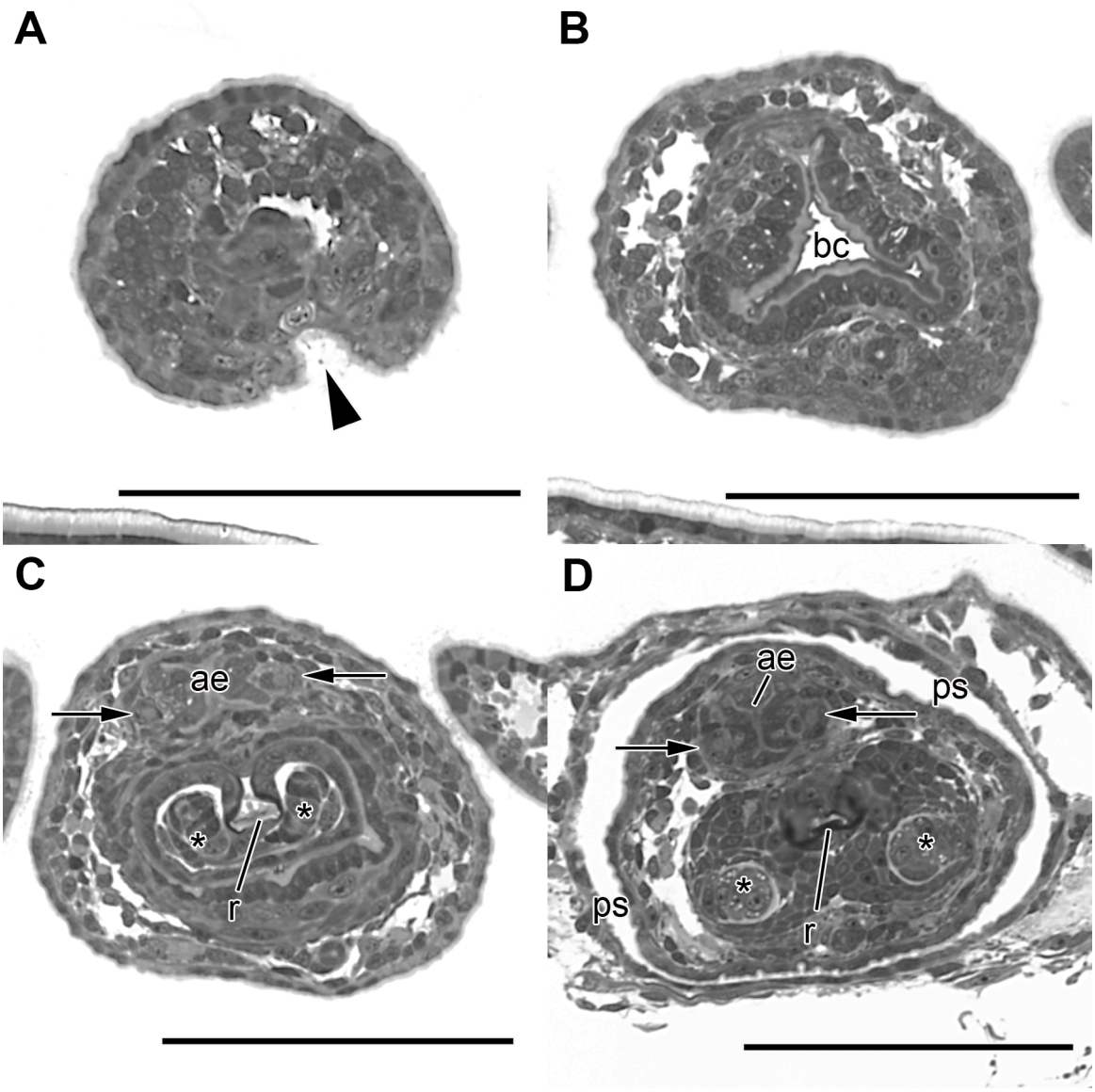

Figure 25. Cross sections through the proboscis and proboscis sac of a recently hatched juvenile of *Nucella ostrina*.

All scale bars= 100 μm. **A.** Mouth (arrowhead) at the terminus of the extended proboscis. **B.** Buccal cavity at the terminus of the extended proboscis, just posterior to the mouth. **C.** Anterior esophagus and radula run down the length of the extended proboscis; arrows indicate the ducts of the acinous salivary glands and the asterisks indicate the odontophoral cartilages that support the radula. **D.** Base of the extended proboscis showing the surrounding proboscis sac. Abbreviations: ae= anterior esophagus, bc= buccal cavity, ps= proboscis sac, r= radula.

