Larval Development and Metamorphosis of *Berthella californica* (Gastropoda, Heterobranchia, Pleurobranchoidea) Including Phylogenetic Implications

by

Nicole Laura LaForge
B.Sc., University of Alberta, 2004

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Abstract

Larval development of *Berthella californica*, a pleurobranchoidean heterobranch from the Northeastern Pacific, is described using histological sectioning, SEM, TEM, and immunolabelling. Current phylogenetic hypotheses place the Pleurobranchoidea as sister to all nudibranchs, or as sister to only the anthobranch nudibranchs. Deciding between these alternatives is difficult due to extensive homoplasy among heterobranchs. Analysis of larval morphology may help resolve this issue by identifying additional phylogenetically informative characters. Larval development has been well documented for many nudibranchs, but there are few studies on pleurobranchoideans. Larvae of *B. californica* dissolve internal shell whorls, a trait uniquely shared with nudibranch larvae. Additionally, mantle fold tissue inflates and reflects dorsally during metamorphosis to form the notum, which also occurs in anthobranchs. *B. californica* possesses several unusual developmental traits, as revealed by a review of current knowledge of heterobranch development. Morphological characters of *B. californica* larvae support hypotheses of nudibranch paraphyly and the clade Pleuroanthobranchea.

Table of Contents

List of Tables

List of Figures

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Dedication

This thesis is dedicated to all of the mentors, friends, and family who have guided and encouraged me throughout my academic endeavours.

Introduction

A phylogenetic hypothesis of the relationships between taxonomic groups is essential for any investigation into the evolution of the groups in question. Studies on adaptations and the direction of evolutionary changes require phylogenies because the ancestral states of the characters must be known. The Heterobranchia is a large group of molluscs characterized primarily by protoconch heterostrophy (Haszprunar, 1985), for which a robust phylogeny has been elusive.

Adult morphology of heterobranchs, the sole source of characters until the relatively recent advent of molecular tools, is rife with characters that may be shared due to distant ancestry, parallelism, or convergence (Gosliner, 1991; Gosliner and Ghiselin, 1984). Traditionally, gastropod classification has been based on shell structure (Schander and Sundberg, 2001). However, since most derived heterobranchs lack shells or other hard structures, the fossil record has been unable to offer many clues to their relationships. Recent phylogenies have been based on either adult morphology (Dayrat and Tillier, 2002; Mikkelsen, 2002; Wägele and Willan, 2000) or molecular characters (Dayrat et al., 2001; Grande et al., 2004a; 2004b; Vonnemann et al., 2005), and have yielded conflicting hypotheses.

Detailed comparative information on the larvae of heterobranchs is a largely overlooked potential source of characters for phylogenetic reconstruction and for studies on the evolution of development. Currently, however, there is a lack of information on larval characters beyond observations of externally visible features of larval development. Furthermore, as noted in Cragg's (1996) overview of phylogenetically informative characters in bivalve larvae, studies "vary considerable in the type of

information they provide". This is also true for studies on heterobranch larvae, which confounds comparisons across clades. This study focuses on describing larval characters at the histological level in order to provide a baseline for future comparative studies, and it suggests phylogenetic implications of these new data.

1.1 Background on Heterobranch Molluscs

Hypotheses about phylogenetic relationships among members of the Gastropoda have recently undergone considerable changes, which reflect the mounting evidence that Thiele's (1929-1935) classification of the Gastropoda into the Prosobranchia, Opisthobranchia and Pulmonata is artificial (Fig. 1A). The revised classification divides the Gastropoda into a number of clades, rather than the previous paraphyletic tripartite scheme. The prosobranchs have been divided into several groups including the Patellogastropoda, Vetigastropoda, and Caenogastropoda, while the former opisthobranchs and pulmonates have been united to form the Heterobranchia (Ponder and Lindberg, 1997) (Fig. 1B). Under this scheme, the heterobranchs are the sister group to the Caenogastropoda.

For over a century, various authors have hypothesized that opisthobranchs and pulmonates form a monophyletic group (Haszprunar, 1985). However, the name given to this monophyletic group, the synapomorphies defining it, and the groups considered to comprise it, have been the subject of much study and debate since the taxon Heterobranchia GRAY, 1840 was proposed (Haszprunar, 1985).

The Heterobranchia is now considered to encompass the opisthobranchs and pulmonates plus a complex of unresolved groups collectively called the Heterostropha,

Allogastropoda, or lower heterobranchs (Haszprunar, 1985; Mikkelsen, 2002) (Fig. 2). Heterobranch clades are united by a number of synapomorphies, including heterostrophy, spiral sperm heads, egg chalazae, and a metanephridium that is located within the mantle fold and is supplied by circulation from pallial vessels (Bieler, 1992).

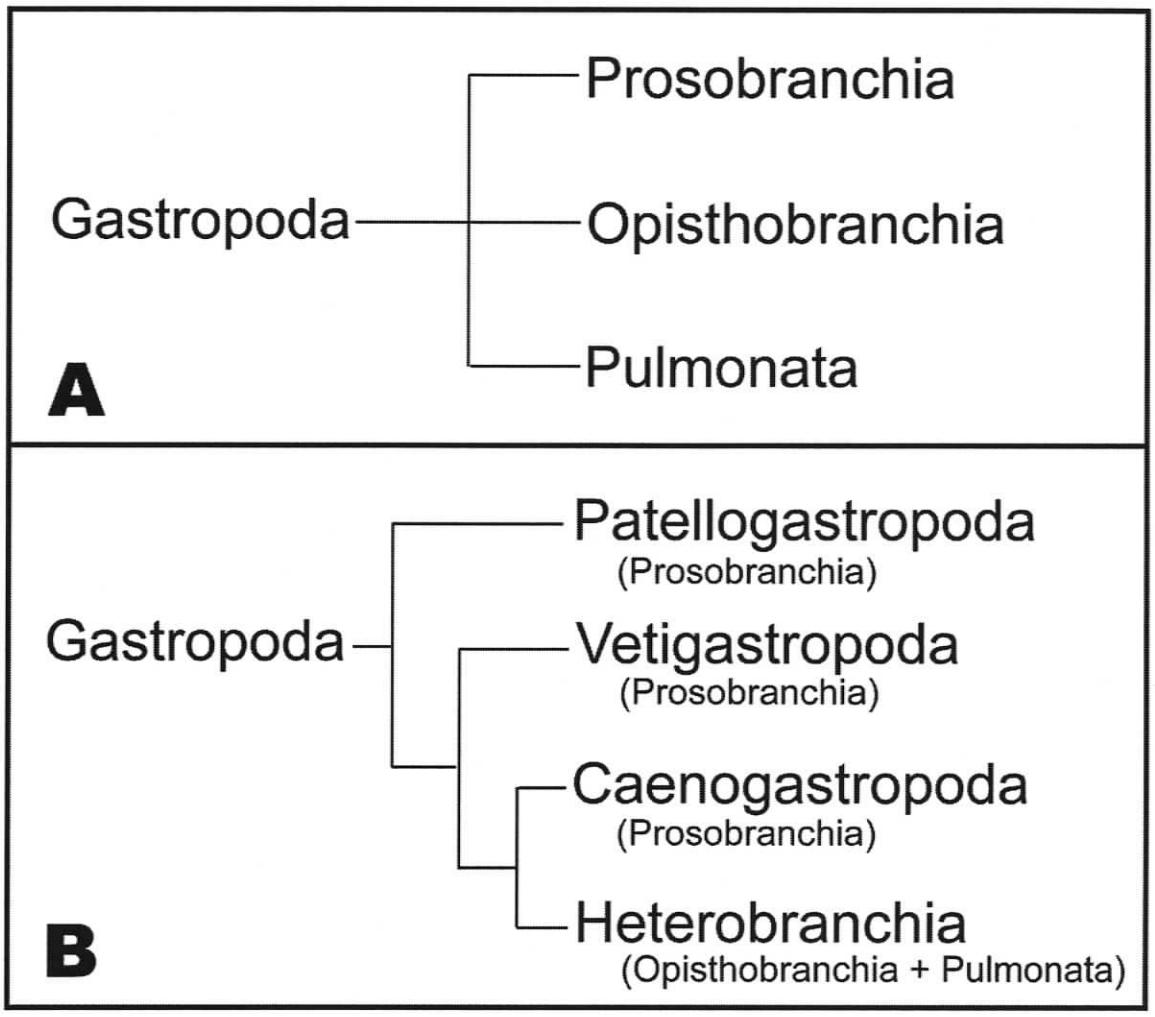

Figure 1. Classification of the Gastropoda.

A: Traditional gastropod classification scheme in use for much of the 20th century (Theile, 1929-1935). **B:** Revised phylogeny of the Gastropoda, simplified from Ponder and Lindberg (1997) with former groupings in parentheses.

Morphologically, heterobranchs are an extremely varied group, ranging in form from shelled animals superficially similar to caenogastropods, to exceedingly derived shell-less varieties. Despite this variation, a number of evolutionary trends are observed in heterobranchs, including detorsion, euthyneury, concentration and fusion of ganglia, post-metamorphic reduction or loss of the shell and operculum, and loss of the ctenidium and development of secondary respiratory structures (Gosliner, 1991).

Detorsion is interpreted as a reversal of the effects of torsion such that the mantle cavity opens on the right side of the body and the intestine is posteriorly situated (Gosliner, 1994). Varying degrees of detorsion occur among heterobranch groups. Some of the architectebranchs, including some members of the genera *Bulla* and *Ringicula,* retain the plesiomorphic condition of the mantle cavity situated anteriorly, over the head. In dorid nudibranchs, the process of detorsion is essentially complete, resulting in animals that are externally bilaterally symmetrical (Gosliner, 1994). The underlying cause of this torsion/detorsion phenomenon, whether due to physical twisting, or asymmetric growth patterns, remains a source of controversy.

The nervous system in the Heterobranchia is characterized by euthyneury, meaning an untwisting of the lateral nerve cords (also called the pleuovisceral nerve loop), which are crossed in the streptoneurous caenogastropods and in less derived heterobranchs (Gosliner, 1994). Euthyneury in pulmonate and opisthobranch herterobranchs is associated with the process of detorsion such that the less torted an organism is, the less twisted and therefore more euthyneurous is its nervous system. However, euthyneury can also be due to shortening of the pleurovisceral nerve loop as observed in the basal heterobranch family, Pyramidellidae (Gosliner, 1994). Throughout

the Heterobranchia, ganglia of the pleurovisceral nerve loop have undergone fusion and condensation to various degrees due to increased cephalization.

Shells are completely absent in the post-metamorphic Nudibranchia and Gymnosomata (Mikkelsen, 1998). In other heterobranch groups, adult shells may be absent, external, or internal, and often in reduced form when present (Mikkelsen, 1998; Poulicek et al., 1991). Reduction of the shell was examined by Poulicek et al. (1991), who found trends of decreased calcification and increased chitin content in the shells of more derived heterobranchs relative to species in more basal clades.

The presence of chemical defense mechanisms derived from food sources is correlated with the absence of shells in many heterobranchs, and is a common alternate way of avoiding predation (Faulkner and Ghiselin, 1983). A wide variety of compounds sequestered by shell-less opisthobranchs have been found to have antifeedant properties, including drimane-based sesquiterpenes found in some dorid nudibranchs which had a chemical structure similar to terrestrial plant-derived insect antifeedants (Schulte and Scheuer, 1982). According to Faulkner and Ghiselin (1983), chemical defenses are a preadaptation that enabled loss of the shell to occur.

Gastropod ctenidia are widely considered to be lost in heterobranchs, and the respiratory organs present are regarded as secondary, independently derived structures. Heterobranchia, meaning 'different gill', refers to the differences from other gastropods and the heterogeneity observed across the clade in the structure and position of gills. Gills of heterobranchs consist of folded lamellae, instead of mono- or bipectinate leaflets, and ciliated tracts and internal supporting elements are both absent (Gosliner, 1994). The

respiratory current is produced by ciliary tracts in the mantle cavity, rather than by cilia arising from the ctenidia themselves as in caenogastropods (Haszprunar, 1985).

Heterobranchs are relevant to people in a variety of important ways. Numerous pulmonate species are agricultural pests, others play host to parasitic diseases that negatively impact millions of human lives per year. Opisthobranchs such as the Aplysiids, *Tritonia diomedea* BERGH 1894, and *Pleurobranchaea californica* MACFARLAND, 1966 are commonly used organisms in neurobiological research (Gillette et al., 1991; Willows et al., 1973). Many nudibranchs are also of interest to medical researchers and pharmaceutical companies as sources of useful drug compounds because they often concentrate secondary metabolites from their prey, making them unpalatable to most predators (Behrens and Hermosillo, 2005). Due to their often striking colouration patterns and unique body forms, opisthobranchs are popular organisms with divers, underwater photographers and aquarists. However, most species are unsuitable for aquaria due to their tendency to be specialist carnivores (Behrens and Hermosillo, 2005).

1.2 Heterobranch Clades

The following is a synopsis of the main groups recognized within the Heterobranchia, including distinguishing characteristics, synapomorphies and member taxa. A current hypothesis of the relationships between many of these groups is presented in Figure 2.

- **Pulmonata**: The Pulmonata is comprised primarily of the freshwater and terrestrial snails and slugs. Synapomorphies include the pneumostome,

pulmonary vessels, the presence of a procerebrum, and dorsal bodies associated with the cerebral ganglia (Dayrat and Tillier, 2002). Pulmonates are divided into three main groups: the Systellemmatophra, Basommatophora, and Eupulmonata (Beesley et al., 1998).

- **Sacoglossa**: These herbivorous sea slugs have several unifying characteristics including uniseriate radulae with unicuspid teeth, asci containing discarded teeth, pharyngeal pouches, and laterally directed eyes (Mikkelsen, 2002). Nine families are recognized including the Volvatellidae, Juliidae, Oxynoidea, Plakobranchidae, Boselliidae, Platyhedylidae, Polybranchiidae, Hermaeidae, and Limapontiidae (Jensen, 1996).

- **Cephalaspidea**: Cephalaspideans are commonly known as bubble shell snails due to their involute shells. Synapomorphies of this group consist of a gizzard with three hardened plates, ciliated strips arising from the mantle epithelium with a flexure near the lateral mantle opening, prepharyngeal ganglionic nerve ring, and the genital ganglion located on the visceral nerve loop rather than separately. This large group is organized into two superfamilies, the Bulloidea and Philinoidea (Mikkelsen, 1996; 2002).

- **Anaspidea**: This clade contains the relatively large-bodied animals known as sea hares. Members have internal shells with little calcification, or lack shells entirely. Synapomorphies include presence of a stomach caecum (shared with pteropods), a filter chamber between the gizzard and stomach, and unique

spermatozoal features (Mikkelsen, 2002), as well as the presence of a purple gland and opaline gland opening into the mantle cavity (Dayrat and Tillier, 2002). A phylogeny based on 28S rRNA by Dayrat et al. (2001) proposed that the Anaspidea form a monophyletic clade with the pteropods (Thecosomata + Gymnosomata). The Anaspidea is comprised of the families Aplysiidae, Dolabriferidae, Notarchidae; and the Akeridae, which were formerly classified as cephalaspideans (Mikkelsen, 2002).

- **Architectibranchia**: Mikkelsen (2002) found no synapomorphies uniting this group, and hypothesized that it represents a paraphyletic assemblage of basal families with no strong affinities to the other, better defined groups. However, a recent molecular analysis found support for the Architectibranchia as a valid monophyletic group (Grande et al., 2004a). Member taxa currently include the Bullinidae, Aplustridae, and the Ringiculidae (Mikkelsen, 2002), although the Ringiculidae were formerly considered cephalaspideans (Beesley et al., 1998).

- **Notaspidea:** The Notaspideans are often called side-gilled sea slugs, due to the prominent gill that runs down the right side of the body, between the notum and the foot. Synapomorphies include longitudinally slit rhinophores, an oral veil, and an anus positioned posterior to the gill (Mikkelsen, 2002). Members of this group also possess a shell, which may be external or internal. This group is currently the subject of considerable controversy. According to some authors, the Notaspidea is paraphyletic (Dayrat et al., 2001; Grande et

al., 2004a; 2004b; Vonnemann et al., 2005). The two superfamilies Umbraculoidea and Pleurobranchoidea have recently been given separate ordinal status, although the clade containing *Umbraculum* and *Tylodina* has been named Tylodinoidea rather than Umbralacoidea due to nomenclature revisions (Valdes, 2001; Willan and Burn, 2003). Throughout this thesis, the former pleurobranchoidean notaspideans are considered to form the clade Pleurobranchoidea. Synapomorphies for the Pleurobranchoidea include the presence of a postero-ventral pedal gland, prebranchial pocket, and an acid secreting gland (Dayrat and Tillier, 2002).

- **Nudibranchia**: Nudibranchs are characterized by the loss of the shell and operculum in the adult stage, and the expansion of the dorsal notum over the head at metamorphosis (Wägele and Willan, 2000). Additional synapomorphies include detorsion resulting in external bilateral symmetry, 2 pairs of cephalic tentacles (oral and rhinophoral), bilaterally symmetrical gills, and 13 chromosomes (Wägele and Willan, 2000). Four suborders are recognized in this large group: the Doridina, Arminina, Aeolidina, and the Dendronotina (Beesley et al., 1998). Recent phylogenies based on mitochondrial genes proposed that the nudibranchs are actually paraphyletic (Grande et al., 2004a; 2004b). The nudibranchs were divided into two groups, Anthobranchia (dorids) and Cladobranchia (aeolids, dentronotids and arminids), and the Pleurobranchoidea were placed as sister to the

anthobranchs (Figs. 2, 3B), to form the taxon Pleuroanthobranchea (Grande et al., 2004a; 2004b).

- **Thecosomata**: The thecosomes are the shelled pteropods. These species are all small, pelagic marine forms that use a modified foot (parapodia) to swim. Synapomorphies include the presence of parapodia and a hypobranchial gland that secretes a mucoid web for suspension feeding (Dayrat and Tillier, 2002). The Thecosomata is comprised of the families Limacinidae, Cavoliniidae, Peraclididae, Cymbulidae, and Desmopteridae (Beesley et al., 1998).

- **Gymnosomata**: Gymnosomes are small, predatory, pteropods that lack a shell. They feed on thecosomes. Currently accepted families include the Pneumodermatidae, Notobranchaeidae, Clionidae, Cliopsidae, and Hydromylidae (Beesley et al., 1998).

- **Acochlidea**: Acochlideans are small, interstitial, vermiform gastropods lacking shells, ctenidia and mantle cavities. This group includes both marine and fresh water forms (Grande et al., 2004a). Bieler (1992) stated that they are likely the sister group to the Sacoglossa, but Dayrat and Tillier (2002) found them to be more closely related to the Gymnosomata. Families currently identified include the Acochlidiidae, Hedylopsidae, Asperspinidae, Microhedylidae, and Ganatidae (Beesley et al., 1998).