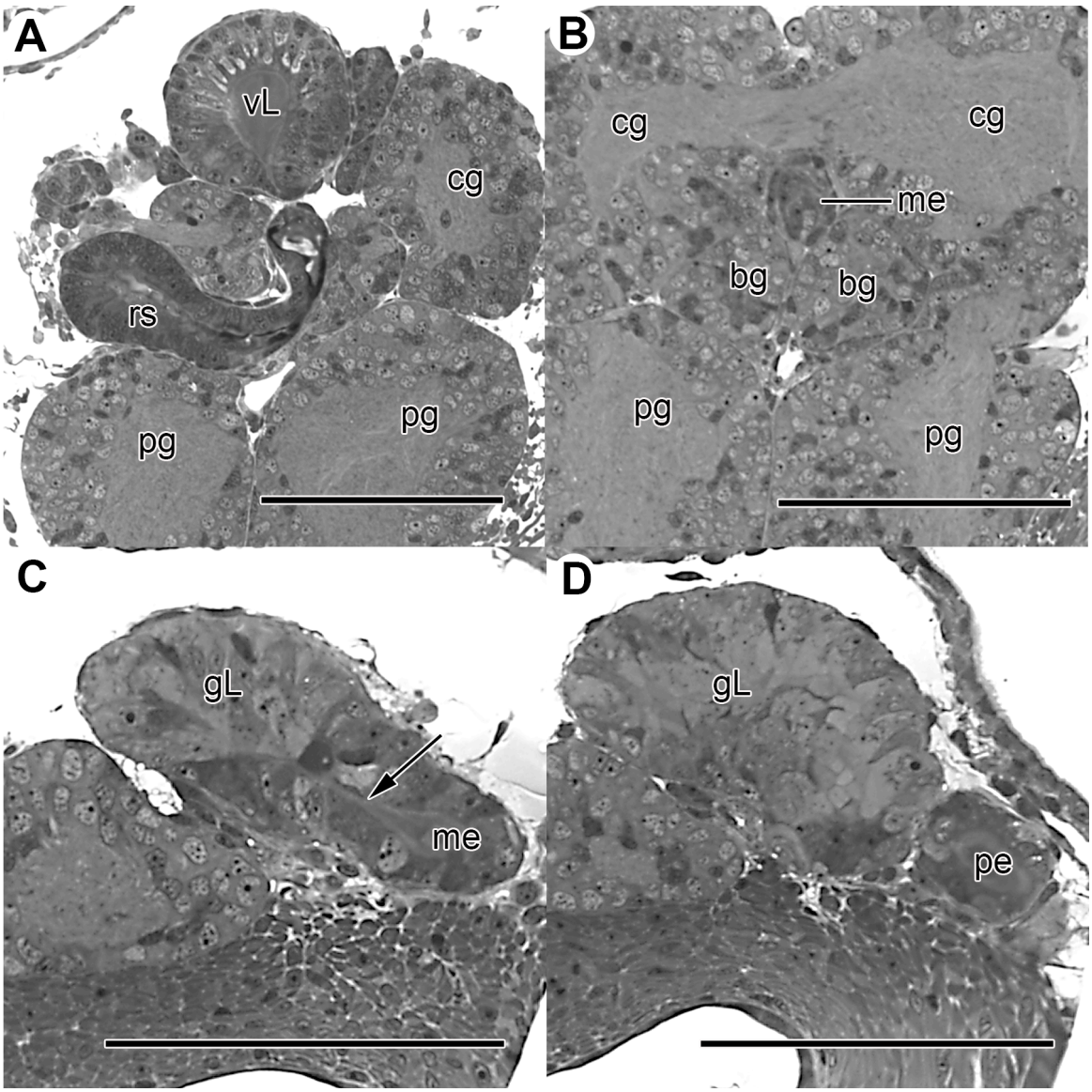

Figure 26. Cross sections through the mid-esophagus of a recently hatched juvenile of *Nucella ostrina*.

All scale bars= 100 μm. **A.** Valve of Leiblein at a dorsal position relative to the nerve ring; the radula coils at a right dorsal position to the nerve ring. **B.** Only the mid-esophagus extends through the nerve ring. **C.** Gland of Leiblein at a right position relative to the mid-esophagus; the arrow indicates the duct that connects the gland of Leiblein to the mid-esophagus. **D.** The gland of Leiblein occupies much of the cephalic hemocoel; the posterior esophagus extends to the stomach. Abbreviations: bg= buccal ganglion, cg= cerebral ganglion, gL= gland of Leiblein, me= mid-esophagus, pe= posterior esophagus, pg= pedal ganglion, rs= radular sac, vL= valve of Leiblein.

3.5 Further confirmation of the fate of the larval esophagus in *Nucella lamellosa* through TEM

Histological sections of pre- and post-metamorphic stages suggested that the distal larval esophagus was destroyed at metamorphosis. Additional TEM analysis of the anterior foregut of pre- and post-metamorphic stages of *N. lamellosa* further supported these findings (compare Figure 27A and B). Comparison of the cross sections through the intact stage 11a larval esophagus and the remnant of the larval esophagus in stage 11b partially illustrated the process of destruction (compare Figure 27C and D). The cells from the wall of the larval esophagus detached from their basal lamina as evidenced by folds in the still intact basal lamina and loose cells within the lumen (Figure 27D). Furthermore, TEM analysis demonstrated the close association between the ducts of acinous salivary glands and the anterior esophagus, not the larval esophagus, in pre-metamorphic stage 11a (Figure 27E). After metamorphosis, the ducts of the acinous salivary glands remained in close proximity to the anterior esophagus (Figure 27F).

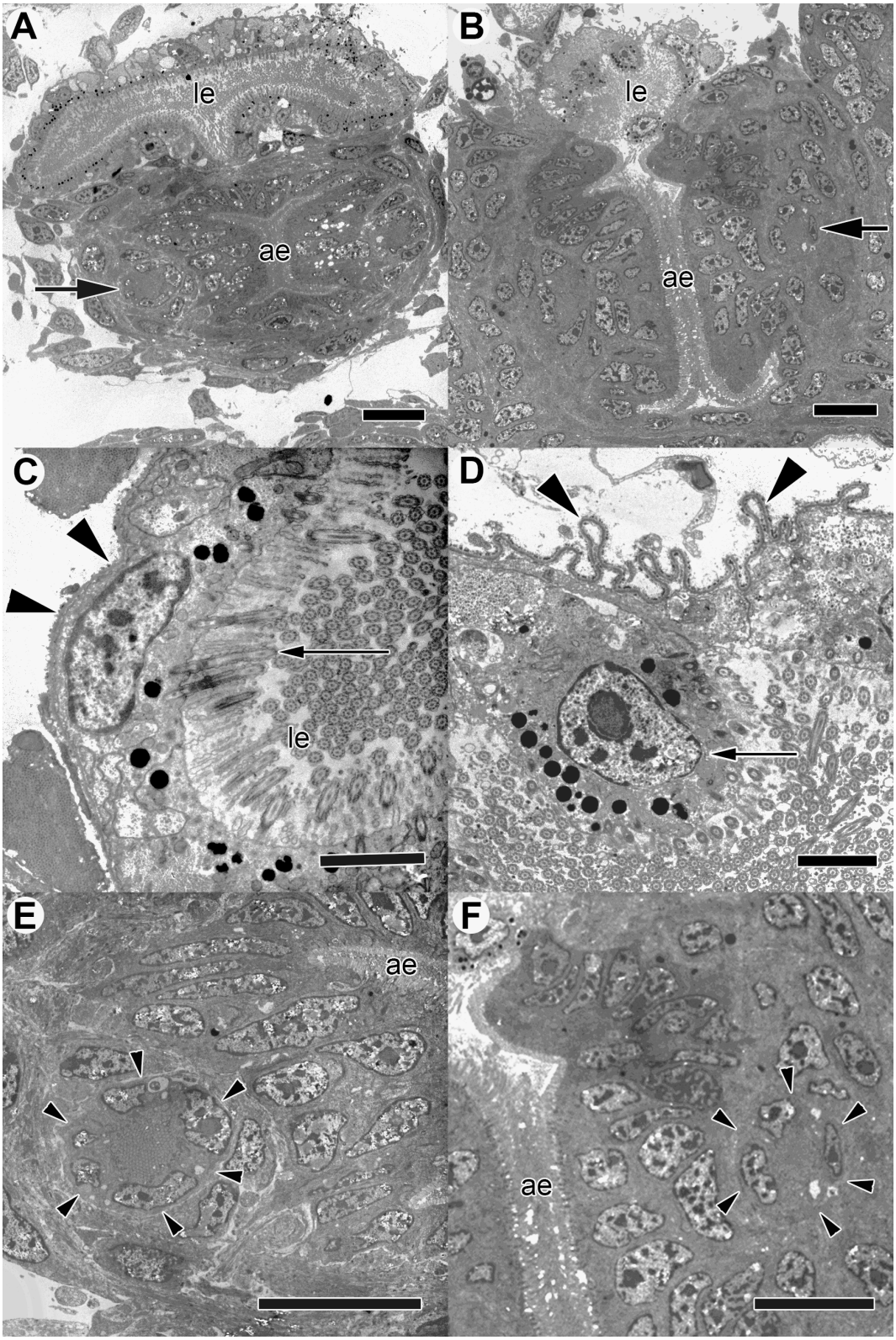

Figure 27. Transmission electron micrographs of cross sections through the foregut of *Nucella lamellosa* before and after metamorphosis.

A. Stage 11a; larval esophagus and anterior esophagus prior to metamorphosis; arrow indicates the duct of the right acinous salivary gland that is enlarged in E; scale bar = 10 μm. **B.** Stage 11b; remnant of the deteriorating larval esophagus and the anterior esophagus after metamorphosis; arrow indicates the duct of the left acinous salivary gland that is enlarged in F; scale bar= 10 μm. **C.** Stage 11a; wall of the larval esophagus showing the basal lamina (arrowheads) and the ciliated lumen (cilium indicated by arrow); scale bar= 2 μm. **D.** Stage 11b; wall of the remnant of the larval esophagus; notice the folded basal lamina (arrowheads) and the cell that had detached from the basal lamina (arrow); scale bar= 2 μm. **E.** Stage 11a; duct of the right acinous salivary gland in close association with the tissues of the future anterior esophagus; scale bar= 10 μm. **F.** Duct of the left acinous salivary gland remains associated with the anterior esophagus post-metamorphosis; scale bar= 10 μm. Abbreviations: ae= anterior esophagus, le= larval esophagus.

4.0 Discussion

4.1 Foregut developmental modules in *Nucella lamellosa* and *N. ostrina*

I demonstrated that foregut development includes dorsal and ventral developmental modules in both *Nucella lamellosa* and *N. ostrina*, two neogastropods (Muricoidea) that have lost a planktotrophic larva from their respective life histories. Furthermore, in both of these species, a vestigial larval esophagus (*i.e.* the dorsal developmental module) was apparant early in development. Subsequently, an out-pocketing of cells arising from the ventral side of the vestigial larval esophagus (*i.e.* the ventral developmental module) gave rise to the post-metamorphic foregut components (Figure 28). The presence of dorsal and ventral components in the developing distal foregut strongly resembles previous descriptions given for caenogastropods with indirect development (*e.g.* Werner, 1955; Fretter, 1969; Thiriot-Quiévreux, 1969, 1974; Page, 2000, 2002, 2005, 2011; Parries and Page, 2003). However, unlike caenogastropods that are herbivorous grazers as adults, the vestigial larval esophagus of *N. lamellosa* and *N. ostrina* was destroyed at metamorphosis and was not retained as the dorsal food groove of the post-metamorphic buccal cavity and anterior esophagus. Destruction of the distal larval esophagus and larval mouth at metamorphosis was previously reported in four other species of predatory gastropods (Page, 2000, 2002, 2005, 2011). Overall, the pattern of foregut development identified in *N. lamellosa* and *N. ostrina* is congruent with previous descriptions given for predatory caenogastropods with indirect development (*i.e. Euspira lewisii,* Naticoidea, Page, 2000; *Marsenina stearnsii,* Lamellaroidea, Page, 2002; *Nassarius mendicus,* Buccinoidea, Neogastropoda, Page, 2000, 2005; *Conus lividus*, Conoidea, Neogastropoda, Page, 2011).

The dorsal and ventral portions of the developing foregut of caenogastropods are suggested to represent distinct developmental modules because they have different developmental trajectories and ontogenetic fates. Raff (1996) defined a developmental module as a component of a developing organism that shows autonomous self-organization, such that a developmental module may change during development without perturbing differentiation of other components of the organism. Developmental modules

are therefore dissociable in time and/or space and are independent of other modules. The best and most widespread evidence for the existence of developmental modules is demonstrated by temporal and spatial dissociability of different components during development, such as was observed in the dorsal and ventral portions of the developing foregut in *N. lamellosa* and *N. ostrina*.

My comparative study on foregut development in two species of *Nucella*, uncovered at least two instances of temporal and spatial shifts in the developmental programs of the dorsal and ventral foregut modules. The first instance involved differences between *N. lamellosa* and *N. ostrina* in the time at which the ventral module (*i.e.* the definitive foregut) began to differentiate relative to the developmental progress of other structures. In *N. lamellosa*, the regional differentiation of the ventral out-pocketing to form the future buccal cavity, radular sac, and anterior esophagus with valve of Leiblein (Figure 28B) began when the veliger-like embryos had round velar lobes, a protoconch that covered the visceral mass, a foot with a propodium, and a cephalic vesicle with initial cephalic tentacle rudiments (stage 6). By contrast, veliger-like embryos of *N. ostrina* that looked externally similar to stage 6 embryos of *N. lamellosa* showed no incipient differentiation of the ventral module of the developing foregut. The ventral module was merely a long trench along the ventral side of the larval esophagus. Differentiation of the ventral foregut module in *N. ostrina* was delayed until after all nurse eggs had been consumed. Nurse egg consumption required a spacious larval esophagus, which left limited space for differentiation of the ventral post-metamorphic foregut during *N. ostrina* development. Thus, in *N. ostrina* space requirements for nurse egg consumption has resulted in a temporal shift (*i.e.* delayed onset of differentiation in the ventral module) relative to the congeneric *N. lamellosa*, which does not feed during development.