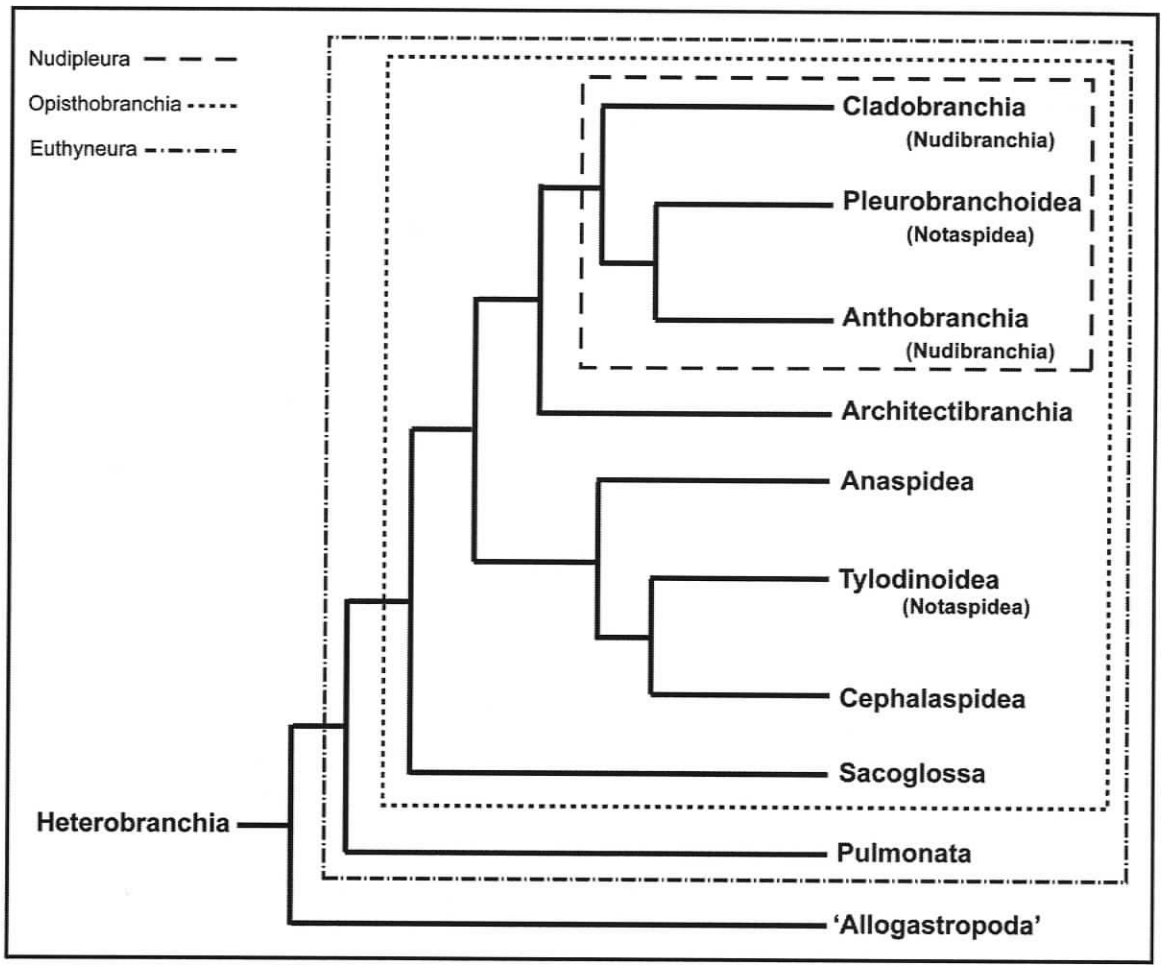

Figure 2. Phylogenetic relationships among a subset of heterobranchs.

Cladogram showing the relationships between the main heterobranch groups based on mitochondrial gene sequences, modified from Grande et al. (2004a), and including the Allogastropoda as used by Haszprunar (1985). Parentheses denote traditional groupings considered paraphyletic in Grande et al. (2004a; 2004b). Boxes indicate members of the groups Nudipleura, Opisthobranchia and Euthyneura.

1.3 Heterobranch Phylogenetic Relationships

Phylogenetic relationships among heterobranch gastropods have been difficult to resolve due to evidence of extensive convergence and parallel evolution within this clade (Gosliner, 1991; Gosliner and Ghiselin, 1984). In recent years, the position of the Notaspidea relative to the Nudibranchia has been a focus of controversy. Classically, notaspideans were considered an intermediate branch between cephalaspideans and nudibranchs (Willan, 1987). A review by Mikkelsen (2002) suggested that the Notaspidea as a whole were most closely related to nudibranchs, but the affinities to other heterobranch groups were unresolved. More recently, a number of authors have proposed a sister group relationship between pleurobranchoideans, a subset of the traditional Notaspidea, and nudibranchs (Salvini-Plawen, 1991; Salvini-Plawen and Steiner, 1996; Schmekel, 1985; Wägele and Willan, 2000) (Fig. 3A). Wägele and Willan (2000) named the clade containing the Notaspidea and Nudibranchia the Nudipleura.

According to Wägele and Willan (2000), synapomorphies of the Nudipleura include the presence of a blood gland and an androdiaulic reproductive tract, and the absence of an osphradium through loss. However, as noted by Grande et al. (2004b), not all nudibranchs possess a blood gland (Minichev, 1970; Wägele, 1998), and androdiaulic reproductive systems are also found in some architectibranchs and sacoglossans (Mikkelsen, 1996). This leaves the lack of an osphradium as the sole remaining morphological synapomorphy currently identified for the Nudipleura (Grande et al., 2004b). Additionally, a helical-loop structure within Domain III of the mitochondrial *rrnL* gene was identified by Grande et al. (2004b), and this feature constitutes a molecular synapomorphy of the Nudipleura.

Historically, the classifications were based primarily on anatomical characters in adult specimens. However, some recent studies have employed molecular characters including mitochondrial DNA (Grande et al., 2004a; Grande et al., 2004b) and ribosomal RNA (Vonnemann et al., 2005). Grande et al. (2004a; 2004b) found both the Notaspidea and the Nudibranchia to be paraphyletic. They divided the nudibranchs into two groups, the Anthobranchia and Cladobranchia, and proposed a sister group relationship between the pleurobranchoidean notaspideans and the anthobranchian nudibranchs (dorids), whereas the other group of former notaspideans (Tylodinoidea) was placed as sister to the Cephalaspidea (Fig. 3B). Vonnemann et al. (2005) also suggested paraphyly for the Notaspidea, but their analysis did not detect paraphyly for the Nudibranchia.

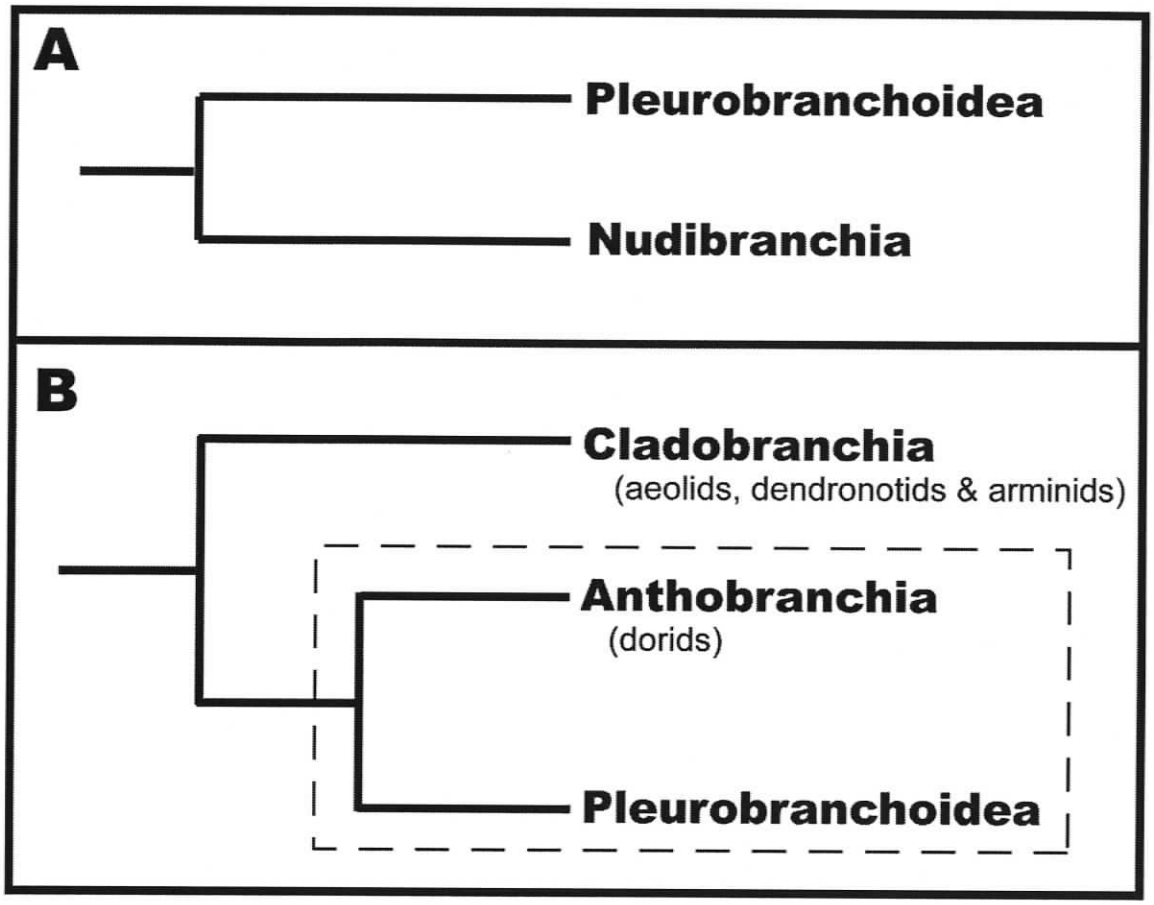

Figure 3. Current hypotheses of nudipleuran classification.

A: The Pleurobranchoidea as sister to the Nudibranchia, based on morphological data from Wägele and Willan (2000). **B:** The Pleurobranchoidea as sister to the anthobranchian nudibranchs, based on mitochondrial gene sequences; modified from Grande et al. (2004a). Dashed box indicates the clade Pleuroanthobranchea.

1.4 Review of Heterobranch Development and Metamorphosis

While this thesis focuses on relationships among the crown group of heterobranchs, the Nudipleura, a review of larval development and metamorphosis in the Heterobranchia is presented here to consolidate the large amount of new information published since the last review of this topic thirty years ago (see Bonar 1978). Despite some differences observed in the development of *B. californica*, much of what was previously known about larval development in heterobranchs is also applicable to this species. Knowledge of the developmental trends exhibited by heterobranchs in general provides the necessary context for evaluating specific details observed in the development of *B. californica*. In addition, the use of larval characteristics to help resolve phylogenetic controversies and the types of potentially useful larval characters suggested by my examination of nudipleurans should be applicable to the Heterobranchia as a whole as well.

1.4.1 Developmental Types and Terminology

Guidelines on developmental terminology for marine invertebrates were published by Giese and Pearse (1974), and are followed throughout this manuscript. Accordingly, the term *embryo* refers to developmental stages occurring within an egg case, while *larvae* are free-swimming stages that undergo metamorphosis to form juveniles. Larvae that must feed in the plankton are termed *planktotrophic*, while those that gain their energy from yolk reserves are called *lecithotrophic. Indirect development* refers to a life history pattern that includes a larval stage, whereas *direct development*

refers to cases where a juvenile hatches from the egg case. A *juvenile* is structurally a miniature adult that occupies the same habitat as the adults do, but has immature reproductive structures (Giese and Pearse, 1974).

Thompson (1961) published a classification system for development in opisthobranchs, categorizing development as planktotrophic, lecithotrophic or direct. This scheme has remained in use, despite criticisms by Bonar (1978a) that it "treats ontogeny as an ecological entity rather than as a morphogenetic one". Bonar (1978a) developed a modification to Thompson's scheme that subdivided direct development into two distinct categories: *capsular metamorphic development* wherein a veliger stage occurs within the egg capsule, and *ametamorphic development* in which the embryo develops directly into a juvenile within the capsule. The justification for this modification was to enable the comparison of species of differing developmental types by both ecological and morphological factors (Bonar, 1978a). Wider use of Bonar's (1978a) classification scheme would allow for more accurate comparisons of species that may exhibit variations in timing of developmental events, but in which the morphogenetic events themselves may be very similar.

Regardless of the system used, some species show intermediate developmental patterns, or even different types of development within the same species (poecilogony), making them difficult to categorize. In the cephalaspidean *Haminoea callidegenita* GIBSON & CHIA, 1989, individuals produce both lecithotrophic larvae and crawl-away juveniles (capsular, metamorphic development) simultaneously from the same egg mass (Gibson and Chia, 1995). The nudibranch *Spurilla neapolitana* (DELLE CHIAJE, 1823) and the sacoglossan *Elysia chlorotica* GOULD, 1870 are also known to have

poecilogonous development, but in these cases developmental type varies among individuals of the species, rather than within the same egg mass (Gibson and Chia, 1995).

1.4.2 Eggs and Embryogenesis

The largely aquatic heterobranchs lay their eggs in capsules surrounded by a gelatinous matrix, which swells on contact with water. Each capsule may contain one or more eggs, and may or may not be organized into a string within the jelly (Hurst, 1967; Soliman, 1991). The egg masses themselves come in a variety of forms.

Hurst (1967) classified opisthobranch egg masses into four types. Type A masses, typical of dorid nudibranchs, form a coiled ribbon shape, with the jelly attached to the substrate along one edge. The pleurobranchoideans *Berthellina citrina* (RÜPPELL AND LEUCKART, 1828) and *Berthella californica* (DALL, 1900) also exhibit Type A egg masses (Strathmann, 1987; Usuki, 1969). Type B egg masses, typical of sacoglossans and aeolids, are in the form of a cord attached to the substrate along one side, and are usually coiled (Hurst, 1967). In type C masses, the capsules are deposited in a gelatinous bag, which is attached to the substrate via a jelly string. This type is commonly exhibited by cephalaspideans such as *Aglaja diomeda* (BERGH, 1894) (Hurst, 1967). In the last type, D, egg capsules are laid in a small sac, and if coiled they do not form a complete circuit. Small aeolid nudibranchs often lay their eggs in this form (Hurst, 1967).

The number of eggs laid by a heterobranch specimen is generally proportional to the size of the adult (Soliman, 1991). Egg sizes are relatively small compared to other gastropods, ranging from 40 to 380 μm, with a mode of 75 μm (Hadfield and Miller, 1987). Large heterobranchs may lay millions of eggs during one spawning event

(Soliman, 1991). Larger egg masses tend to be laid by individuals at the beginning of the breeding season, so size of egg masses is variable, but egg sizes vary little within species (Thompson, 1967). Another trend in heterobranch development is that larger eggs usually require a longer period to develop, and the larvae are larger at hatching than those from smaller eggs (Thompson, 1967).

1.4.3 Larval Shell

Shells of heterobranch larvae may be either coiled or inflated (type 1 and 2 respectively) (Thompson, 1961). Type 1 coiled protoconchs are the most common, with type 2 protoconchs occurring only in certain dendronotid and aeolid nudibranch families (Hurst, 1967; Tardy, 1991). Considerable growth of the protoconch occurs in planktotrophic larvae until just prior to metamorphic competence, when growth arrests; this can be a good indicator that metamorphic competence is approaching (Bickell and Chia, 1979a; Bickell and Kempf, 1983; Gibson and Chia, 1989; Switzer-Dunlap, 1978). This phenomenon of arresting growth of the protoconch prior to competence is ubiquitous within the Heterobranchia, except for an observation on the nudibranch *Hermissenda crassicornis* (ESCHSCHOLTZ, 1831), where growth continued right up to metamorphosis (Avila et al., 1997).

Shell coiling direction reverses at metamorphosis in heterobranch species that retain the shell, changing from hyperstrophic in larvae to orthostrophic in adults. The change in coiling direction is called heterostrophy (Robertson, 1993). Although the reason for hyperstrophy remains unknown, Smith (1967) suggested that it may be due to

hypertrophy of the left digestive gland in early development while the shell gland is forming, resulting in the shell apex conforming to the shape of the visceral mass.

Protoconch loss in heterobranchs is common and occurs at metamorphosis in all members of the Nudibranchia, Acochildiacea, and Gymnosomata (Bonar, 1978a), and also in some members of most other heterobranch groups (Gibson, 2003; Mikkelsen, 2002; Switzer-Dunlap, 1978; Tsubokawa and Okutani, 1991). The mechanism by which these species loose their protoconchs differs among the various groups. In most nudibranchs, the larval retractor muscle detaches from the protoconch and the visceral mass is either pulled from the larval shell using these muscles, or the larval shell is pushed off via reflexion of the mantle fold (Bonar, 1978a). This process is described in detail by Bickell (1978) for *Corambe [Doridella] steinbergae* (LANCE, 1962) and by Bonar (1976) for *Phestilla sibogae* (BERGH, 1905). Alternatively, the protoconch may be dissolved away or reabsorbed, as is the case in the direct developing nudibranch *Cadlina laevis* LINNEAUS, 1767 (Thompson, 1967) and the pleurobranchoidean *Pleurobranchaea maculata* (QUOY AND GAIMARD, 1832) (Gibson, 2003).

While dissolution is an important mechanism for protoconch loss, it was also found to be a means of altering the larval shell prior to metamorphosis within the Nudibranchia. Page (2000) found that nudibranch larvae with type 1 protoconchs dissolved the internal whorls that had been overgrown such that the internal space at metamorphic competence was a hollow cup very similar to that of nudibranch larvae with type 2 protoconchs. This phenomenon of internal dissolution was not observed in other heterobranch species with type 1 larval shells, and was hypothesized to allow uncoiling of the visceral mass prior to metamorphosis (Page, 2000).

Sculpture patterns on the protoconch, while common in other groups, are usually lacking in heterobranch species (Bonar, 1978a; Soliman, 1991). However, Hurst (1967) reported distinctive sculpture patterns covering the entire external surface of the larval shell in the cephalaspidean *Gastropteron pacificum* BERGH, 1894. Additionally, newly hatched larvae of *Pleurobranchaea maculata [japonica]* have four to six distinct ridges present on the inner lip of the protoconch (Tsubokawa and Okutani, 1991). Red or yellow pigmentation of the larval shell is reported for aplysiids (Switzer-Dunlap, 1978) and *Busatella leachii plei* RANG, 1828 (Paige, 1988). Besides these few exceptions, the protoconchs of heterobranch veligers are typically described as smooth and transparent (Bonar and Hadfield, 1974; Carroll and Kempf, 1990; Goddard, 1996; Gohar and Abul Ela, 1957; Hurst, 1967).

Opercula are found on the dorsal surface of the foot in most heterobranch larvae, but they vary in size among species and many do not completely close the shell aperture when the foot is retracted (Hurst, 1967). Notable exceptions where opercula are entirely absent in planktonic larvae include all pleurobranchoideans whose larvae have been studied to date (Gibson, 2003; Goddard, 2001a; Goddard, 2001b; Gohar and Abul Ela, 1957; Thiriot-Quiévreux, 1967; Tsubokawa and Okutani, 1991; Usuki, 1969) and the dorid nudibranchs *Aegires albopunctatus* MACFARLAND, 1905 and *A. punctilucens* (D'ORBIGNY, 1837) (Goddard, 2001a; Goddard, 2001b) .

According to Soliman (1991), opercula are generally absent in direct developing heterobranchs. However, most pleurobranchoideans for which development has been described are planktotrophic, and opercula are absent in all species that have been examined (Gibson, 2003; Thiriot-Quiévreux, 1967; Tsubokawa and Okutani, 1991). Also,

Smith (1967) described the presence of an operculum in *Retusa obtusa* MONTAGU 1803, a direct developing cephalaspidean. Therefore, a trend towards loss of the operculum may exist in direct developing heterobranch larvae, but its absence is not always associated with direct development. Nevertheless, in most heterobranchs, the operculum is present during larval life and lost at metamorphosis, even in species retaining the shell in the adult stage.

1.4.4 Velum

As in the veligers of other gastropods, the velar lobes of heterobranchs are ciliated expansions of cephalic epithelium that are used for feeding and swimming. The edge of each lobe is formed by a row of ciliated cells, termed the prototroch or preoral ciliated band, and the cilia beat in metachronal rhythm to produce water currents for locomotion and feeding (Bonar, 1978a). A second row of ciliated cells form the metatroch or postoral ciliated band, located beneath and separated from the preoral band by the food groove. The postoral band and often the whole velum may be reduced or absent in species that develop directly, such as *Retusa obtusa* and *Cadlina laevis* (Smith, 1967; Thompson, 1967), or lecithotrophically as in *Doto amyra* MARCUS, 1961 (Goddard, 1996).

The velum of heterobranchs is typically bilobed and generally does not become as large or complex as that of caenogastropods, where it is often subdivided into additional lobes (Bonar, 1978a; Soliman, 1991). Only one instance is known of a heterobranch veliger having subdivisions of the velar lobes; the cephalaspidean *Philine denticulata* (ADAMS, 1800) has a pair of small indentations that give the appearance of having four lobes (Bonar, 1978a; Soliman, 1991).

The mouth of veliger larvae is located mid-ventrally in the groove between the two ciliated bands, which beat in opposite directions such that particles are concentrated and moved along the food groove (Strathmann and Leise, 1979). This is the case for all known veligers except in a study of *Pleurobranchaea maculata* (Gibson, 2003) where a structure identified as the mouth was beneath the postoral ciliated band. However, the mouth in other pleurobranchoideans studied was in the usual location (Gohar and Abul Ela, 1957; Tsubokawa and Okutani, 1991; Usuki, 1969), and the atypical position observed in *Pleurobranchaea maculata* requires confirmation.

1.4.5 Foot

The foot of a developing heterobranch veliger is ciliated and contains many glands. During young larval stages, a tract of cilia extending down the middle of the foot's ventral surface creates a rejection current for non-food particles in planktotrophic larvae, while longer sensory cilia are found on the peripheral margin (Bickell and Chia, 1979b; Bonar, 1978a; Bonar and Hadfield, 1974). An additional ciliary tract, which may function to draw water away from the anus, is found on the right lateral portion of the foot in nudibranchs and was also observed in *Pleurobranchaea maculata* (Gibson, 2003). The entire ventral surface of the foot becomes densely ciliated as larvae develop.

Glandular secretions are necessary both for benthic adult life and for attachment to the substrate during metamorphosis. Hypertrophy of the pedal mucus glands was observed to correlate with swim-crawl search behaviour shortly before metamorphosis, and is responsible for the enlargement of the propodium associated with metamorphic competence (Bickell and Kempf, 1983; Bonar, 1978a). Many authors have found that an

enlarged, and in some cases, darkly pigmented propodium is a good indication that metamorphic competence has been achieved (Bonar, 1978a; Gibson and Chia, 1989; Goddard, 1996; Paige, 1988; Switzer-Dunlap, 1978; Tsubokawa and Okutani, 1991).

The metapodial glands, located on the posterior part of the foot, were found to disappear following metamorphosis, indicating that their role is primarily for attachment during metamorphosis (Bickell, 1978; Bonar and Hadfield, 1974). In addition to gland hypertrophy, the foot also widens and lengthens at metamorphosis, which is consistent with the transition from pelagic to benthic life (Bonar, 1978a; Carroll and Kempf, 1990).

1.4.6 Musculature

Ultrastructural studies have found that nudibranch larvae possess three shell-attached muscles and a subepithelial network of fibres (Bonar and Hadfield, 1974; Page, 1995b). The larval retractor muscle (Page, 1995b) or right larval retractor muscle (Bonar and Hadfield, 1974) arises from an attachment plaque on the left posterior portion of the shell, arches over the junction between the left digestive gland and the stomach, and then fans out to the velum, mantle fold and oral regions. This muscle acts to withdraw the head and velum into the shell, and is the only shell-attached muscle present in newly hatched larvae (Page, 1995b). The right pedal muscle (Page, 1995b) is likely equivalent to the opercular muscle described by Bonar and Hadfield (1974), and develops midway through the larval period. This muscle originates on the ventral rim of the shell aperture, passes over the hinge between the operculum and shell, and inserts on the epithelium of the foot beneath the operculum (Page, 1995b).

Bonar and Hadfield's (1974) left larval retractor muscle corresponds to Page's (1995b) left pedal muscle, which also develops midway through larval life. It inserts on the left dorsal side of the larval retractor muscle plaque in larvae with type 1 shells, and follows the path of the larval retractor muscle anteriorly before diverging from its left ventral side and inserting on the epithelium underlying the operculum on the left side of the foot (Page, 1995b). In *Phestilla sibogae*, a nudibranch with a type 2 protoconch, the left pedal/retractor muscle has a separate origin on the shell to the left of the right retractor muscle (Bonar and Hadfield, 1974).The subepithelial network of muscle fibres is not attached to the shell, but is associated with the foot, velum and gut of nudibranch larvae (Bickell and Chia, 1979b; Bonar and Hadfield, 1974; Page, 1995b).

Less is known regarding larval musculature in non-nudibranch heterobranchs. The presence of a larval retractor muscle has been documented in many opisthobranch species including, *Pleurobranchaea maculata* (Gibson, 2003; Tsubokawa and Okutani, 1991) and *Aplysia californica* COOPER, 1865 (Kriegstein, 1977b), but they were not described in detail nor followed through development.

1.4.7 Hearts

The larval heart, when present, is composed of a thin, pulsatile membrane and develops beneath the floor of the mantle cavity, however, the timing of its development varies by species (Gibson, 2003; Switzer-Dunlap, 1978). Earlier authors reported that a larval heart was only occasionally present in heterobranch veligers (Bonar, 1978a; Soliman, 1991). However a larval heart has now been observed in a wide variety of species. A possible reason for this discrepancy is that in early studies it was not possible

to successfully rear planktotrophic larvae through development. More recent studies have shown that the larval heart develops only after 1/2 to 3/4 of larval development in heterobranchs, often requiring several weeks of development under laboratory culture conditions (Bickell and Chia, 1979b; Bickell and Kempf, 1983; Gibson, 2003; Gibson and Chia, 1989; Switzer-Dunlap, 1978; Tsubokawa and Okutani, 1991). The larvae in early studies likely did not survive long enough for this observation to be made. Nevertheless, Bonar and Hadfield (1974) did not observe a larval heart in the aeolid *Phestilla sibogae*, which they reared through metamorphosis.

In aplysiids (Switzer-Dunlap, 1978) and *Pleurobranchaea maculata [japonica]* (Tsubokawa and Okutani, 1991), the adult or definitive heart was observed to develop late in larval life, and both hearts briefly beat asynchronously before the larval heart degenerated. In other species, including *Corambe [Doridella] steinbergae*, and *Phestilla sibogae,* the definitive heart developed after metamorphosis (Bickell and Chia, 1979b; Bonar and Hadfield, 1974).

1.4.8 Nervous System

Nervous systems of heterobranchs consist of a number of paired and unpaired ganglia that develop in a similar order throughout the clade. However, in more derived groups such as the Nudibranchia, the ganglia are closer together, or even fused (Bonar, 1978a; Gosliner, 1991; Gosliner, 1994). Therefore, the number of ganglia and distance between ganglia differs among heterobranch groups.

In general, the ganglia are linked by connectives and arranged in a loop surrounding the esophagus. The paired cerebral ganglia are generally located dorsal to the

esophagus and are eventually associated with the eyespots. The cerebral commissure, which passes dorsally over the esophagus, connects the cerebral ganglia to each other (Bickell and Chia, 1979b; Kriegstein, 1977a; Page, 1992b). The unpaired supraesophageal or supraintestinal ganglion is found on the right side of the esophagus, while the subesophageal/subintestinal ganglion is located on the larva's left (Kriegstein, 1977a). Paired pedal ganglia are located ventral to the esophagus, associated with the statocysts, and have connectives to each other as well as to the cerebral ganglia (Bickell and Chia, 1979b; Bickell and Kempf, 1983; Kriegstein, 1977a). Additionally, buccal ganglia, which are found on either side of the buccal mass, connect to each other as well as to the cerebral ganglia (Gosliner, 1991; Gosliner, 1994; Kriegstein, 1977a).

An abdominal ganglion as described in *Aplysia californica*, is comprised of the fused supraintestinal, visceral and subintestinal ganglia (Kriegstein, 1977a). Additionally, an osphradial ganglion, which connects to the supraintestinal ganglion, is found in most heterobranchs, with the notable exception of the nudibranchs and pleurobranchoideans, which lack osphradia in the adult stage (Gosliner, 1994). A number of studies are available for more detailed information on ultrastructure of developing nervous systems in opisthobranchs including: Kempf and Page (2005), Kempf et al. (1997), Kriegstein (1977a), Marois and Carew (1997a; 1997b; 1997c), and Page (1992a; 1992b).

1.4.9 Sensory Structures

Heterobranch veliger larvae typically possess a number of sensory structures that develop throughout larval life. These may include statocysts, eyes, osphradia, and apical

sensory organs (ASO), however, the order of development varies, and not all of these organs are found in larvae of all species (Bonar, 1978a).

Statocysts are paired gravity sensing structures found in all heterobranch larvae that are composed of a sphere of ciliated cells, containing single or multiple statoliths. The statocysts are located beside the pedal ganglia, but they are innervated directly from the cerebral ganglia (Bonar, 1978a). These are the earliest sensory structures to develop, forming during early embryogenesis, and are retained throughout adult life (Bonar, 1978a; Soliman, 1991).

Eyes usually develop late in larval life, often just prior to the onset of metamorphic competence (Bonar, 1978a). However, eyespots in hatching larvae occur in some nudibranchs with type 2 shells such as *Crenata albocrusta* (MACFARLAND, 1966) (Hurst, 1967), and even more rarely in other planktotrophic heterobranch species, including *Berthella californica* (Strathmann, 1987) and *Aegires albopunctatus* (Goddard, 2001a).

Osphradia are chemosensory organs usually found in association with the ctenidia in gastropods. In the adult stage they are found throughout the Heterobranchia, with the notable exception of the Nudibranchia and Pleurobranchoidea (Bullock and Horridge, 1965). Development of osphradia in heterobranchs was described for *Aplysia californica* by Kriegstein (1977a), and for the cephalaspidean *Retusa obtusa* by Smith (1967).

The apical sensory organ (ASO) of opisthobranch larvae has been characterized by Page (2002) as a ganglion located over the cerebral commissure. The ganglion consists of sensory neurons and presumed interneurons and includes five neuronal somata expressing serotonin-like immunoreactivity. The neurons with serotonin-like

immunoreactivity are arranged with one medial sensory neuron flanked by pairs consisting of one sensory and one non-sensory neuron. In addition, the ganglion contains 4-6 ampullary sensory neurons that each have a deep internal pocket filled with cilia. The cilia in these cells have normal 9+2 axonemes, whereas the ampullary neurons within the larval ASO of other gastropod groups have aberrant axonemes and the cells are much more numerous (Page, 2002).

Based on its position and structure in *Phestilla sibogae*, Bonar (1978b) speculated that the ASO may be a chemosensory structure, possibly functioning to detect inducers for metamorphosis. Chia and Koss (1984) examined the ultrastructure of the ASO in *Rostanga pulchra* and hypothesised that it may function in chemo- and mechanoreception related to settlement and feeding. A subsequent ultrastructural study indicated that functions of the ASO in heterobranch larvae may include neural integration, endocrine activity, modulation of velar muscles, and control of velum function (Kempf et al., 1997). Hadfield et al. (2000) provided experimental confirmation of the ASO's role as a receptor for settlement and metamorphic cues.

1.4.10 Digestive System

The larval foregut includes the mouth situated between the preoral and postoral ciliated bands of the velum, and an esophagus. The stomach and a pair of digestive glands comprise the midgut, while the hindgut consists only of the intestine ending with an anus opening on the right side of the mantle cavity.

Algal food particles are swept into the mouth via the action of the postoral cilia (Strathmann and Leise, 1979). Later in larval development, in preparation for metamorphosis, the radula develops in a ventral diverticulum of the foregut in most

heterobranchs (Bonar, 1978a). Jaws may also develop at the anterior end of the esophagus late in larval life (Tsubokawa and Okutani, 1991).

The ventral portion of the stomach is lined by a gastric shield, and in heterobranchs the tissue contains many hyaline rods oriented perpendicular to the wall of the stomach (Bickell and Chia, 1979b; Gibson, 2003). During larval development the left digestive gland enlarges and acquires a greenish colour in feeding larvae, while the right digestive gland remains small and undifferentiated, and may even atrophy until it disappears completely (Bickell and Chia, 1979b; Bonar, 1978a; Smith, 1967).

1.4.11 Excretory Organs

Larvae of heterobranchs possess one or possibly two types of excretory structures: protonephridia or nephrocysts and a pair of anal cells or excretory vesicles (Bonar, 1978a; Soliman, 1991). A third organ, formerly called the larval or secondary kidney, is now known to be composed of glandular tissues, now termed the pigmented mantle organ (PMO) and is unrelated to excretion (Robertson, 1985). Additionally, the definitive kidney or metanephridium is often observed to begin development prior to metamorphosis (Bickell and Chia, 1979b; Thompson, 1976).

The paired protonephridia or nephrocysts develop from ectodermal tissue in larvae (Thompson, 1976). These structures are situated just beneath the mantle fold epithelium, on either side of the esophagus and each consists of only a single cell with many vacuoles (Bickell and Chia, 1979b; Bickell and Kempf, 1983; Bonar and Hadfield, 1974). Thompson (1967) noted that the nephrocysts were absent in the direct developing *Cadlina laevis*.

The anal cells or excretory vesicles are also paired structures. They appear early in embryogenesis and indicate the location of the future anal invagination, which comprises the terminal end of the digestive tract (Smith, 1967; Thompson, 1976). The morphogenesis of the anal cells in *Haminoea navicula* (COSTA, 1778) was studied in detail by Schaefer (1997), who observed that the intestine makes contact with the anal cells before the anus breaks through, and the anal cells subsequently become incorporated into the epithelium surrounding the anus. Very little is known about the role of these cells in excretion, but in some species of nudibranchs they have been observed to extrude hyaline droplets (Soliman, 1991).

1.5 Objectives and Justification

Heterobranch development has not been reviewed for almost 30 years (Bonar, 1978a), and since that time, much new information with respect to more clades has come to light. There have also been major revisions to hypotheses about valid heterobranch clades and sister-group relationships among these clades. Nudipleura (nudibranchs + pleurobranchoideans) has emerged as a clade in studies using either morphological or molecular characters (Grande et al., 2004a; 2004b; Vonnemann et al., 2005; Wägele and Willan, 2000). According to extensive studies by Grande et al. (2004a; 2004b), the pleurobranchoideans are the sister-group to the anthobranch (dorid) nudibranchs (Pleuroanthobranchea) and this clade is the sister-group to the cladobranch nudibranchs (aeolids + dendronotids + arminids) (Fig. 2). Therefore, pleurobranchoideans have a previously unrecognized significance within the evolutionary radiation of the

Heterobranchia, and may retain ancestral characters common to the Pleuroanthobranchea and Nudipleura.

Resolving the remaining phylogenetic controversy about the exact placement of the pleurobranchoideans relative to the nudibranchs requires further investigation using additional characters. A possible source for new characters to add to this analysis are studies of larval development, about which very little is currently known for members of the Pleurobranchoidea. Increased knowledge of development in these organisms is also necessary in order to gain a better understanding of the trends in morphological evolution and to enable the detection of similarities among groups due to homoplasy (Bieler, 1992; Gibson, 2003).

To date, published descriptions of larval development from egg through metamorphosis in pleurobranchoideans are limited to two species. Planktotrophic development has been recently described in *Pleurobranchaea maculata* (Gibson, 2003), and *Pleurobranchaea japonica* THIELE, 1925 (Tsubokawa and Okutani, 1991). However, a review of the family Pleurobranchaeidae by Marcus and Gosliner (1984), suggests that *P. japonica* is a synonym of *P. maculata*. Therefore, it appears that one species has been studied twice, rather than two different species studied. In light of this information the species will be referred to as *P. maculata* in the remainder of this thesis. Direct development was documented in *Berthellina citrina* (Gohar and Abul Ela, 1957; Usuki, 1969). However, all of these studies described only externally visible morphological features, as histological sectioning was not used to analyze development.

Development at the histological level has not previously been described for any pleurobranchoidean species. I will describe and compare larval and metamorphic

development in *B. californica* to that of other heterobranch larvae, using data from histological sections and scanning and transmission electron microscopy. These data will contribute to the search for larval synapomorphies of the Nudipleura and Pleuroanthobranchea.

1.6 Description and Natural History of *Berthella californica*

Berthella californica is a wide-ranging species from the Northeastern Pacific that is similar in appearance to an anthobranch nudibranch. This species was originally described as *Pleurobranchus californicus* DALL, 1900 and underwent several name changes before Gosliner and Bertsch (1988) extensively reviewed all members of the genus *Berthella* from the North American Pacific coast. The body of *B. californica* lacks tubercles, and is translucent white, with opaque white spots scattered over the dorsal surface and a white band around the margin of the notum. Adults reach 50 mm in length and have a thin, flattened internal shell (Beeman and Williams, 1980). Specimens have been reported from Point Craven, Alaska, south through California, and recently the range was extended to include Panama, the Galapagos Islands, and eastern Russia (Behrens and Hermosillo, 2005; Camacho-Garcia et al., 2005). A colour morph with a brownish notum occurs in California (Behrens and Hermosillo, 2005). Although some other members of the genus are known to eat various species of sponges in the class Demospongiae, the prey of adult *B. californica* is currently unknown (Willan, 1984).

Strathmann (1987) reported that egg masses are laid from February through July in the San Juan Islands, Washington, in the form of a broad, coiled ribbon conforming to Hurst's (1967) type A egg mass. The form of the egg mass and appearance of newly

hatched veliger larvae of *B. californica* were described by Strathmann (1987); however, development beyond hatching was unknown prior to the present study.