The second instance of a developmental difference involving the ventral foregut modules of *N. lamellosa* and *N. ostrina* involved the degree of spatial separation between the larval esophagus and the anterior esophagus. In *N. lamellosa*, the lumens of the larval esophagus and the post-metamorphic esophagus were completely separated just prior to metamorphosis (Figure 28C, D, and E). The pre-metamorphic foregut of *N. lamellosa* strongly resembled that of *Nassarius mendicus*, a species with a planktotrophic larva.

However, in *Nassarius mendicus* the tube of the larval esophagus is separate from the tube of the post-metamorphic anterior esophagus throughout pre-metamorphic development (Page, 2000, 2005), whereas this separation did not occur until a late pre-metamorphic stage in *N. lamellosa* (stage 11a). Conversely, in *N. ostrina*, the lumens of the larval esophagus and the post-metamorphic anterior esophagus never completely separated (Figure 28C and D); the larval esophagus was destroyed by dissociation of cells while its lumen was fully continuous with that of the definitive anterior esophagus. In fact, the pre-metamorphic stage in *N. ostrina* strongly resembled that of *Nucella lapillus*, a congeneric that also feeds on nurse eggs during development (Ball *et al.,* 1997a, b; Ball, 2002).

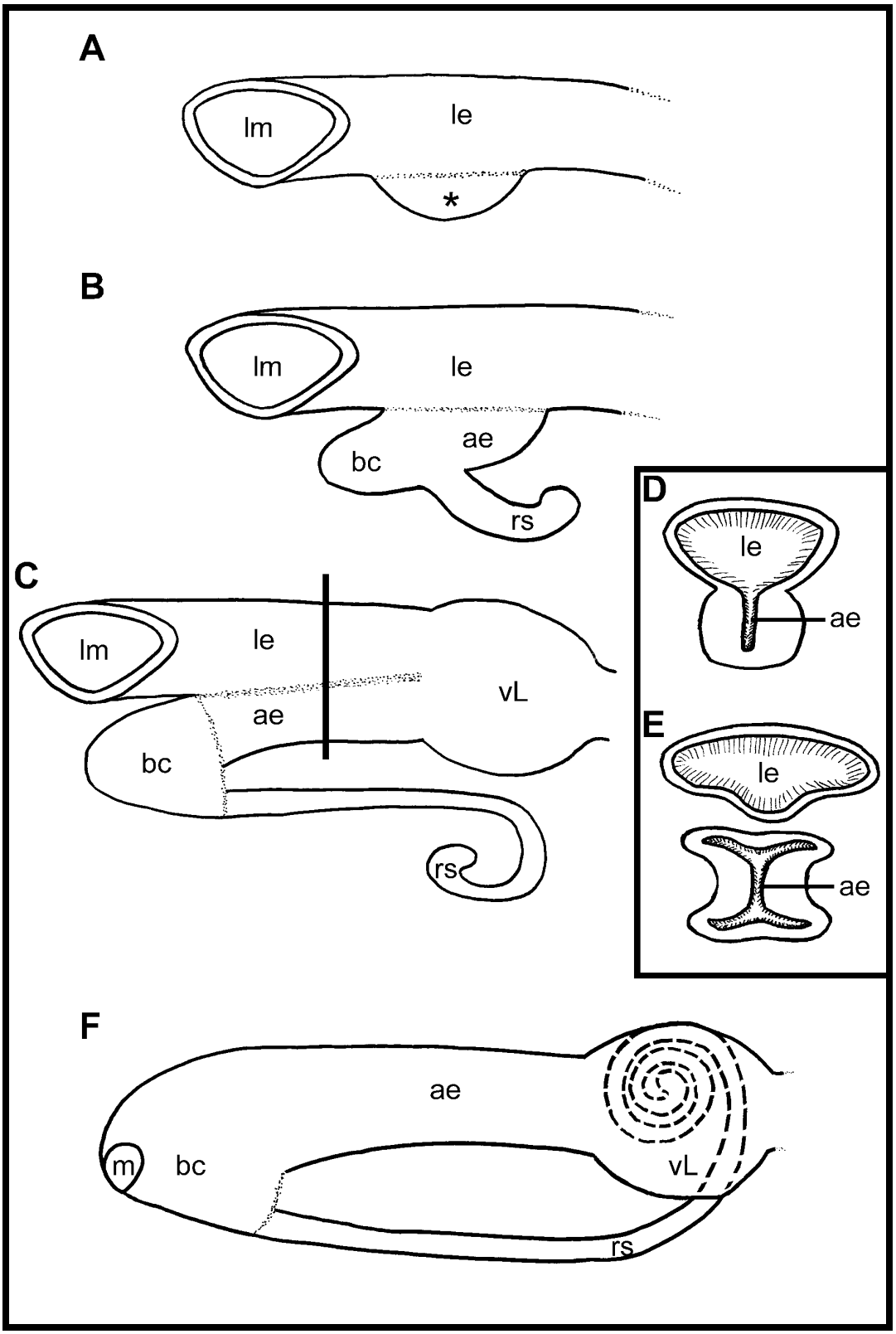

Figure 28. Summary of foregut development in *Nucella lamellosa* and *N. ostrina*.

A. Formation of the larval esophagus and ventral out-pocketing (asterisk). **B.** Regional differentiation of the ventral out-pocketing to form the buccal cavity, definitive anterior esophagus, and radular sac; note that the definitive anterior esophagus and larval esophagus remain connected along a mid-sagittal trench. **C.** Pre-metamorphic foregut showing the larval esophagus and the post-metamorphic foregut at an advanced stage beneath; line indicates the cross sections in D and E; the larval esophagus and definitive anterior esophagus remain connected along a mid-sagittal trench in stage 8 of *N. lamellosa* and stage 6a of *N. ostrina* (cross section D); the larval esophagus and anterior esophagus are two separate tubes in stage 11a of *N. lamellosa* (cross section E). **D.** Cross section showing the connection between the larval esophagus and the anterior esophagus in stage 8 of *N. lamellosa* and stage 6a of *N. ostrina*. **E.** Cross section through the larval esophagus and anterior esophagus of *N. lamellosa* showing the separation that occurs prior to metamorphosis in stage 11a of this species. **F.** Juvenile of *N. lamellosa* and *N. ostrina*; no larval esophagus and a new mouth. Abbreviations: ae= anterior esophagus, bc= buccal cavity, le= larval esophagus, lm= larval mouth, m= post-metamorphic mouth, rs= radular sac, vL= valve of Leiblein.