Materials and Methods

2.1 Specimen Collection and Maintenance

Adult *B. californica* were collected from shore at Ogden Point, Victoria in July 2004 and May 2005 during low tides of -0.10 m and -0.13 m respectively. Specimens were found on rocks located near the water level on the inside of the breakwater. Adults were maintained in aquaria in the recirculating seawater system at the University of Victoria at a temperature of 11-12°C and allowed to lay egg masses.

A small piece of each egg mass used for larval culture or embryological observations was removed from the aquarium after oviposition and cultured in a 150 ml dish of seawater that was changed daily. Egg masses were observed and photographed on a daily basis.

2.2 Larval Culture

Larvae were cultured in prefiltered (Pall Corporation, type A/E) seawater maintained at 12°C in an incubator. They were fed a diet consisting of 1:1 *Isochrysis* sp. (CCMP1324) and *Pavlova lutheri* (CCMP1325) at a concentration of 2×10^4 algal cells per ml, which was calculated using a hemacytometer to determine the volume of algae required to yield the desired concentration in the larval cultures. To prevent growth of bacteria, the antibiotics penicillin G and streptomycin sulfate (Sigma Chemical Company) were added to the cultures at concentrations of 60 µg/ml and 50 µg/ml respectively. Algae were obtained from Provasoli – Guillard National Center for Culture of Marine Phytoplankton (CCMP), Bigelow Laboratory for Ocean Sciences. Algal

cultures were maintained in Provasoli's E.S. enrichment medium with constant illumination and aeration. Prior to adding algae to the larval cultures, it was centrifuged for 10 minutes at 1 500 rpm and then re-suspended in prefiltered seawater to remove the Provasoli's medium.

To initially culture large numbers of larvae, cultures were maintained in 500 ml Erlenmyer flasks (Pyrex), full to the top and covered with Parafilm to prevent the hydrophobic shells from becoming trapped in the surface tension. Larval density was less than two per ml. The culture medium was changed every 2-3 days by pouring the culture slowly through a 49 μm pore size Nitex sieve within a small dish, such that the larvae were retained in the sieve along with a small amount of seawater. The larvae were then rinsed gently with fresh pre-filtered seawater, and were pipetted into a clean flask containing fresh prefiltered seawater, algae and antibiotics.

When the number of larvae decreased due to mortality and sampling, cultures were transferred to 150 ml dishes with less than 100 larvae per dish. Flakes of cetyl alcohol were sprinkled on the water surface to reduce the surface tension and prevent the larvae from becoming trapped. The media was changed in these cultures every 2-3 days by individually pipetting the larvae into a clean culture dish containing fresh prefiltered seawater, algae, antibiotics and sprinkled with a small amount of cetyl alcohol flakes.

2.3 Histological Techniques

At various points during development, larvae were fixed for histological sectioning. Initially, larvae were relaxed by several changes of artificial seawater solution with increased Mg^{2+} and decreased Ca^{2+} concentrations, to prevent withdrawal of the

cephalopedal mass into the shell. The relaxation procedure occurred over a period of 2 hours in an 8 ml fixation vial, and the larvae were kept cool on a layer of crushed ice during this time. Further relaxation to prevent contraction of tissues during fixation was accomplished by adding 3 drops of chlorotone six times at 1.5 minute intervals (for a total of 18 drops) while swirling the vial and maintaining it at 0°C on a layer of ice. The anaesthetizing solution was then removed with a pipet and replaced with the primary fixative, consisting of 2.5% glutaraldehyde (EM Science) in a phosphate buffer (pH 7.6). Larvae were stored in the primary fixative overnight at 8°C and then decalcified in a 1:1 solution of 2.5% glutaraldehyde and 10 % ethylene diaminotetraacetic acid (disodium salt) (EM Science) for 2.5-4 hours (depending on age/size of larvae), with solution changes every 45 minutes.

Following decalcification, larvae were rinsed three times for 10 minutes in 2.5% $NaHCO_3$ (Sigma Chemical Company) at pH 7.2. The specimens were post-fixed for 1 hour in a 1:1 solution of 4% OsO_4 (Stevens Metallurgical) and 2.5% $NaHCO_3$. Finally, specimens were dehydrated in a graded series of acetone and embedded in Epon 812 plastic resin (Taab Electron Microscopy Supplies). Juveniles were also fixed and embedded according to the technique described above.

Histological sections 1 μm thick were cut from specimens using glass knives made on a Leica EMKMR2 knife maker. Sectioning was done at a knife angle of 8° on a Reichert Ultracut E microtome. Sections were mounted on glass slides, stained with a mixture of methylene blue and sodium borate (Richardson et al., 1960) and protected by applying a cover glass affixed with Permount® (Fisher Scientific, SP15-100).

Ultrathin sections 85 nm thick were also cut on a Reichert Ultracut E microtome using a diamond knife (Diatome) at 6°. Ribbons of sectioned material were mounted on 100 mesh hexagonal copper grids (Soquelec Ltd. D10900) coated with Formvar and dusted with carbon using a Polaron Range High Vacuum Evaporator (BOC Edwards). The sections were stained using 5% uranyl acetate in 50% ethanol for 10 minutes followed by staining in 5% lead citrate for 4 minutes. The grids were rinsed 5 times with distilled water after each staining and allowed to dry before being examined using a Hitachi S-7000 Transmission Electron Microscope (TEM).

2.4 Preparation of Specimens for SEM

Larvae and juveniles were prepared for scanning electron microscopy (SEM) by following the previously described fixation procedure, except the dehydration was performed with a graded series of ethanol. Specimens were then dried using an EM Sciences 850 critical point drier, mounted on SEM stubs using an eyelash tool and double-sided tape, and gold coated using a sputter coater (Edwards S150B). Specimens were examined using a Hitachi S-3500N SEM.

Shells of larvae at various stages were also examined by both SEM and compound microscopes. Shells were prepared by rinsing larvae in distilled water to remove seawater, and incubating them in a solution of 2.5% sodium hypochlorite for 30-45 minutes, until soft tissues were removed from the shells. Shells were then rinsed 3 times with distilled water and dehydrated in a graded series of acetone. For SEM, shells were treated as described above, with the omission of the critical point drying step. Shells were pipetted onto lens paper over a layer of filter paper, and the acetone was allowed to

evaporate. Once dry, the shells were mounted and sputter coated with gold as above. For light microscopy, shells were pipetted into a drop of unset gelatin (8 g Knox gelatin dissolved in 400 ml of warm distilled water) on a slide and oriented in the desired position using a pin.

2.5 Immunohistochemistry

Newly hatched larvae were immunolabelled and visualized using fluorescence and confocal scanning laser microscopy (CSLM) to obtain images of ASO structure. Larvae were anesthetized and fixed as previously described, except that 4% paraformaldehyde was used as the primary fixative instead of glutaraldehyde. The fixed specimens were rinsed 4 times in a solution of phosphate-buffered saline (PBS, pH 7.3) and 0.1% sodium azide.

After several weeks of storage, specimens were incubated at 6° C for 4 hours in a blocking medium consisting of PBS with 0.1% sodium azide, 0.1% Triton X-100, and 4% goat serum. The specimens were then incubated for 24 hours in monoclonal antibodies against mouse acetylated α-tubulin (Sigma T-6793) and rabbit polyclonal antibodies against serotonin (Immunostar 20080) diluted 1:1000 in blocking solution. Following the primary antibody incubation, the specimens were rinsed with PBS+azide+Triton X solution 6 times over the course of 8 hours prior to the addition of secondary antibodies for a further incubation of 20 hours. The mix of secondary antibodies consisted of goat anti-rabbit IgG conjugated to Alexa 488 flourophore (Molecular Probes) and goat anti-mouse IgG conjugated to Alexa 568 flourophore (Molecular Probes) at a dilution of 1:250 in blocking medium. All incubations involving

antibodies were done at 6°C on an orbital shaker. After rinsing 4 times over 4 hours, the specimens were viewed with a Zeiss Axioskop compound microscope with epiflourescence capacity and a Zeiss LSM410 scanning laser confocal microscope.

2.6 Imaging Methodology

Throughout development, light micrographs of live larvae and embryos were obtained using a Zeiss Axioskop compound microscope with a DVC digital camera operated by Northern Eclipse software (v. 5.0). After relaxation, larvae were pipetted in a drop of water onto a microscope slide. A coverslip with plasticine feet on the corners was placed over the water droplet in order to hold the larvae in place without squashing them. A similar technique was used to photograph developing embryos, however, no relaxation was necessary. Northern Eclipse (v. 5.0) software was used to measure larval shell dimensions.

Thick sections were photographed using a DVC digital camera mounted on a Zeiss Axioskop compound microscope. Ultrathin sections were photographed with the TEM using a plate camera. Live juvenile *B. californica* were photographed using an Olympus SZX9 dissecting microscope equipped with a Sony Power Had 3CCD colour video camera. Photographs of adult specimens were taken with a Sony A360 digital camera. Contrast and brightness of digital images were adjusted using Adobe Photoshop CS2 (v. 9.0) software.

Results

3.1 Egg Masses and Embryogenesis

The egg mass of *B. californica* is characteristic of Hurst's (1967) type A egg mass, consisting of a coiled egg ribbon that is attached to the substrate along one edge. The ribbon is wide and flattened, with a thickness of approximately 1 mm. In the laboratory, egg masses were laid over a period of several hours on the sides of glass aquaria, and consecutive egg masses laid by the same individual showed a trend to decrease in size.

Within the transparent, gelatinous egg ribbon, the white primary egg capsules had an unorganized distribution. Each egg capsule had an oval shape with a diameter of 165-185 μm and contained 1 or 2 embryos (Fig. 4A). The ova within the capsules had a diameter of 95-98 μm. The first cleavage occurred 5.5 hours after oviposition at 11°C, and was equal. The second and third cleavages followed at 8.5 and 13.5 hours after oviposition respectively (Fig. 4B).

During days 2-7 of embryogenesis, the embryo passed through the blastula and gastrula stages. By day 9, the embryos had reached the early trochophore stage, with cilia and the larval shell (protoconch) visible. During the 10^{th} day the velar cilia began rotating the embryo within the capsule via their rhythmic beating, and by day 11 the ciliary beating could be arrested (Fig. 4C). The statocysts, each containing one statolith, and the pigmented mantle organ became visible on day 12, followed by the eyespots on day 13. The foot, which lacked an operculum, also became visible at this time. On the 14^{th} day of

development, the larval retractor muscle became visible. By day 15, the protoconch was enlarged and obvious (Fig. 4D), and the larval retractor muscle became functional.

Hatching finally occurred on day 19 of development. The gelatinous matrix of the egg ribbon deteriorated, and the veligers swam out of the egg capsules. The timing of the main embryological events that occurred at a temperature of 11°C in *B. californica* is summarized in Table 1.

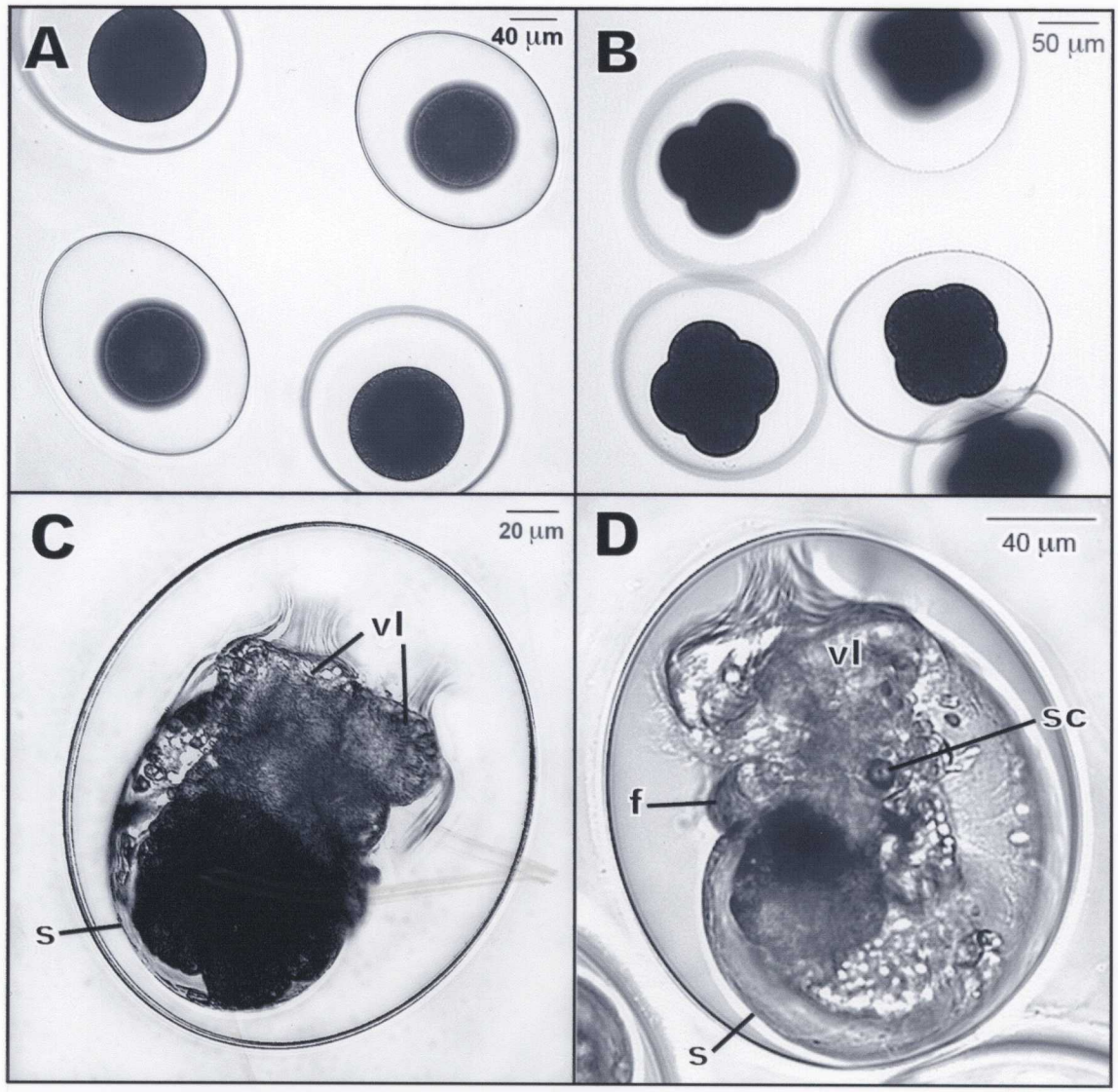

Figure 4. Embryological stages of *B. californica*.

A: Recently deposited egg capsules containing uncleaved ova. **B**: Embryos in 4-cell stage, just after second cleavage. **C:** Embryo 11 days after oviposition showing cilia on the developing velar lobes (vl) and shell (s). **D:** Embryo 15 days after oviposition showing the left statocyst (sc), foot (f), and prominent shell (s).

Table 1: Embryogenesis in *B. californica* at 11°C

Time	Event
0 h	oviposition
5.5 h	1st cleavage
8.5 h	2nd cleavage
13.5 h	3rd cleavage
2-7 d	blastula - gastrula
8 d	apical cilia visible
9 d	shell visible
10 d	embryos rotating via beating of velar cilia
11 d	intermittent arrest of velar cilia observed
12 d	velar lobes distinct, statocysts & PMO visible
13 d	eyespots visible, individual organs of digestive system visible
14 d	larval retractor muscle visible
15 d	larval retractor muscle functional
19 d	hatching

3.2 Growth and Development

3.2.1 Overview of Development and Metamorphosis

Newly hatched veligers of *B. californica* were unusual among heterobranchs because they possessed eyespots but lacked an operculum on the foot (Fig. 5A). Young larvae began to feed on unicellular algae immediately after hatching and swam actively towards the surface. They tended to get caught on the surface tension due to the hydrophobic properties of their shells. The larval heart appeared on day 17 and continued to pulse for the remainder of the larval period. By 50 days post-hatching, the larvae began to spend more time at rest on the bottom of culture bowls, swimming up only when disturbed. At this stage, shell growth arrested, and the propodium of the foot became darkly pigmented and enlarged (Fig. 5B).

Larvae 58 days old were observed lying on the bottom of their glass dishes, despite having a very mobile foot. Crawling, an indication of metamorphic competence, was not observed until the larvae were 72 days post-hatching (Fig. 5C). The mantle fold did not begin to grow over the shell during larval life as in other pleurobranchoideans (Gibson, 2003; Gohar and Abul Ela, 1957; Tsubokawa and Okutani, 1991), but by 72 days post-hatching the mantle fold had retracted from the rim of the shell aperture.

The process of metamorphosis, in which competent *B. californica* larvae (Fig. 5C) make the transition to the juvenile stage (Figs. 6A, 6B), required approximately 2 days at a temperature of 12°C. None of the larvae in standard culture conditions underwent metamorphosis. However, larvae that were placed on a piece of bivalve shell that hosted a diverse assemblage of invertebrate fauna did metamorphose. It remains unknown which

organism or chemical compound present on the shell actually induced the metamorphic process.

Metamorphosis was induced in larvae that were at least 79 days post-hatching. The metamorphic process in this species involved the expansion and reflection of the mantle fold over the larval shell, resulting in internalization of the shell, loss of the velar lobes, and development of the rhinophores (Figs. 6A, 6B). The rhinophores were not visible in larvae prior to metamorphosis. Additionally, newly metamorphosed juveniles did not yet possess the bipectinate gill that is characteristic of adult pleurobranchoideans.

When larvae were initially placed on the bivalve shell, they crawled around actively on its surface. However, after 1-2 hours they ceased crawling and remained stationary, attached to the shell by the foot. After 8 hours initial stages in the formation of the notum were visible. The notum is the mantle fold of the larva after it has reflected dorsally over the lip of the protoconch. Twenty-four hours after being placed on the shell, the notum covered approximately half of the protoconch. After 48 hours, the notum completely covered the protoconch, and the new juveniles resumed active crawling.