With the results from this study in mind, an analysis of histological sections and diagrams of foregut development in *Nucella lapillus* from Ball *et al.* (1997a, b) and Ball (2002) shows structures that strongly resemble homologues of the larval mouth and larval esophagus *i.e.* the dorsal module, as described here for *N. lamellosa* and *N. ostrina*. Ball *et al.* (1997a, b) and Ball (2002) most likely incorrectly identified these structures as the definitive mouth and oral tube/dorsal food groove, respectively. Because Ball *et al.* (1997a, b) and Ball (2002) did not investigate early developmental stages in *N. lapillus* the obvious larval esophagus, which was used to feed on nurse eggs, was missed. Instead, Ball *et al.* (1997a, b) identified the homologue of the larval esophagus in later developmental stages as two different structures: the oral tube and the dorsal food groove. However, as demonstrated in both *N. lamellosa* and *N. ostrina*, the oral tube, which connects the mouth to the buccal cavity, does not develop until after metamorphosis. Even the elongate oral tube that runs the length of the proboscis in *C. lividus* did not develop until after metamorphosis (Page, 2011). The interpretation by Ball *et al.* (1997a,b) and Ball (2002) for late developmental stages in *N. lapillus* was understandable, but is now shown to be flawed given new information from the investigation of the congenerics: *N. lamellosa* and *N. ostrina* (present study).

Furthermore, the portion of the larval esophagus that was connected to the anterior esophagus in early stages of foregut development in *N. lamellosa* and up to metamorphosis in *N. ostrina,* was correctly identified as the dorsal food groove in *N. lapillus* (recall that the dorsal food groove and larval esophagus are thought to be homologous structures). However, the pre-metamorphic mouth and the homologue of the larval esophagus were most likely destroyed at metamorphosis in *N. lapillus*, but Ball *et al.* (1997a, b) did not describe this process. Because the spatial separation of the dorsal module (larval esophagus) and the ventral module (post-metamorphic anterior esophagus) was absent in *N. lapillus*, similar to *N. ostrina,* identification of distinct developmental modules was missed. Additionally, the destruction of the distal portion of the dorsal module in *N. lapillus* was perhaps obfuscated due to the lack of spatial separation of the dorsal and ventral modules.

By comparing foregut development in a neogastropod with indirect development, *Nassarius mendicus* (Buccinoidea), and the direct developing neogastropods: *Nucella*

lamellosa, N. ostrina, and *N. lapillus* (Muricoidea), a pattern of reduced spatial separation between the dorsal and ventral foregut developmental modules emerges. In *Nassarius mendicus* the larval esophagus and anterior esophagus remain separate throughout development, whereas in *N. ostrina* and *N. lapillus* the larval esophagus and anterior esophagus remain connected throughout development. Separation of the distal portion of the larval esophagus from the anterior esophagus does eventually occur in *N. lamellosa*, but only in the stage immediately prior to metamorphosis. Therefore, foregut development in *N. lamellosa* appears to occupy a transitional stage in the loss of the spatial uncoupling of the dorsal and ventral developmental modules.

A proposed phylogeny (based on 718 base pairs of nucleotide sequence from the mitochondrial cytochrome b gene) for the genus *Nucella* places *N. lapillus* in a more ancestral position compared to *N. lamellosa* and *N. ostrina* and places *N. ostrina* in the most derived position (Collins *et al.,* 1996). According to this phylogeny, the ancestral life history for northern hemisphere species of *Nucella* is adelphophagic direct development, with nurse egg production lost in *N. lamellosa*. In fact, *N. lamellosa* is the only member of the clade that does not have adelphophagic development. Therefore, the loss of spatial separation between the dorsal and ventral modules during foregut development may be the basal condition for the genus *Nucella*. If this is the case, then spatial separation of the dorsal and ventral modules during foregut development in *N. lamellosa* must be regarded as an atavism *i.e.* reacquisition of the presumed ancestral developmental pattern for neogastropods.

Results from this study, however, would indicate that *N. lamellosa* has partially retained the ancestral condition, which was subsequently lost in other members of the genus, such as *N. ostrina* and *N. lapillus.* According to Page (2000), the physical separation of the larval esophagus and the ventral post-metamorphic foregut was initially selected based on the need to maintain larval feeding while concurrently developing an elaborate post-metamorphic foregut in species with indirect planktotrophic development. Selection for separation of the larval esophagus and anterior esophagus into two tubes is clearly favorable in species with a feeding larval stage. At the same time, the loss of spatial separation observed in *N. ostrina* and *N. lapillus* is likely the result of reduced selective pressure for separation associated with the shift in life history. The size

restrictions due to nurse egg consumption may have selected against physical separation and thus against the concurrent development of the foregut modules. However, reacquisition of spatial separation of the dorsal and ventral modules yields no obvious benefits for *N. lamellosa*. From the comparative developmental data, the spatial separation in *N. lamellosa* would be better interpreted as retention rather than reaquisition of an ancestral trait. Thus, on the basis of findings from this study, revisting the phylogeny of the genus *Nucella* may be an interesting pursuit.

4.2 Evolutionary significance of foregut developmental modules in caenogastropods

Accumulated comparative data on foregut development in caenogastropods argues for the existence of separate dorsal and ventral developmental modules. The dissociability of these modules during development appears to have played a key role in the evolutionary history of the Caenogastropoda by facilitating two important novelties: the emergence of larval planktotrophy and the transition to predatory feeding. The early formation of the dorsal module to form the larval esophagus was essential to larval planktotrophy. Presumably, this larval esophagus was the dorsal food groove of the ancestral, post-metamorphic foregut that was co-opted for use in the larval stage. In caenogastropods that become herbivorous adults, the larval esophagus reverts to its original function of dorsal food groove after metamorphosis. However, in predatory gastropods that have been studied through development, the distal part of the larval esophagus is destroyed at metamorphosis. Subsequently, the isolation of the ventral module from the dorsal module during foregut development enabled the post-metamorphic ventral foregut components to specialize free from the morphological constraints of the larval mouth and esophagus. That is, the distal portion of the post-metamorphic foregut was not a remodelled version of the simple, ciliated larval mouth and esophagus. The ventral module forms an out-pocketing in the distal larval esophagus, which is elaborated to form the major components of the post-metamorphic foregut, such as the buccal cavity, radular sac, and acinous salivary glands. In predatory neogastropods, the entire post-metamorphic distal foregut is derived from this ventral out-pocketing. At the mid-region of the foregut, the dorsal module *i.e.* the larval esophagus, becomes the

main channel of the mid-esophagus after metamorphosis. In predatory neogastropods, the ventral module in the mid-esophagus consists of a ventral trench of densely staining cells during development, which differentiate into the post-metamorphic gland of Leiblein in neogastropods such as *Nucella* and forms the venom gland in *Conus*.

The direct transformation of the larval esophagus into the dorsal food groove at metamorphosis in *Lacuna vincta* (Page, 2000) strongly suggests that the larval esophagus and the dorsal food groove are developmentally homologous structures. As suggested by Page (2000), the destruction of the larval esophagus at metamorphosis in predatory caenogastropods may simply be an upregulation in the reduction of cells from the larval esophagus, which accompanies metamorphosis in the herbivorous grazers such as *L. vincta*. This upregulation could explain the complete loss of the distal larval esophagus at metamorphosis and thus loss of the post-metamorphic dorsal food groove in predatory neogastropods such as: *Nassarius mendicus* (Page, 2000, 2005) and *N. lamellosa,* and *N. ostrina* (present study). Nevertheless, Graham (1941) identified a putative dorsal food groove in the foregut of adult *N. lapillus*. However, in light of the more recent developmental information, the suggestion of a dorsal food groove running down the anterior esophagus of *N. lapillus* should be reconsidered. As indicated by both light microscopy and TEM, the portion of the larval esophagus (*i.e.* the dorsal food groove) from the valve of Leiblein forward was destroyed at metamorphosis in *N. lamellosa* and *N. ostrina*, and was most likely also destroyed in the congeneric *N. lapillus*. Longitudinal folds in the wall of an adult gastropod foregut could easily be interpreted as the dorsal food groove, but would not necessarily represent a true homology. Thus developmental studies can be useful for testing hypotheses about homologous structures. Previous identifications of the dorsal food groove in predatory neogastropods need to be revisited.

Ponder (1973) suggested that the anterior esophagus of predatory neogastropods can be interpreted as a posterior extension of the buccal cavity. He noted that the buccal ganglia (and the commissure that connects them) lie beneath the junction of the buccal cavity and the esophagus in most gastropods; however, in muricoidean neogastropods the buccal ganglia and connecting commissure lie beneath the junction of the valve of Leiblein and the mid-esophagus. In both *N. lamellosa* and *N. ostrina*, the buccal ganglia and commissure were present beneath the start of the mid-esophagus, immediately

posterior to the valve of Leiblein. Further, the buccal cavity in the ancestral herbivorous grazer, *Lacuna vincta*, was derived from a ventral out-pocketing embedded in the larval esophagus (Fretter, 1969; Page, 2000). The developmental derivation of the anterior esophagus and valve of Leiblein from the ventral out-pocketing in *Nassarius mendicus* (Page, 2000, 2005), *N. lamellosa*, and *N. ostrina* further corroborates Ponder's (1973) theory concerning the evolutionary derivation of the anterior esophagus.

The present study provides additional support for the hypothesis that the mid-esophageal gland (elaborated into the gland of Leiblein in some neogastropods) is homologous with the venom gland in members of the genus *Conus* (Page, 2012). This homology is suggested by the equivalent developmental derivation of the two structures. The venom gland in *Conus lividus* developed from a ventral trench of more densely staining cells, which pinched off from the mid-esophagus after metamorphosis. The pinching off of the venom gland was thought to be a unique developmental feature of this structure (Page, 2012). However, development of the gland of Leiblein in *N. lamellosa* and *N. ostrina* demonstrated a similar pinching off after metamorphosis. Similar to venom gland development in *C. lividus*, the gland of Leiblein in *N. lamellosa* and *N. ostrina* originated from a ventral trench of more densely staining cells in the mid-esophagus. After metamorphosis the greatly enlarged gland of Leiblein pinched off the main channel of the mid-esophagus and remained connected by only a narrow passage.

Heterobranchs provide an excellent group for comparison to the caenogastropods because of their sister group relationship (Figure 3). Although predatory feeding has emerged in this group, the specialized foregut of predatory heterobranchs does not develop via physical separation of dorsal and ventral modules during foregut development. For example, in the nudibranch *Doridella steinbergae*, a predatory species that feeds on bryozoans in the genus *Membranipora*, foregut development during the larval stages consists of formation of a ciliated larval esophagus and the radular apparatus develops from a diverticulum beneath (similar to caenogastropods). Cells that gave rise to the radular sac, radular teeth, and radular musculature were derived from an isolated out-pocketing permitting both development of the radula and unobstructed larval microherbivory (Bickell and Chia, 1979; Bickell *et al.,* 1981). However, the larval mouth and larval esophagus are not destroyed at metamorphosis, but are remodelled and

elaborated into the adult feeding structures after metamorphosis (*e.g.* the specialized buccal pump) (Bickell and Chia, 1979; Bickell *et al.,* 1981). If larval planktotrophy was acquired once in the ancestor to the neritimorphs, heterobranchs, and caenogastropods, then the esophagus present during larval stages of heterobranchs is most likely homologous to the larval esophagus present in caenogastropods. The ventral out-pocketing in the wall of the larval esophagus, that forms the radular apparatus during larval stages, is most likely homologous with the ventral module described in caenogastropods. However, the specialization of the ventral module in isolation from the dorsal module (larval esophagus), such as the derivation of the post-metamorphic buccal cavity from the ventral out-pocketing, appears to be an important feature in the evolution of caenogastropod foregut development. The predatory heterobranch described above developed its specialized feeding structure after metamorphosis. The spatial separation of dorsal and ventral modules during foregut development does not appear to have influenced the emergence of predatory feeding in the heterobranchs.