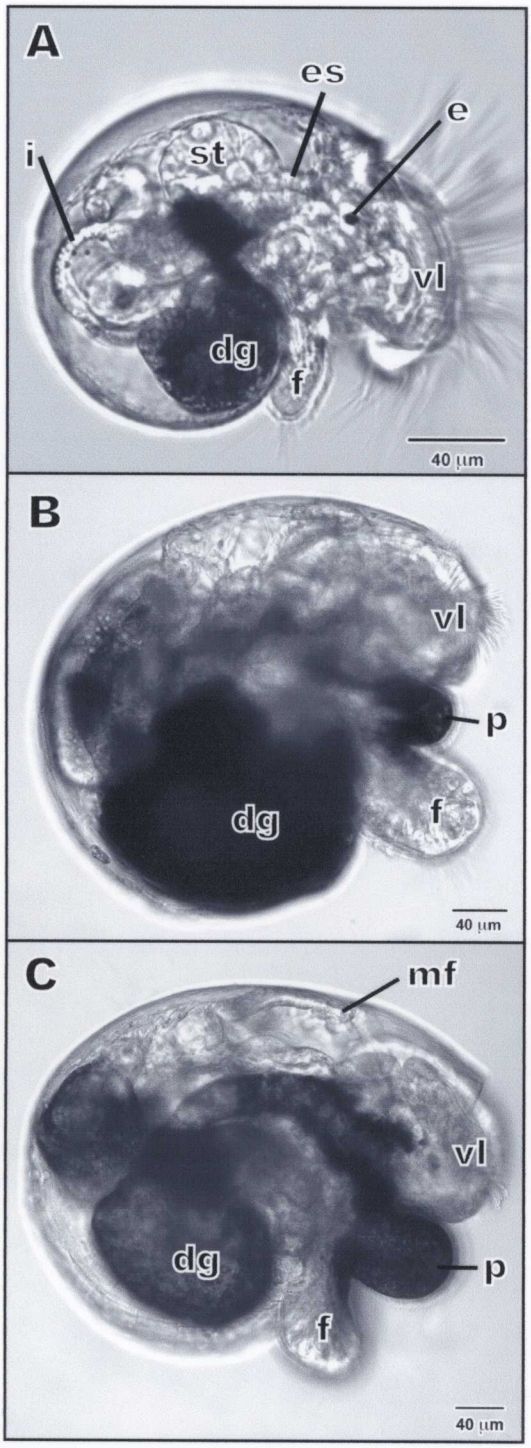

Figure 5. Light micrographs of *B. californica* larval stages.

A: Newly hatched larva showing digesive system, eyespot (e) and absence of operculum on the foot (f). **B:** 45-day larva showing enlarged, dark propodium (p). **C:** 79-day larva showing greatly enlarged propodium and retracted mantle fold (mf). dg, digestive gland; es, esophagus; i, intestine; st, stomach; vl, velar lobe.

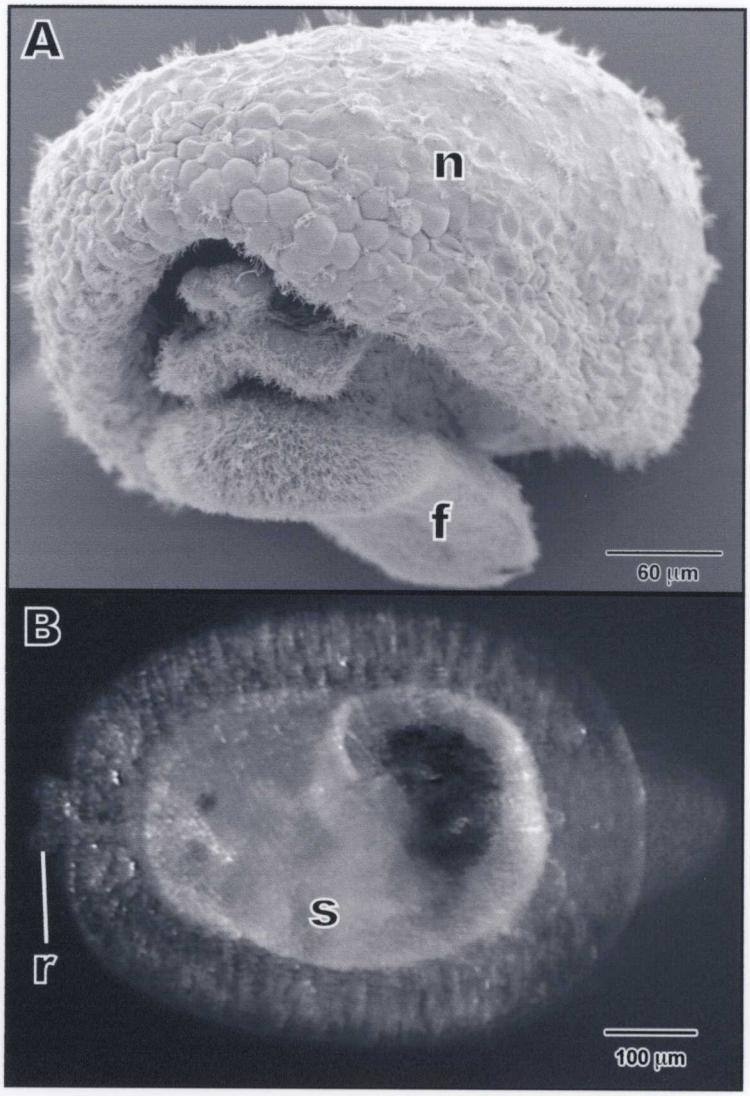

Figure 6. Juveniles of *B. californica*.

A: SEM micrograph showing densely ciliated foot (f) and notum (n) formed by reflection and expansion of the mantle fold over the protoconch. **B:** Light micrograph showing the rhinophores (r) and internalized shell (s) covering the visceral organs.

3.2.2 Protoconch

The larvae of *B. californica* had a coiled type 1 protoconch that grew significantly during the relatively long larval stage. The protoconch was smooth over most of its surface, but had a reticulated sculpture pattern on the umbo just ventral to the shell aperture (Fig. 7A). Protoconch coiling was hyperstrophic, as in the larvae of other heterobranchs. At hatching, the protoconch consisted of 1/2 of a whorl and had a mean shell length of 146.6 μm (range 145.15 - 148.46 μm, n=3) (Figs. 7A, 8A).

The larvae continued to feed and grow until approximately 50 days after hatching, by which time they attained a mean shell length of 336.0 μm (range 333.62 – 339.92, n=4). A raised pattern of sculpture was observed just ventral to the shell aperture in larvae at least 31 days post-hatching (Figs. 7B-E). During the later growth period, the protoconch coiling became nearly planispiral, and the internal whorls of the shell were dissolved away (Figs. 8B, 9A). Dissolution of the internal shell whorls is also known to occur in nudibranchs (Fig. 9B), but does not occur in at least one other species of heterobranch, the sacoglossan *Olea hansinensis* that looses the larval shell at metamorphosis (Fig. 8C).

The protoconch ceased to grow after approximately 50 days of development. In the shells of competent 79-day larvae, both the right (Fig. 7D) and left sides (Fig. 7E) of the outer whorl accreted to the lateral margin of the previous whorl, rather than tracking the dorsal edge, as in most heterobranch larvae such as *Onchidoris bilamellata* (Anthobranchia) (Fig. 7F). Following metamorphosis, the shell in *B. californica* was not shed or dissolved away, but resumed growth, and was thin, flattened, and poorly calcified in adult specimens.

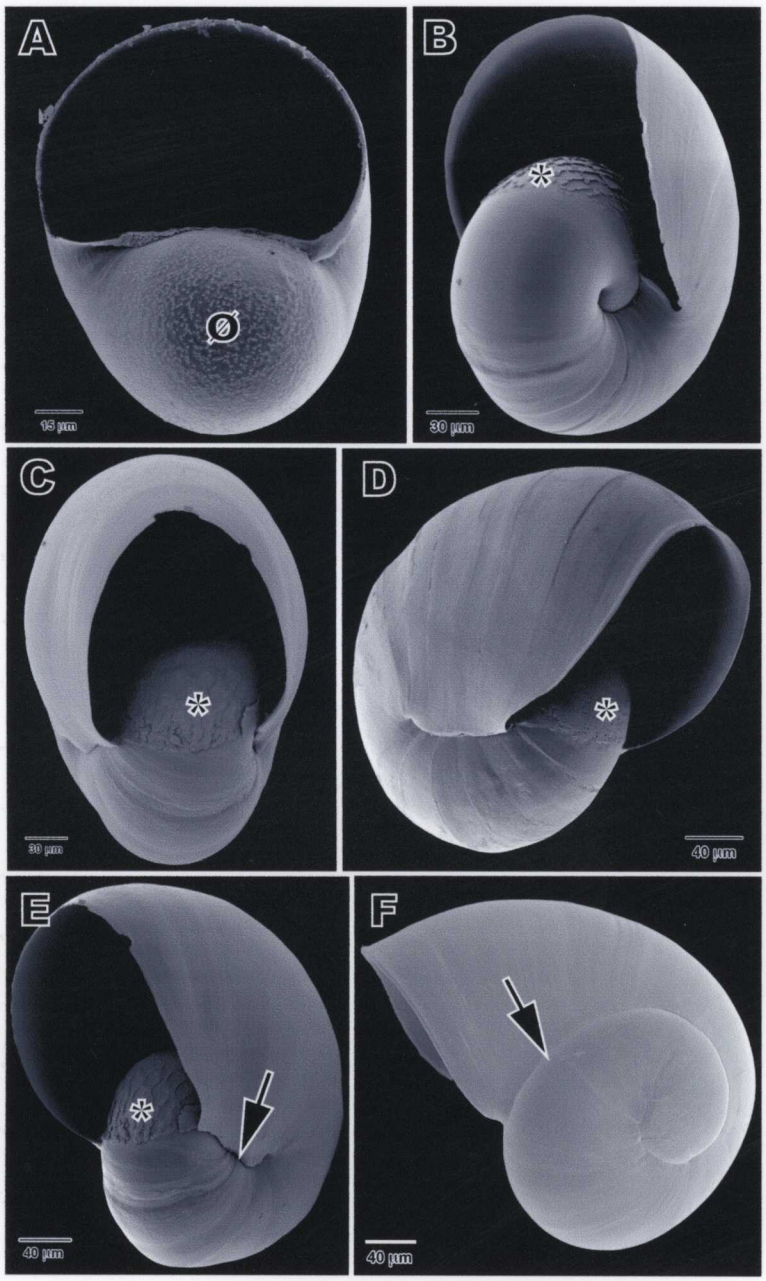

Figure 7. SEM images of protoconch growth in *B. californica.*

A: Protoconch of a hatching larva showing incipient hyperstrophic coiling and reticulated sculpture near aperture (ø). **B:** Protoconch of a 31-day larva with prominent raised sculpture (*) on the ventral aperture. **C:** Protoconch of a 79-day larva showing near-planispiral coiling. **D:** Right view of a 79-day protoconch. **E:** Left view of the protoconch of a 79-day larva showing outer whorl accreted to the side of previous whorl (arrow). **F:** Left view of a typical heterobranch protoconch (*Onchidoris bilamellata*), showing outer whorl accreted to the top of previous whorl (arrow). Image F courtesy of L. Page.

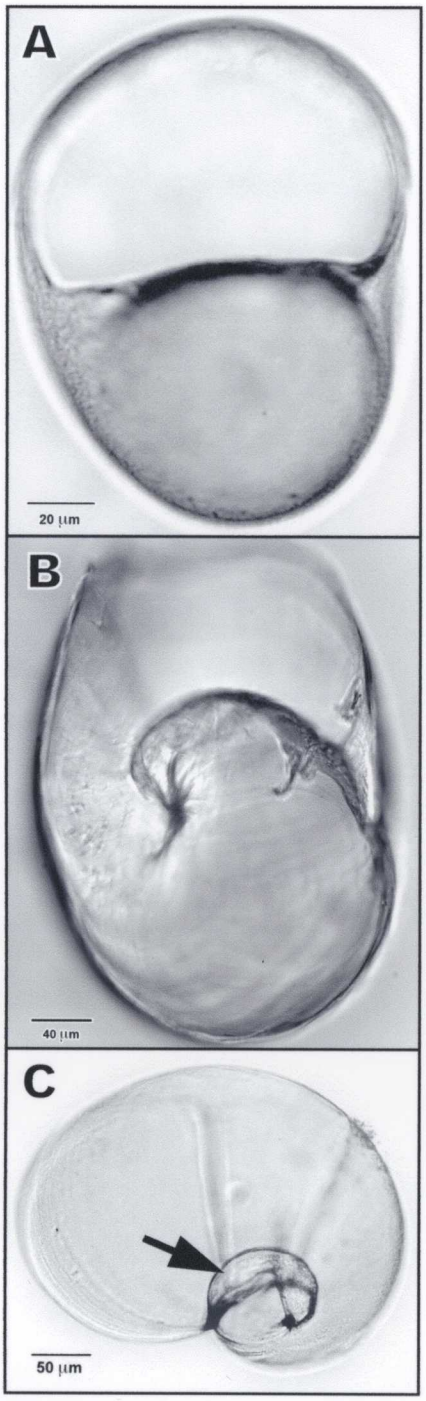

Figure 8. Light micrographs of protoconchs.

A: Ventral view of the protoconch of a newly hatched *B. californica.* **B:** Ventral view of the protoconch of a 79-day *B. californica* showing the absence of internal shell whorls. **C:** Dorsal view of the protoconch of the sacoglossan *Olea hansinensis* showing intact internal shell whorls (arrow); this shell was discarded by a metamorphosing larva. Image C provided by L. Page.

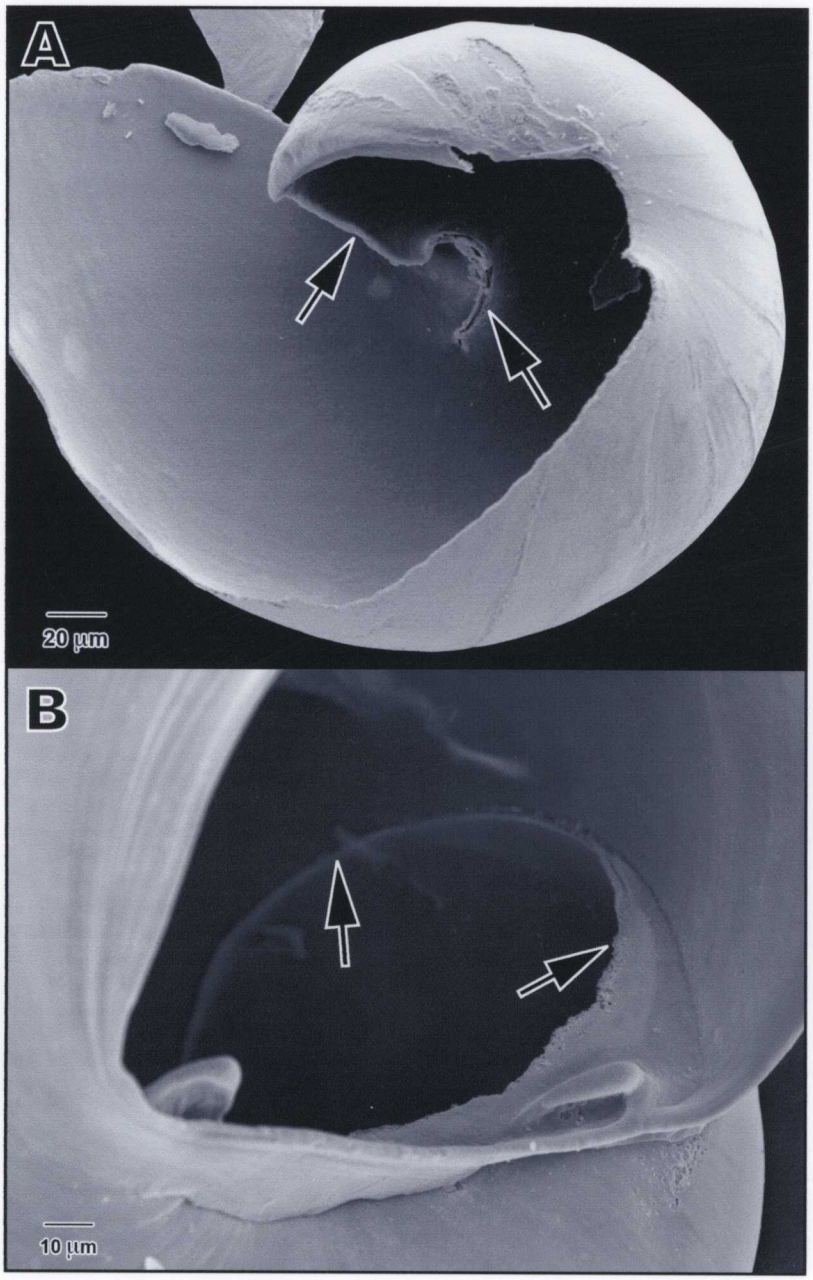

Figure 9. Dissolution of internal shell whorls.

A: Opened shell of *B. californica* showing line marking dissolved internal shell whorl (arrows). **B:** Apertural view inside the protoconch of the anthobranch *Onchidoris bilamellata* showing line of dissolution. Image B provided by L. Page.

3.2.3 Mantle Morphogenesis

The inner and outer epithelia of the mantle fold were very thin for most of the larval phase. At metamorphic competence, the mantle fold consisted of an inner layer of columnar epithelium thrown into many folds and an outer layer of squamous epithelium lining the inner wall of the larval shell (Figs. 10D, 10F). During metamorphosis, this tissue expanded greatly and reflected dorsally over the lip of the protoconch such that the inner epithelial layer of the larva's mantle fold became the dorsal epithelium or notum of the juvenile (Figs. 10A-C). The notum of the juveniles consisted of expanded cuboidal epithelial cells containing large vacuoles overlying a thin layer of squamous epithelium (Figs. 10E, 10G).

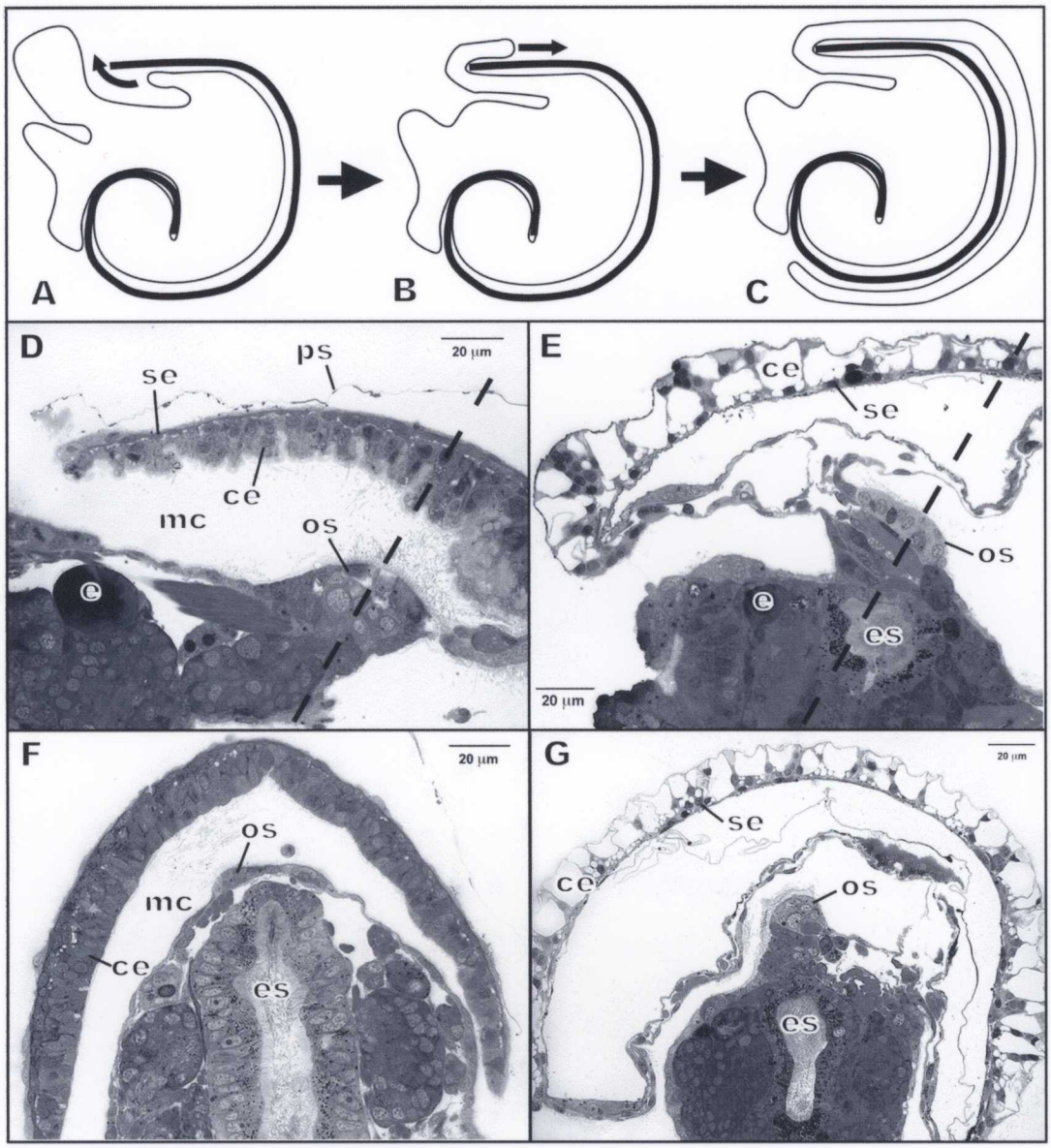

Figure 10. Mantle morphogenesis in *B. californica*.