4.3 Proboscis development and foregut rearrangements in juveniles of *Nucella lamellosa* and *N. ostrina*

Despite differences in foregut development, proboscis development in *N. lamellosa* and *N. ostrina* closely followed the pattern identified in previous studies of both pleurembolic and intraembolic proboscis types (*i.e. Nucella lapillus,* Ball *et al.*, 1997a; *Conus anemone,* Ball *et al.,* 2002; *Marsenina stearnsii*, Page, 2002; *Nassarius mendicus,* Page, 2000; Page, 2005; *Conus lividus,* Page, 2011). In all species, proboscis development involved the formation of epithelial thickenings dorsal and ventral to the larval mouth and formation of dorsal and ventral invaginations of the developing proboscis sac. Initial invagination of the proboscis sac appears to vary slightly between species. In *N. lapillus* the proboscis sac initially formed as two dorsal and two ventral invaginations, whereas in *N. lamellosa* the proboscis sac started as two dorsal and one ventral invagination and in *N. ostrina* the proboscis sac started as one dorsal and one ventral invagination. In *N. lapillus, N. lamellosa,* and *N. ostrina* the dorsal invaginations were much deeper than the ventral invaginations. In all cases, loss of the velar lobes at metamorphosis allowed the proboscis sac to fully surround the proboscis and

subsequently the proboscis elongated. Although Golding *et al.* (2009) has suggested multiple origins of the various proboscis types, the similarities in neogastropod early proboscis development may suggest a common origin for the neogastropod intraembolic and pleurembolic proboscis types.

Foregut rearrangements that accompanied the elongation of the proboscis in *N. lamellosa* and *N. ostrina* were very similar to the description by Ball *et al.* (1997a) for *N. lapillus*. As Ball *et al.* (1997a) stated, the neogastropod foregut arrangement was not realized until after metamorphosis. Neogastropod foregut synapomorphies include: the position of the acinous salivary glands in front of the circumesophageal nerve ring, two histologically distinct salivary glands, and a valve of Leiblein anterior to the nerve ring (Taylor and Morris, 1988; Kantor, 1996). In juveniles of both species these synapomorphies were present, however, in pre-metamorphic stages the acinous salivary glands and the valve of Leiblein extended through the circumesophageal nerve ring. It was only after metamorphosis in these species that the acinous salivary glands and the valve of Leiblein were pulled anteriorly out of the nerve ring, which was the result of proboscis elongation. Proboscis elongation also pulled the radula out of the nerve ring. The radula then coiled anterior to the nerve ring, on the right side. Only the portion of the mid-esophagus posterior to the valve of Leiblein passed through the nerve ring after metamorphosis. Growth of the ganglia that composed the circumesophageal nerve ring reduced the internal aperture and thus greatly restricted the structures that could pass through the nerve ring. Overall, despite differences in the early foregut development of *N. lamellosa* and *N. ostrina*, the rearrangements of the foregut after metamorphosis produced strikingly similar juvenile foregut morphology.

4.4 Other aspects of developmental evolution in *Nucella lamellosa* and *N. ostrina*

Recently laid egg capsules of *N. lamellosa* contained approximately 20-50 embryos that were approximately 500-600 μm in diameter, whereas recently laid egg capsules of *N. ostrina* contained hundreds of eggs that were approximately 200 μm in diameter. These results agree with previous work that determined the average number of embryos per egg capsule and the egg diameter for *N. lamellosa* to be 19.3 ±8.1 and 590-

638 μm, respectively; egg capsules of *N. ostrina* were estimated to contain 300-1000 eggs with a diameter of 180 μm (Strathmann, 1987). Despite marked differences in ovum size and number between *N. lamellosa* and *N. ostrina*, juvenile hatching size was comparable between these species. Most of the eggs present in the early egg capsules of *N. ostrina* became nurse eggs, which were all consumed by developing embryos. From the capsules that I opened, only about 10-20 juveniles of *N. ostrina* hatched. Juveniles of *N. lamellosa* had an approximate shell length of 1 mm, whereas juveniles of *N. ostrina* had an approximate shell length of 1-1.5 mm. Again this agrees with the previously published estimates for juvenile shell length of ~1 mm for *N. lamellosa* and 0.9-1.8 mm for *N. ostrina* (Strathmann, 1987). In general, *N. lamellosa* had much larger, but fewer eggs per egg capsule, whereas *N. ostrina* had numerous small eggs in each egg capsule.

Interestingly, an egg capsule of *N. ostrina* was found that contained a few very small embryos, which did not appear to feed on nurse eggs. The other embryos that did consume nurse eggs appeared to be proportionately larger based on the unequal sharing. Nonetheless, all of the embryos were at the same developmental stage, just different sizes. Either the larger more robust embryos outcompeted the small embryos for resources or the small embryos never attempted to feed on nurse eggs, as occurs in some polychaete species with poecilogony (Gibson and Carver, 2013).

Nurse egg consumption may select for reduced velar lobes. For example the caenogastropod *Petaloconchus montereyensis* (Vermetidae), a species that attaches itself to a large mass of yolk, has highly reduced velar lobes that are modified into a single wide ciliated band (Hadfield and Iaea, 1989). *Nucella ostrina* had smaller velar lobes than *N. lamellosa,* but they did not appear to be highly modified for feeding on nurse eggs like the velar lobes of *P. montereyensis*. The velar lobes of *Nucella lapillus,* however, are highly reduced (see Figure 4A and B in Ball *et al.,* 1997a) and they consist of a prototrochal ciliary band that protrudes slightly dorsally and laterally from the mouth (Stockmann-Bosbach, 1988). Large velar lobes may obstruct adelphophagy when embryos feed on a large mass of nurse eggs during ingestion. Therefore, selection may favor reduced velar lobes in *N. ostrina* and *N. lapillus*, compared to *N. lamellosa*.