A-C: Sketches depicting notum formation via reflection of mantle fold over the larval shell during metamorphosis. **D:** Longitudinal section (L.S.) of a 79-day larva with mantle fold consisting of outer squamous epithelium (se) and inner columnar epithelium (ce). **E:** L.S. of a juvenile showing notum composed of columnar epithelium over squamous epithelium. **F**: Cross section (C.S) of a 79-day larva showing columnar epithelial cells of mantle fold prior to reflexion over the shell. **G:** C.S. of a juvenile with columnar epithelial cells greatly expanded by large intracellular vacuoles. e, eyespot; es, esophagus; f, foot; mc, mantle cavity; os, osphradium; ps, periostracum. Dashed lines in D and E indicate positions of cross sections F and G.

3.2.4 Foot

The foot of newly hatched *B. californica* larvae consisted of a thin metapodium with a ciliated cuboidal epithelial layer (Fig. 11A). As the larvae grew, the propodium developed and began to accumulate dark pigment around 40 days after hatching. The propodium continued to enlarge and darken until metamorphic competence was attained (Fig. 5C). Histological sections through the foot of a 79-day old larva revealed a densely ciliated ventral epithelium, with many large vacuoles concentrated dorsally at the anterior and posterior ends of the foot (Fig. 11B). In the juvenile (Fig. 11C) however, the vacuoles were reduced in number, indicating that the secretory product they contained in live larvae may be of greater importance during metamorphosis. No operculum was observed on the foot during any stage of development in *B. californica.*

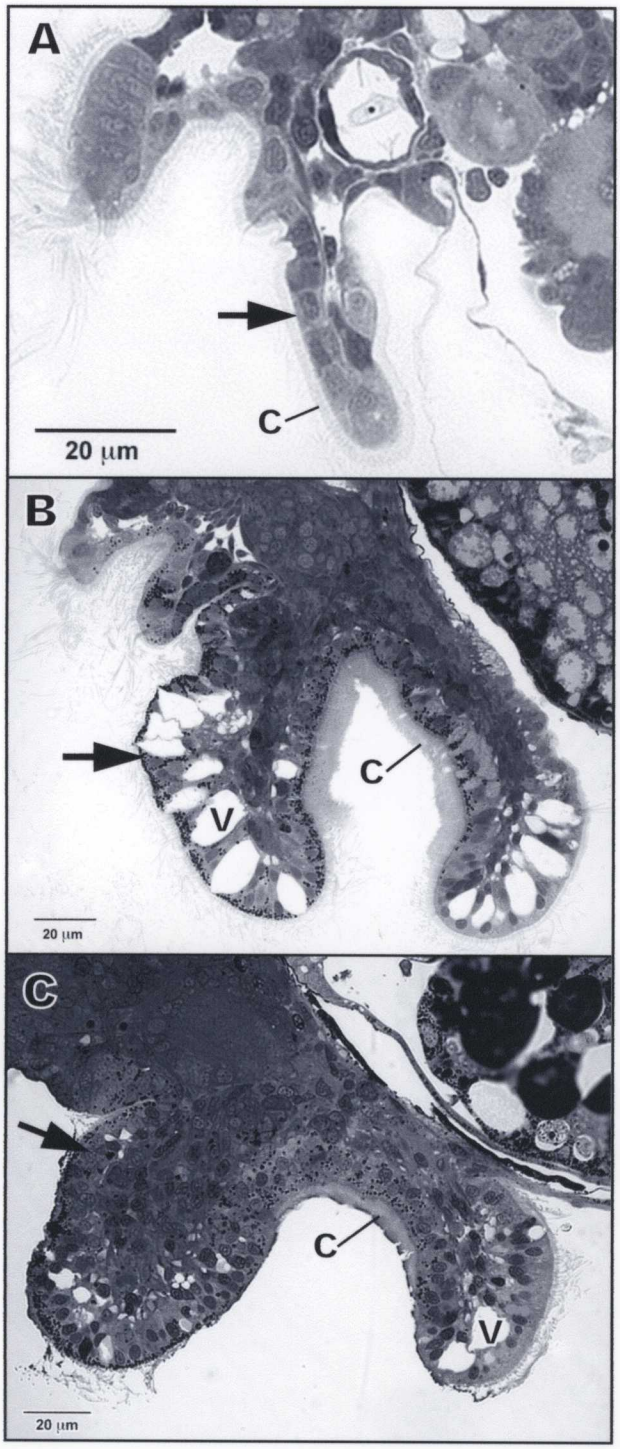

Figure 11. Longitudinal sections through the foot of *B. californica* at various stages. **A:** Foot of a newly hatched larva consisting of a metapodium (arrow) with cilia (c) in the pedal groove. **B**: 79-day larva showing enlarged propodium (arrow) with vacuoles (v). **C:** Foot of a juvenile with ciliated sole (c) and reduced number of vacuoles (v), arrow indicates propodium.

3.2.5 Musculature

Hatching larvae possessed a larval retractor muscle that attached to the inner wall of the protoconch via the perivisceral membrane. The perivisceral membrane is the mantle epithelium lining the inner surface of the shell (Thompson, 1958). The attachment is at the posterior region of the protoconch, just left of the midline. The larval retractor muscle extended anteriorly over the left digestive gland and ran parallel to the esophagus, into the cephalopedal area (Fig. 12A). Fibres from the larval retractor muscle also extended into the velar lobes and attached to the inner epithelial layer of the velum, enabling its retraction (Fig. 12C). In 79-day larvae, the larval retractor muscle was hypertrophied (Fig. 12B) relative to its condition in hatching larvae. Muscle fibres were also visible in the foot (Fig. 12D) of 79-day larvae, but it was not clear whether they extended from the larval retractor muscle or from a separate pedal muscle.

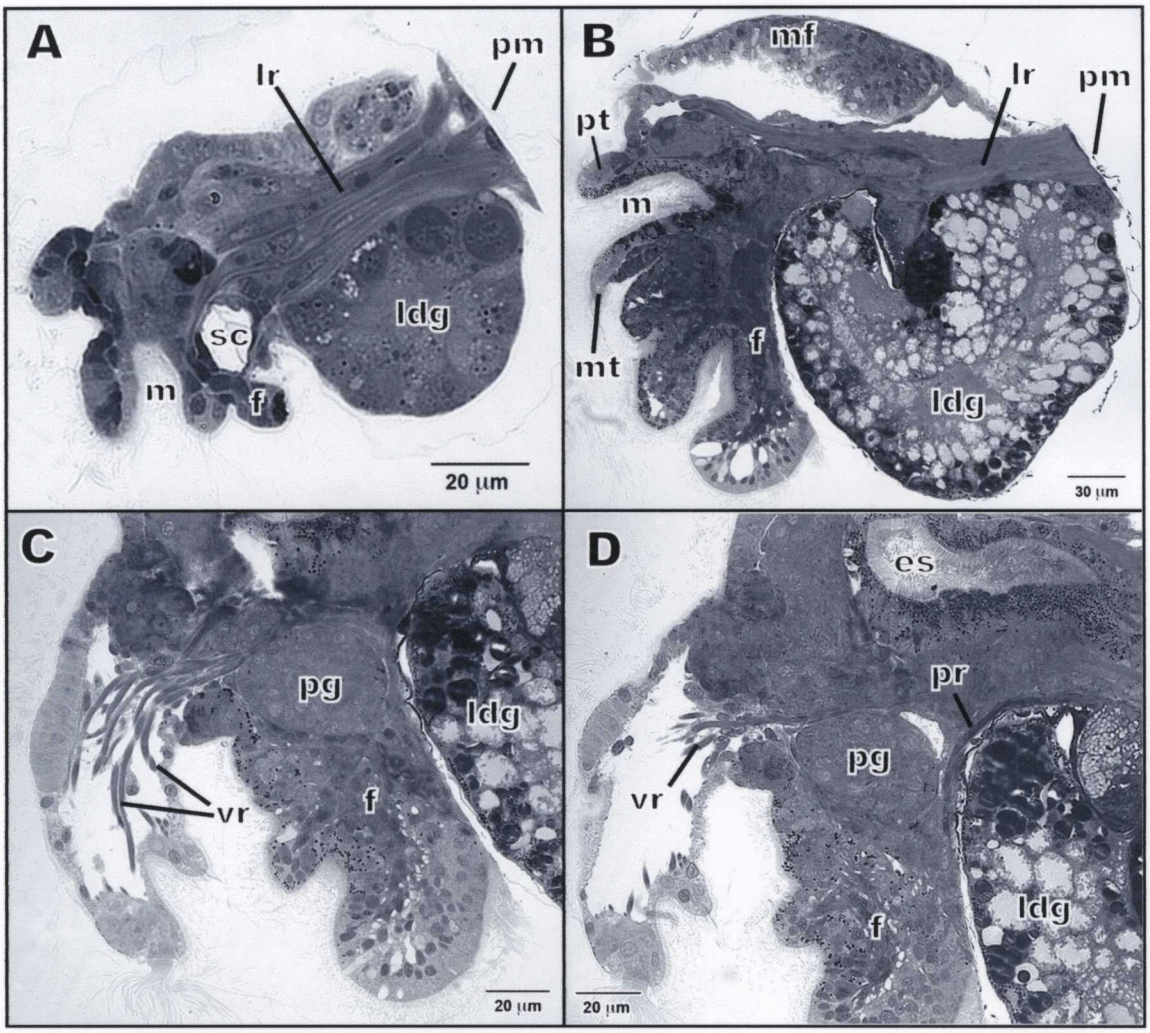

Figure 12. Musculature of *B. californica* larvae.

A: L.S. through a newly hatched larva showing the larval retractor muscle (lr) that originates on the perivisceral membrane (pm). **B:** L.S. through a 79-day larva showing the hypertrophied larval retractor muscle (lr) and mouth (m) positioned between the prototroch (pt) and metatroch (mt). **C:** L.S. of a 79-day larva showing the velar retractor fibres (vr), which extend from the larval retractor muscle. **D:** L.S. through a 79-day larva showing pedal retractor (pr) fibres inserting on the dorsal surface of the metapodium. es, esophagus; f, foot; ldg, left digestive gland; m, mouth; mf, mantle fold; pg, pedal ganglion; sc, statocyst.

3.2.6 Nervous System and Sensory Structures

Ganglia, Eyespots, and Statocysts

Hatching larvae of *B. californica* possessed paired cerebral ganglia, which were associated with the eyespots and situated on the dorsolateral aspect of the esophagus. The cerebral ganglia were connected to each other via the cerebral commissure, which passed dorsally over the esophagus (Fig. 13A). Newly hatched larvae also possessed paired pedal ganglia (Fig. 13B), which were located on the ventrolateral side of the statocysts (Fig. 13B, inset) and connected to each other via the pedal commissure which passes ventral to the esophagus.

In 79-day larvae, both the cerebral and pedal ganglia had increased substantially in size (Figs. 13C, 13D) and additional ganglia had developed. The pleural ganglia were not distinct, but were visible as a posterio-dorsal lobe of the cerebral ganglia. The supraesophageal ganglion was located on the right side of the esophagus, adjacent to the osphradium (Fig. 13D). Primary sensory neurons forming an osphradial ganglion were visible in the basal region of the osphradium, beneath the sensory epithelial layer (Fig. 13D, 14B, 14C).

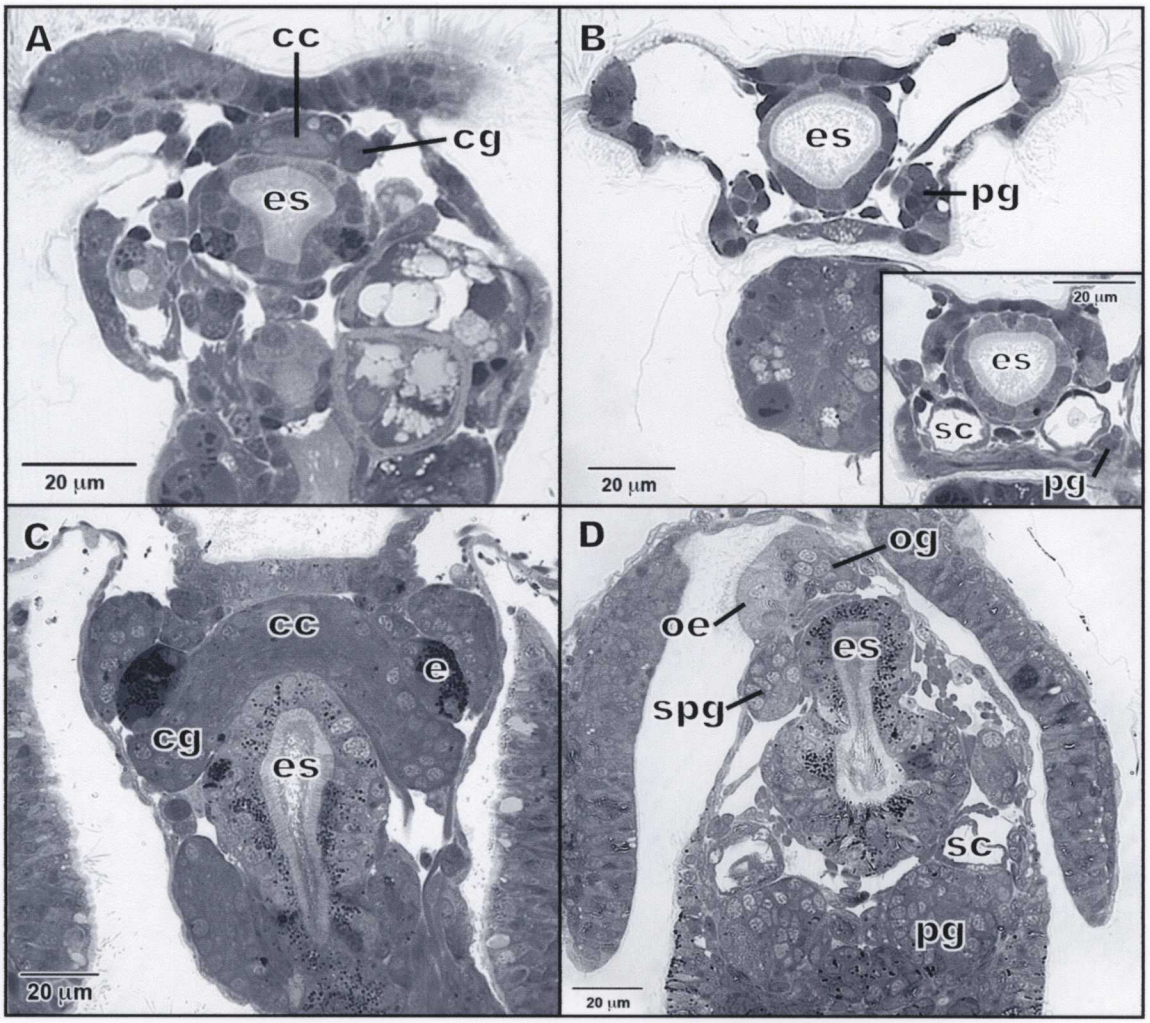

Figure 13. Ganglia and sensory organs in *B. californica* larvae.

A: F.S. through a newly hatched larva showing the cerebral ganglia (cg) and the cerebral commissure (cc). **B:** F.S. through a newly hatched larva showing the pedal ganglia (pg), situated ventral to the statocysts (sc), shown in the inset. **C:** 79-day larva in F.S. showing enlarged cerebral ganglia with associated eyespots (e) and cerebral commissure. **D:** C.S. of a 79-day larva showing the supraesophageal ganglion (spg) adjacent to the osphradial sensory epithelium (oe) with associated osphradial ganglion (og), and the statocysts with the associated pedal ganglia. es, esophagus.

Osphradial Ganglion

An osphradial ganglion and associated sensory epithelium were found in the floor of the mantle cavity of *B. californica* larvae. The structure was located on the right side of the mantle cavity, and consisted of a layer of sensory epithelium overlaying an osphradial ganglion (Fig. 14A). Primary sensory neurons in the epithelium were connected via axons to the osphradial ganglion, which was situated beneath the left side of the ciliated epithelium (Figs. 13D, 14B). Neurotubules in the axons of the ganglion were also visible (Fig. 14C). Adult *B. californica* are not known to possess osphradia, and it was previously unknown that their larvae retained this structure.

Apical Sensory Organ

The structure of the apical sensory organ was examined in *B. californica* larvae by immunolabelling newly hatched larvae with antibodies against serotonin and acetylated α-tubulin. The ASO was located between the eyespots of the larvae, just under the velar epithelium. It consisted of one central serotonergic neuron with paired lateral serotonergic neurons on each side, connected via neuropil (Fig. 15A). The sensory neurons each possessed an apical dendrite with a terminal swelling (Fig. 15A). Four ampullary sensory neurons were also present, located just dorsal to the serotonergic neurons (Fig. 15B). Ampullary neurons can be detected with antibodies against α-tubulin because these sensory neurons are characterised by a deep invagination of the apical cell membrane that is filled with cilia arising from the wall of the invagination (Kempf and Page, 2005).

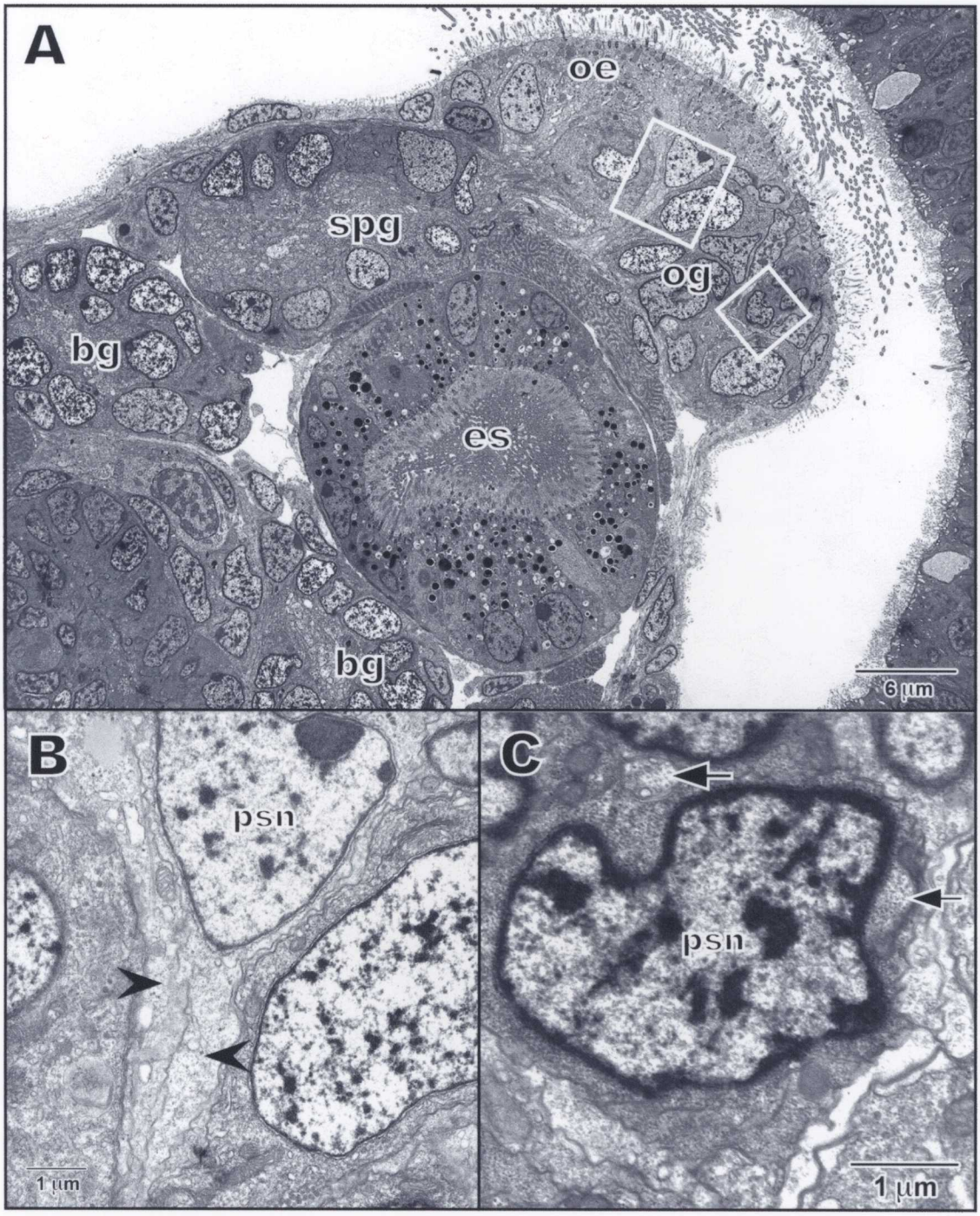

Figure 14. TEM micrographs of osphradial epithelium in *B. californica.*

A: Ciliated osphradial sensory epithelium (oe) with the osphradial ganglion (og) and supraesophageal ganglion (spg) within the mantle cavity, boxes indicate areas enlarged in B and C. **B:** Axons (arrowheads) emerging from a primary sensory neuron (psn). **C:** Neurotubules in cross section (arrows) in the osphradial ganglion. bg, buccal ganglion; es, esophagus.

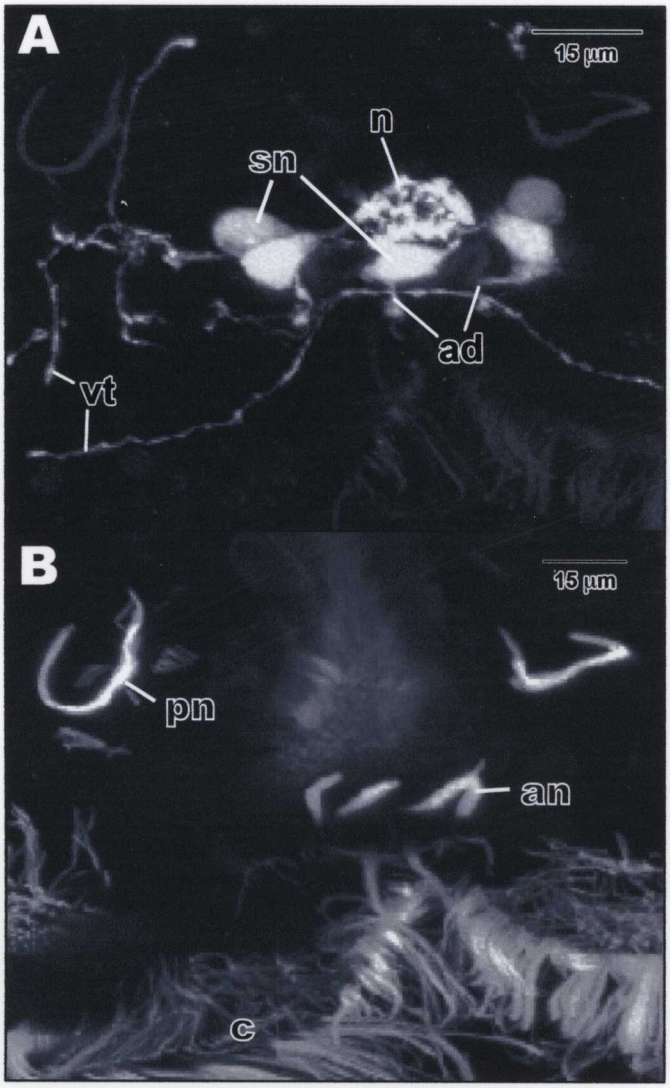

Figure 15. Confocal laser scanning micrographs of the ASO in *B. californica*.
A: ASO immunolabelled with antibodies against serotonin showing central and paired lateral serotonergic neurons (sn) connected via neuropil (n), and apical dendrites (ad) with terminal swellings. **B:** ASO immunolabelled with antibodies against α-tubulin showing 4 ampullary neurons (an). c, cilia; pn, protonephridium; vt, velar tracts of sensory neurons.

3.2.7 Digestive System

The alimentary tract of *B. californica* larvae consisted of a mouth opening between the prototrochal and metatrochal ciliated bands of the velum (Fig. 12B), an esophagus that led to the two-chambered stomach, and an intestine that terminated in an anus (Figs.16A, 16B). A large left digestive gland and small right digestive gland opened into the stomach (Fig. 16A).

The tubular esophagus was lined with cuboidal epithelial cells bearing cilia that serve to transport the algal food particles from the mouth to the stomach (Fig. 16B). Thin, transparent structures were visible in the lumen of the ventral esophagus in 79-day larvae (Fig. 16E). These structures likely represent radular teeth or possibly jaw elements. Thin sectioning would be required to resolve whether they originate in the radula sac, which would indicate that they are indeed radula teeth.

The esophagus and left digestive gland emptied into the ventral chamber of the stomach (Figs. 16A-D), which is lined with a cuticle that has hyaline rods embedded in it perpendicular to the stomach wall (Fig. 16B inset). The dorsal chamber of the stomach is lined with a ciliated band (Fig. 16B), which was observed to rotate the bolus of food particles in live veligers. The dorsal stomach emptied into the intestine, which consisted of a ciliated tube that emptied into the right side of the mantle cavity (Figs. 16A, 16D).

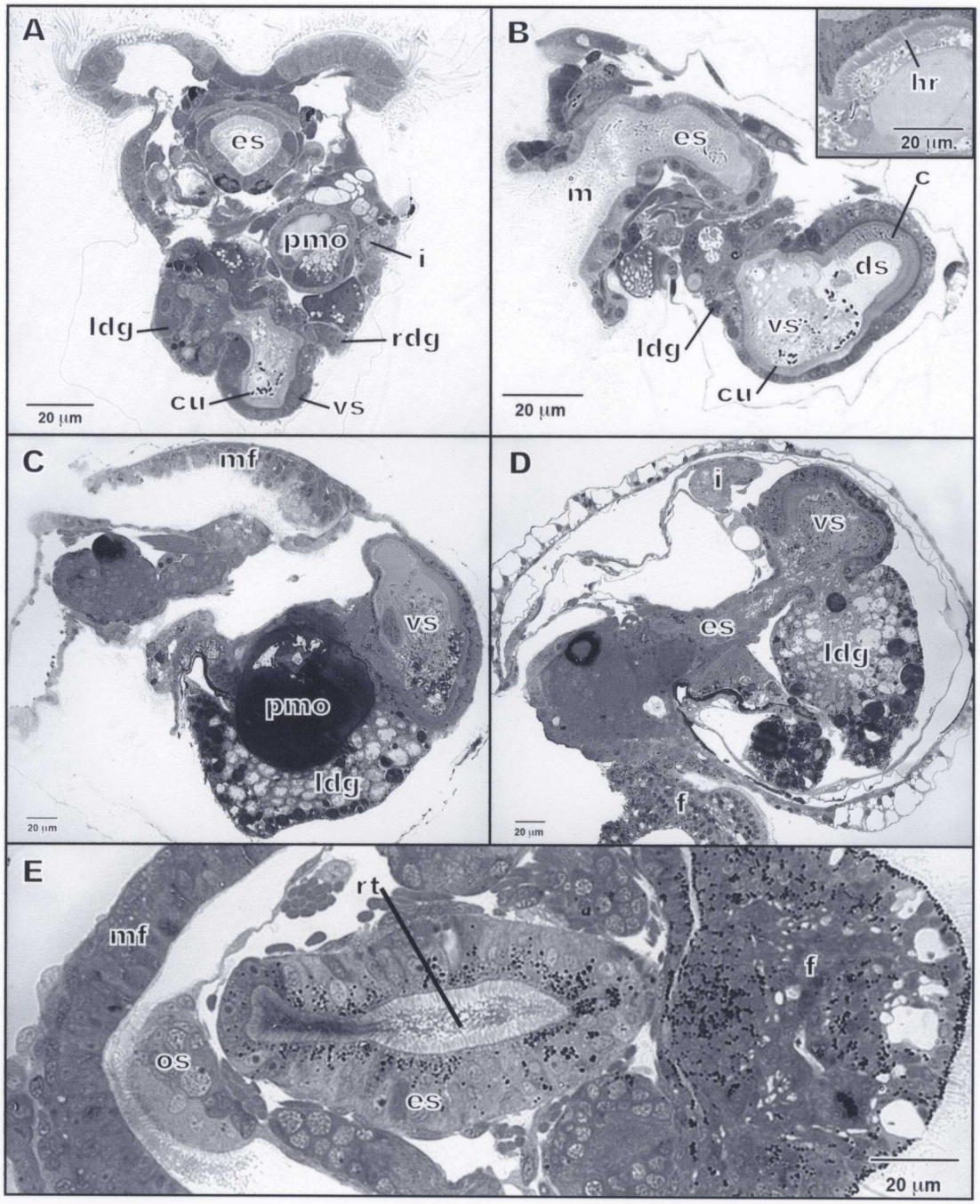

Figure 16. Digestive system in *B. californica* larvae and juveniles.

A: F.S. through hatching larva. **B:** L.S. of the digestive system in hatching larva. **C:** L.S. showing components of the digestive system in a 79-day larva. **D:** L.S. of a juvenile. **E:** C.S. of a 79-day larva showing the radula teeth (rt). c, ciliated band; cu, cuticle; ds, dorsal stomach; es, esophagus; f, foot; hr, hyaline rods; i, intestine; ldg, left digestive gland; m, mouth; mf, mantle fold; os, osphradium; pmo, pigmented mantle organ; rdg, right digestive gland; vs, ventral stomach.

3.2.8 Pigmented Mantle Organ

The pigmented mantle organ (PMO) of *B. californica* larvae appeared to consist of three distinct types of tissues, a-c. Type a stained a very dark black (with Richardson's stain), and was the most ventrally situated of the tissues visible in longitudinal section (Figs. 17A-C). In later larval stages, type a tissue sectioned very poorly, and appeared to consist of a substance that was dense and brittle when fixed. Tissue b was located on the anterior side of the pigmented mantle organ and consisted of large vacuolated cells that appeared empty in hatching larvae (Fig. 17A), but stained a uniform dark colour in later stages (Figs. 17B, 17C). Tissue c was not visible in newly hatched larvae, but in later stages was situated most dorsally in the PMO, adjacent to the metanephridium (Fig. 17C). Tissue c contained many tiny, round, darkly stained granules (Figs.17B, 17C).

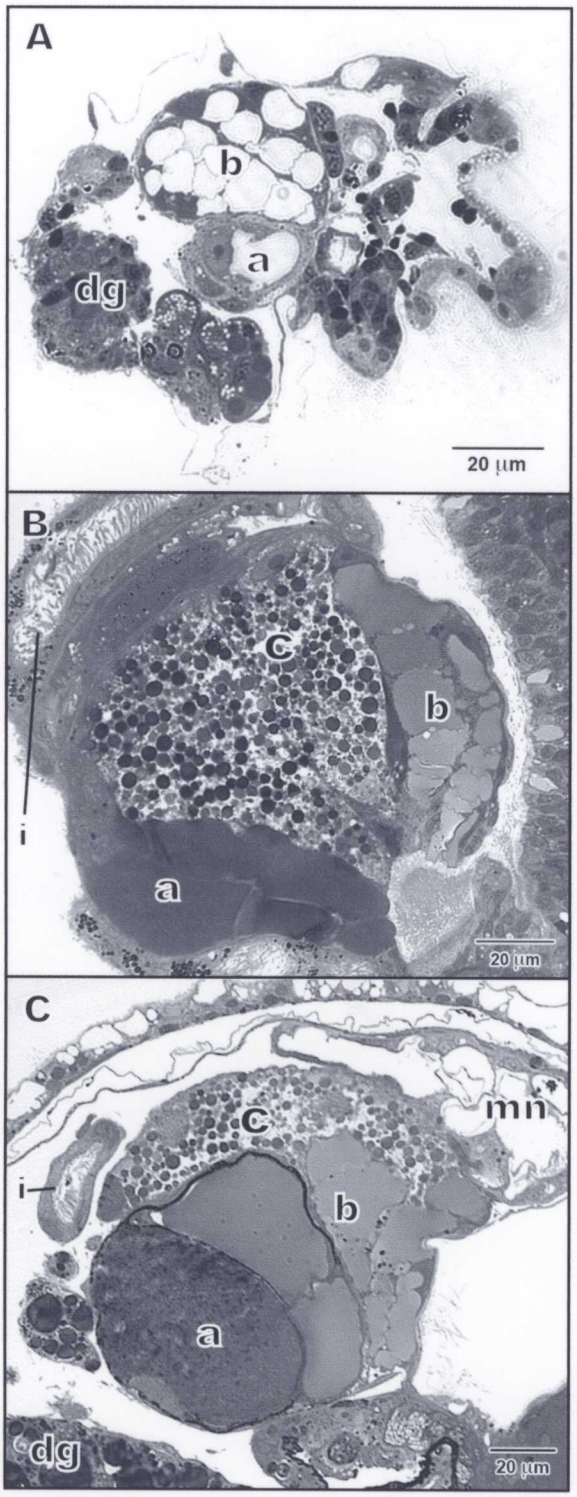

Figure 17. Tissue types a-c in the pigmented mantle organ of *B. californica*.
A: Newly hatched larva in L.S. showing PMO tissues a and b. **B:** 79-day larva in L.S. with tissues a-c. **C:** L.S. through a juvenile showing tissue types a-c in the PMO. dg, digestive gland; i, intestine; mn, metanephridium.

Discussion

4.1 Larval Structure and Development – Comparison to other Heterobranchs

Heterobranch veliger larvae differ from veligers of their caenogastropod sister group in numerous respects. Caenogastropod veligers typically have orthostrophically coiled, sculptured protoconchs, vela with multiple lobes, highly modified protonephridia involved in albumen uptake within the egg capsule, deep mantle cavities, and possess eyespots and tentacles prior to hatching (Page, 1995a; Ponder and Lindberg, 1997). In contrast, heterobranch veligers characteristically possess smooth, hyperstrophically coiled protoconchs, simple vela, protonephridia which function in excretion, shallow mantle cavities with a mantle fold that detaches from the shell aperture during larval development, and they do not develop eyespots or rhinophores until the second half of the larval stage (Page, 1995a; Ponder and Lindberg, 1997). Additionally, the presence of an operculum, at least in the larval stage, was thought to be common to all gastropods (Ponder and Lindberg, 1997).

Veligers of *B. californica* are clearly recognizable as heterobranch larvae because they exhibit the classic larval characteristics of heterobranchs as described above, with two significant exceptions that are apparent at the time of hatching. *B. californica* larvae develop eyespots prior to hatching as in caenogastropods, and completely lack opercula unlike the vast majority of other gastropod larvae. As the larvae develop, other notable differences become manifest, such as the unusual growth pattern of the protoconch and the relatively long larval period as compared to other pleurobranchoideans. This section of my thesis will compare structure and development between *B. californica* larvae and

the larvae of other heterobranchs, highlight the major differences that I observed, and discuss the phylogenetic implications of these new data.

4.1.1 Protoconch

The pattern of protoconch growth in *B. californica* differed substantially from that of any other heterobranch larva studied to date. Rather than tracking previous whorls on the left side of the larval shell and accreting to the dorsal margin of the whorl beneath it, growth of the outer whorl in *B. californica* began to widen markedly around 30 days post hatching (Fig. 7B). Thus, the outer whorl covered and accreted to the lateral margins of the previous whorl on both the left and right sides of the protoconch, yielding a shell that looked superficially planispiral (Figs. 7C-E). Drawings of the protoconch of *Berthellina citrina*, a pleurobranchoidean species that exhibits capsular metamorphic development, do not indicate that *B. citrina* shares this peculiar shell growth pattern (Gohar and Abul Ela, 1957; Usuki, 1969). Unfortunately, details of the form of the protoconch were not described in the studies of *Pleurobranchaea maculata*. It would be valuable to examine with SEM the protoconchs of *P. maculata* and of other species of *Berthella* to see if the protoconch of any other species share the growth pattern observed in *B. californica*.

Dissolution of internal shell whorls was previously known to occur only in nudibranchs with type 1 protoconchs (Page, 2000). The discovery of this internal dissolution process occurring in *B. californica* (Figs. 8B, 9A) supports a close relationship between pleurobranchoideans and nudibranchs, the clade named Nudipleura by Wägele and Willan (2000). Page (2000) suggested that the functional significance of internal protoconch dissolution in nudibranchs may be to allow the visceral mass of

larvae to take on an uncoiled adult-like morphology, facilitating its rapid incorporation into the dorsal area of the foot at metamorphosis. In *B. californica*, the shell is retained in adults rather than lost at metamorphosis, but due to its flattened shape the visceral mass also has an uncoiled conformation (Figs. 12A, 12B, 16A-D) as in nudibranchs. The functional significance of internal whorl dissolution in nudipleurans remains unclear since other heterobranchs such as *Olea hansenensis* (Sacoglossa) that loose the shell at metamorphosis do not exhibit internal whorl dissolution (Fig. 8C). Further sampling throughout the Heterobranchia should be carried out in order to determine if internal whorl dissolution is ubiquitous among nudipleurans, and to confirm that it is unique to the Nudipleura.