Other direct developing caenogastropods maintain well-developed velar lobes and even use of the opposed band feeding mechanism (*e.g.* Chaparro *et al.,* 2002). Despite the

shift to direct development, *N. lamellosa* and *N. ostrina* had velar lobes with all three ciliary bands (prototroch, metatroch, and food groove); however, the metatroch present in veliger-like embryos of *N. ostrina* was reduced relative to the metatroch in veliger-like embryos of *N. lamellosa*. Furthermore, *N. lamellosa* may be capable of particle capture using an opposed band feeding mechanism. This was evidenced by pieces of the fragile larval kidney complex (damaged during the opening of egg capsules) found in the guts of sectioned stage 6 individuals. However, particle capture was not directly observed and thus it is uncertain whether or not the opposed band feeding mechanism was used. Nonetheless, although the velar lobes allowed the encapsulated embryos of both *N. lamellosa* and *N. ostrina* to rotate in the egg capsule, neither were capable of swimming in the water column when liberated from their egg capsules.

The retention of a prototroch in encapsulated embryos is advantageous because the long cilia can be used to circulate fluid within the egg capsule; the retention of a metatroch and food groove, however, has no obvious role other than providing plasticity in the reacquisition of larval planktotrophy (Strathmann, 1978). Because *N. ostrina* feeds on nurse eggs by attaching and engorging itself with yolk, the metatroch and food groove do not play an obvious role in consuming nurse eggs. Additionally, the metatroch and food groove certainly play no obvious role in the non-feeding veliger-like embryos of *N. lamellosa*. The phylogeny proposed by Collins *et al*. (1996) suggests that adelphophagy is ancestral for nucellids, however, it is unclear what velar lobe structure is ancestral for this group. The presence of planktotroph-like velar lobes in both *N. lamellosa* and *N. ostrina* suggests that this trait has been retained (not reacquired) in these species, but more members of the genus must be examined.

By metamorphosis in both *N. lamellosa* and *N. ostrina* the velar lobes were completely absorbed into the sides of the head. However, by the juvenile stage these masses of tissue were no longer apparent in either species. Reabsorbed cells of the velar lobes may be capable of dedifferentiation and redifferentiation to feed cells to the rapidly growing proboscis. Muricoideans can regenerate the distal foregut and the wall of the proboscis (Carriker *et al.*, 1972), which suggests that cells in this region (perhaps including the reabsorbed cells of the velar lobes) may be capable of dedifferentiation and redifferentiation for rapid growth. Additionally, the first meal of the post-metamorphic

stage appeared to be the larval esophagus as the cells from this degenerating structure were present in the gut after metamorphosis. Larval structures that were lost at metamorphosis appear to be recycled in the juvenile.

Juvenile feeding in *N. lamellosa* was observed two days after barnacle prey was provided. However, newly metamorphosed barnacles were collected approximately two weeks after juveniles of *N. lamellosa* began to hatch. Thus, juveniles can survive at least two weeks before their first meal, but the time period (if any) required for juveniles to become capable of feeding after hatching was not determined. Juveniles of *N. ostrina* were not reared in the lab and thus time to feeding was not determined for this species. However, because the foregut of a newly hatched juvenile of *N. ostrina* looked nearly identical to the foregut of a feeding juvenile of *N. lamellosa*, *N. ostrina* may be capable of feeding at hatching. At the same time, yolk from previously ingested nurse eggs was observed in the guts of juveniles of *N. ostrina*, suggesting that they could survive without food for at least as long or perhaps longer than *N. lamellosa*.

4.5 Summary

Through histological sectioning (light microscopy and TEM), 3D reconstructions, and SEM the present study has demonstrated that the direct developing neogastropods, *Nucella lamellosa* and *Nucella ostrina*, have retained remnants of the foregut developmental novelties that facilitated both larval feeding and predatory feeding in their ancestors. In both species, development of the foregut involved dorsal and ventral modules that had different ontogenetic fates and the modules were dissociable both temporally and spatially. Vestiges of the larval esophagus were present in both species. That is, the temporal separation between the dorsal and ventral modules was retained, resulting in early formation of the dorsal module *i.e.* the homologue of the larval esophagus. The larval esophagus in the non-feeding veliger-like embryos of *N. lamellosa* had no obvious role aside from forming the progenitor tissue for the ventral foregut module. Conversely, retention of the larval esophagus was essential to adelphophagy in *N. ostrina.* At metamorphosis both species destroyed the larval mouth and the distal larval esophagus and formed a new mouth after metamorphosis. Thus, the dorsal food groove was not present in the post-metamorphic stages of either species.

Differences in foregut development related to the physical separation of the dorsal and ventral developmental modules exist between *N. lamellosa* and *N. ostrina*. Whereas the larval esophagus and the definitive anterior esophagus are separate tubes before metamorphosis in *N. lamellosa*, the lumens of the larval esophagus and the definitive anterior esophagus remain connected prior to metamorphosis in *N. ostrina*. My observations help reconcile the disparate patterns of foregut development described previously for neogastropods with and without a planktotrophic larva, because *N. lamellosa* shows an intermediate condition between the pattern described for *Nassarius mendicus* (planktotrophic larvae) and the highly derived pattern seen in *Nucella lapillus* (Ball *et al.*, 1997a,b and Ball, 2002) and *Nucella ostrina* (present study). However, based on the phylogeny of the genus *Nucella* (Collins *et al.*, 1996) the spatial separation between the larval esophagus and the post-metamorphic anterior esophagus that was observed in *N. lamellosa* is an atavism. Nonetheless, my results suggest that differences in foregut development observed in species with and without a planktotrophic larva were associated with the shift in life history pattern rather than a difference in the origin of predatory feeding.

Moreover, my research lends additional support for the hypothesis that a developmental novelty promoted the diversification of predatory caenogastropods. Whereas the temporal separation between development of the dorsal food groove and development of the rest of the definitive foregut may have enabled larval feeding, the spatial separation between development of the dorsal larval esophagus and the ventral post-metamorphic foregut may have facilitated both larval feeding and a fast transition to a novel feeding strategy in the juvenile. Furthermore, the isolation of the post-metamorphic foregut may have enabled predators to evolve unconventional designs for the definitive foregut. The destruction of the larval mouth and distal larval esophagus together with formation of a new mouth after metamorphosis enabled the post-metamorphic foregut to evolve free from larval constraints. This developmental freedom, which was possible because of dissociable developmental modules, may have facilitated the rapid diversification of predatory species, culminating as the highly successful neogastropods.

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