The raised sculpture pattern observed on the protoconchs of older larvae (Figs. 7B-E) was localised to the area in contact with the dorsal surface of the metapodium in living specimens. It is therefore possible that the foot secreted this material, rather than an operculum as occurs in most other heterobranch larvae. In *Pleurobranchaea maculata*, a similar raised sculpture pattern consisting of 4-6 ridges spaced approximately 10 μm apart was observed in the same location on the protoconch (Tsubokawa and Okutani, 1991).

The fate of the protoconch after metamorphosis appears to be variable among pleurobranchoideans. In *Berthella californica* and *Berthellina citrina* (Gohar and Abul Ela, 1957; Usuki, 1969) the shell is retained throughout life, while in *Pleurobranchaea maculata* it is dissolved away after metamorphosis (Gibson, 2003; Tsubokawa and Okutani, 1991). The functional significance of this variation in protoconch fate is currently unknown.

4.1.2 Mantle Morphogenesis

The formation of the notum in *B. californica* (Fig. 10) and in other pleurobranchoideans (Gibson, 2003; Gohar and Abul Ela, 1957; Thiriot-Quiévreux, 1967; Tsubokawa and Okutani, 1991) via reflexion of the mantle fold is notable because this method of notum formation occurs in anthobranchs as well (Bickell, 1978; Chia and Koss, 1988; Thompson, 1967; Thompson, 1976) and appears to be a synapomorphy of the Pleuroanthobranchea. In cladobranchs such as *Melibe leonina* (Bickell and Kempf, 1983), *Hermissenda crassicornis* (Harrigan and Alkon, 1978), and *Phestilla sibogae* (Bonar and Hadfield, 1974) reflexion of the mantle fold does not occur.

The mantle fold in *B. californica* retracted from the apertural lip of the protoconch in older larvae after the protoconch stopped growing (Fig. 5C). Cells of the mantle fold are responsible for shell growth because they secrete the new material at the apertural edge of the shell, therefore growth arrests when the mantle fold retracts from the lip of the protoconch, as commonly occurs during the latter part of the larval period in planktotrophic heterobranchs (Fretter and Graham, 1962).

In the related pleurobranchoidean species *Pleurobranchaea maculata* (Gibson, 2003; Tsubokawa and Okutani, 1991) and *P. meckelii* (Thiriot-Quiévreux, 1967), the mantle fold gradually began to expand and reflect over the lip of the protoconch well before the larvae settled and shed the velum. At the onset of metamorphic competence in *P. maculata*, reflected mantle fold epithelium had completely covered the protoconch. This process occurred abruptly in *B. californica* and concurrently with the other metamorphic changes. Despite this difference, in both species the shell continues to grow after the mantle reflects dorsally to cover it.

Gibson (2003) stated that the mantle fold of *P. maculata* was lost when the mantle overgrew the shell, and thus the new mantle region that remained in contact with the shell aperture must also have shell-secretion abilities. I believe this may be a misinterpretation of the process, since it seems more likely that shell-depositing cells of the outer mantle fold epithelium remain in place, while the inner epithelial layer grows out and reflects dorsally to overgrow the shell.

4.1.3 Foot

The development of the foot in larvae of *B. californica* was comparable to that observed in other heterobranch larvae. The thin metapodium of the newly hatched veligers (Fig. 11A) grew and expanded due to the development of glands to form the propodium as described in *Phestilla sibogae* (Bonar and Hadfield, 1974), *Corambe [Doridella] steinbergae* (Bickell and Chia, 1979b), and *Melibe leonina* (Bickell and Kempf, 1983).

The most notable features of the foot in *B. californica* larvae were the large, numerous vacuolated cells present in the later larvae (Fig. 11B). Although these vacuoles appeared empty in the sectioned material, it is reasonable to assume that they would contain a secretory product important for attachment and crawling in the metamorphosing larvae and juveniles based on tests of the chemical composition of pedal vacuole contents in the larvae of other heterobranchs (Bonar and Hadfield, 1974).

Opercula are thought to function in predator defence, enabling the shell to be closed when the body and foot are withdrawn inside (Fretter and Graham, 1962; Norton, 1988). While opercula are absent in a few capsular ametamorphic (direct developing)

species, they are present in late embryos and larval stages of most other heterobranch larvae studied to date, with the exception of the dorid nudibranchs *Aegires albopunctata* and *A. punctilucens* (Goddard, 2001a). It is so rare for a larval gastropod to lack an operculum that Ponder and Lindberg (1997) cited presence of a larval operculum as a synapomorphy of the Gastropoda. However, the absence of an operculum was expected for *B. californica*, since the larvae of all pleurobranchoideans observed to date lack opercula, including *Berthellina citrina* (Gohar and Abul Ela, 1957; Usuki, 1969), *Berthellina ilisima [engeli]* (MARCUS AND MARCUS, 1967) (Goddard, 2001a), *Pleurobranchoidaea maculata* (Gibson, 2003; Tsubokawa and Okutani, 1991), *Pleurobranchaea meckelii* LEUE, 1813 (THIRIOT-QUIÉVREUX, 1967) and *P. californica* (Page 2006, University of Victoria; pers. comm.). Therefore, the absence of a larval operculum appears to be a synapomorphy of the Pleurobranchoidea.

Goddard (2001a) compared the larvae of *Aegires albopunctata* to *Pleurobranchaea maculata*, since both had large velar lobes, lacked opercula, and had a mantle fold which grew over the shell during larval life, although in *A. albopunctata* the protoconch was cast off prior to settling. He proposed that opercula were useless in these species because the combination of the large velum and precocious mantle growth prevented complete withdrawal of the body into the protoconch (Goddard, 2001a). However, *B. californica* does not share these characteristics, and is able to fully withdraw into its protoconch, while still lacking an operculum. Thus, the reason for the absence of the operculum in pleurobranchoidean larvae remains without a satisfactory explanation.

4.1.4 Musculature

Larvae of *B. californica* appeared to have one main retractor muscle that increased in size as the larvae grew, and had fibres that inserted on the velar lobes and in the foot (Figs. 12A-D), although the fibres observed in the foot may be from a separate muscle. This arrangement of musculature differs from that described for nudibranchs, which have three larval muscles that develop asynchronously during the larval stage (Bonar and Hadfield, 1974; Page, 1995b). However, thin sectioning was required to trace the muscle origins in nudibranch larvae (Page, 1995b), so it is possible that the retractor muscle observed in *B. californica* actually represents more than one muscle as well.

4.1.5 Nervous System and Sensory Structures

The larvae of heterobranchs such as *B. californica* are very small compared to larvae of caenogastropods, and as a result the components of the nervous system are very compacted and difficult to resolve using light microscopy of thick sections. I was able to resolve large ganglia including the paired cerebrals and pedals in 1 μm sections (Figs. 13A-D), but the connectives between ganglia were difficult to resolve without thin sectioning and TEM (Fig. 14A). Previous studies of pleurobranchoideans did not include any histological sectioning; therefore nothing is currently known about the nervous system in larvae of other species. However, developing nervous systems of nudibranchs and *Aplysia californica* have been well studied, and the arrangement of the ganglia is quite similar to that observed in *B. californica*.

Larvae of *B. californica* are unusual among planktotrophic heterobranch larvae because they possess eyespots prior to hatching. Goddard (2001a) claimed that both

Aegires albopunctata (Anthobranchia) and *Pleurobranchaea maculata* possess eyespots at hatching, however both studies of larval development in *P. maculata* state that hatching larvae lack eyespots (Gibson, 2003; Tsubokawa and Okutani, 1991). The congeneric species *P. californica* also lacked eyespots at hatching (Page, 2006, University of Victoria; pers. comm.). An observation on the larvae of a Hawaiian species of *Pleurobranchus* sp. (Ostergaard, 1950) is the only other record of eyespots present at hatching in Pleurobranchoideans to date. Some species of cladobranch nudibranchs that have type 2 protoconchs hatch with eyespots (Goddard, 2001a), but in most other species of heterobranchs eyespots develop during the second half of larval life (Bonar, 1978a; Hadfield and Switzer-Dunlap, 1984). Why *B. californica* larvae should hatch with eyespots, while the vast majority of other planktotrophic heterobranch veligers succeed without them remains unknown. The hatching larvae of other species of *Berthella* should be examined to determine if the presence of eyespots at hatching is characteristic of the genus.

The discovery of an osphradium in the larvae of *B. californica* (Figs. 14A-C, 18A) was surprising because adult pleurobranchoideans are not known to possess osphradia. In fact, Grande et al. (2004b) stated that "the lack of osphradium is the only well-defined synapomorphy of the Nudipleura". However, thick sections cut though larvae of the anthobranch *Onchidoris bilamellata* (LINNAEUS, 1767) indicate that an osphradium is present in this species as well.

Although the osphradium is smaller in *Onchidoris bilamellata* (Fig. 18B-D) than in *B. californica* (Fig. 18A), the morphology and location of the structure, arising from epithelium lining the floor of the mantle cavity are very similar, despite the structure

occurring further to the larva's left side in *O. bilamellata*. However, axon tracts and neurotubules cannot be resolved in thick sections, so serial thin sections viewed with a TEM would be required to confirm the identification of the structure as an osphradium. My identification of the ganglia associated with the osphradial epithelium in *Onchidoris bilamellata* should therefore be regarded as tentative.

In light of these new data, the statement by Grande et al (2004b) regarding the lack of an osphradium as a synapomorphy of the Nudipleura should be amended to clarify that adult nudipleurans lack osphradia, but the larvae of at least two species of pleuroanthobranchs appear to retain this structure. It would therefore be reasonable to look at the late stage larvae of other species specifically to assess whether there is evidence for the retention of osphradia, or even osphradial rudiments, in any other members of the Nudipleura.

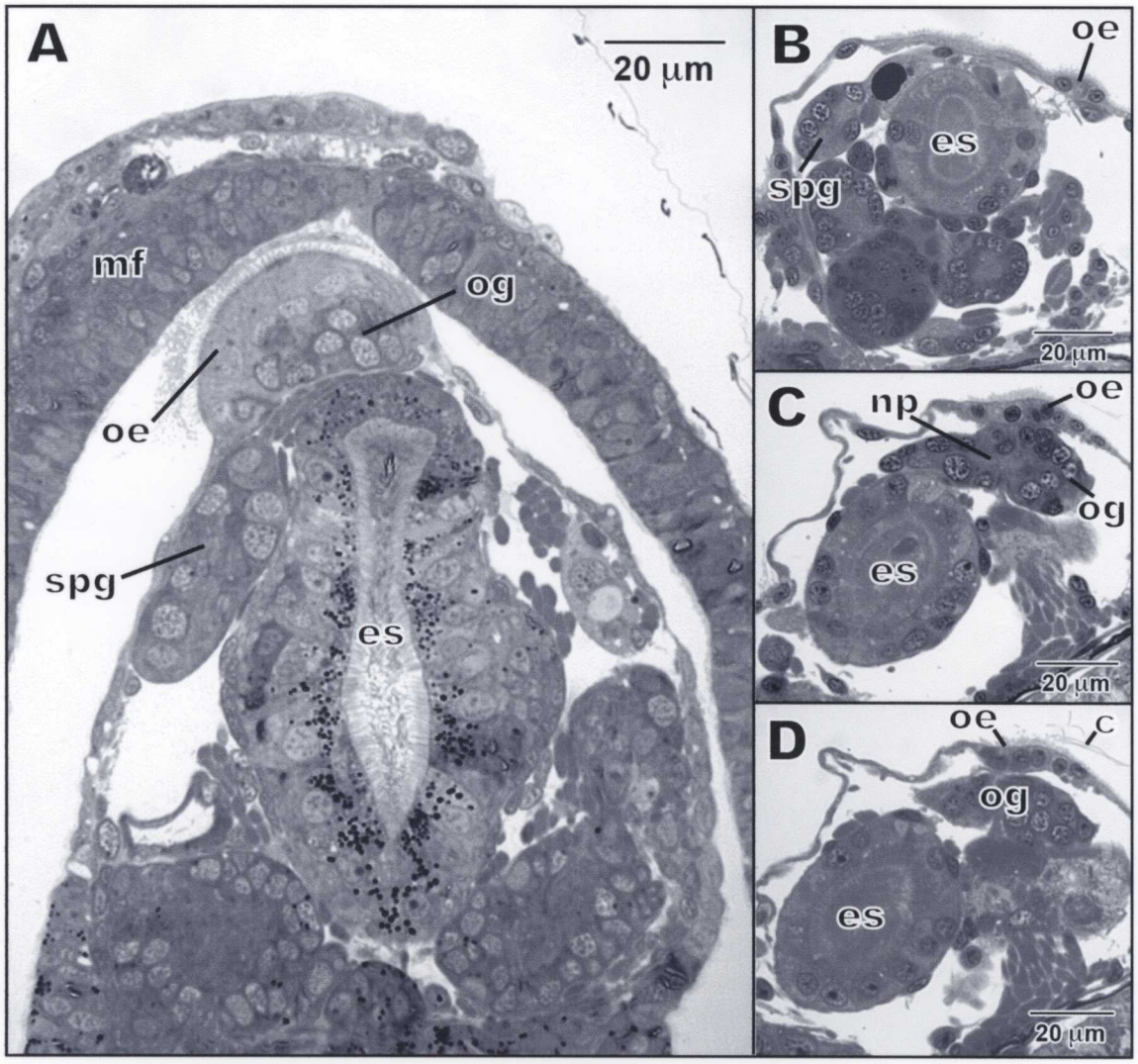

Figure 18. Osphradia in the larvae of *B. californica* and *Onchidoris bilamellata*. **A:** C.S. showing the osphradial epithelium (oe), osphradial ganglion (og) and supraesophageal ganglion (spg) in a 79-day larva of *B. californica*. **B:** C.S. of the osphradial epithelium and supraesophageal ganglion in 45-day competent *O. bilamellata*. **C:** C.S. through *O. bilamellata* showing the osphradial ganglion with neuropil (np), in association with the osphradial epithelium. **D:** Cilia (c) emerging from the osphradial epithelium in *O. bilamellata*. es, esophagus; mf, mantle fold.

The apical sensory organ of larval gastropods is a ganglion situated dorsal to the cerebral ganglia, which consists of putative sensory and non-sensory neurons and neuropile (Bonar, 1978b; Chia and Koss, 1984; Kempf and Page, 2005; Kempf et al., 1997; Marois and Carew, 1997a; Page, 2002; Page and Parries, 2000). The main types of cells observed in the ASO of heterobranchs include ciliary tuft cells, ampullary sensory neurons with ciliated invaginations, and three types of serotonergic neurons: lateral sensory, medial sensory (both sometimes termed para-ampullary), and lateral non-sensory (Marois and Carew, 1997a; Page, 2002). The arrangement of the serotonergic neurons in *B. californica* larvae, with one medial and a pair each of lateral sensory and lateral non-sensory on each side (Fig. 15A), is congruent with the configuration observed in most other larval heterobranchs (Kempf et al., 1997; Marois and Carew, 1997a; Marois and Carew, 1997b; Page, 2002; Schaefer and Ruthensteiner, 2001).

The number of ampullary neurons in the ASO has been examined in a number of cladobranch species, and all consistently had five ampullary neurons (Kempf et al., 1997). Within the Anthobranchia however, the number of ampullary neurons in the ASO has only been documented previously in 2 species. *Rostanga pulchra* MACFARLAND, 1905 possessed four ampullary neurons in its ASO according to Chia and Koss (1984), as did *Diaulula sandiegensis* (COOPER, 1862) (Page 2006, University of Victoria; pers. comm.). The presence of four ampullary neurons in the ASO of *B. californica* (Fig. 15B) is consistent with these observations, since the pleurobranchoideans were proposed to be the sister group to the anthobranchs (Grande et al., 2004a; Grande et al., 2004b). The presence of 4 ampullary neurons in the ASO was also observed in the cephalaspidean *Haninoea vesicula* (GOULD, 1855) (Page 2006, University of Victoria; pers. comm.) and

the anaspideans *Aplysia californica* (Marois and Carew, 1997a) and *Phyllaplysia taylori* DALL, 1900 (Kempf and Page, 2005). Based on these observations it appears that the presence of 4 ampullary neurons in the ASO may be a plesiomorphy of opisthobranchs, while the development of a fifth ampullary neuron may be an apomorphy of the Cladobranchia, although further sampling of this trait throughout the Heterobranchia will be required to confirm this hypothesis.

Rhinophores are the pair of chemosensory tentacles found on the dorsal cephalic region of most derived heterobranchs including nudibranchs, pleurobranchoideans, anaspideans and sacoglossans. They appear to have originated independently at least three times in these clades (Gosliner, 1994). Rhinophores did not develop in *B. californica* until metamorphosis, whereas in *P. maculata,* they were visible by the second week after hatching (Gibson, 2003; Tsubokawa and Okutani, 1991) and they were also present prior to metamorphosis in *P. meckelii* (Thiriot-Quiévreux, 1967).

4.1.6 Digestive System

The digestive system of *B. californica* was very similar to those of many other planktotrophic heterobranchs that have been studied (Bickell and Chia, 1979b; Bickell and Kempf, 1983; Kriegstein, 1977b; Tsubokawa and Okutani, 1991). The mouth in most planktotrophic gastropod veligers, including *B. californica*, is positioned mid-ventrally in the food groove between the prototrochal and metatrochal ciliated velar bands (Fig. 12B) such that food particles are concentrated and moved towards the mouth by the beating action of the velar cilia (Strathmann and Leise, 1979). One previous study on another pleurobranchoidean species, *Pleurobranchaea maculata*, described the mouth as being

located ventral to the metatrochal band (Gibson, 2003); however the feeding mechanism of veligers makes this unusual position highly unlikely, and an aberrant mouth position for pleurobranchoidean veligers was not confirmed by the present study (Fig. 12B). Additionally, a previous study on *P. maculata [japonica]* observed the larval mouth in the usual position between the prototroch and metatroch (Tsubokawa and Okutani, 1991).

In the competent larvae of most heterobranchs, the radula, which juveniles use for feeding, is visible in the radula sac located just ventral to the esophagus. The radula teeth in adult *B. californica* are elongate and hook-like (Gosliner and Bertsch, 1988). In sections through late larvae and juveniles the radula teeth were very difficult to see due to their thin profile (Fig. 16E). Better resolution of the radula teeth in larval and juvenile *B. californica* could likely be achieved using thin sections viewed on a TEM, however, the thin profile of the teeth would still make it problematic to see much of each tooth in a single section.

4.1.7 Pigmented Mantle Organ

The term pigmented mantle organ (PMO) was coined by Robertson (1985) to describe the "conspicuously pigmented organ … located in the dorsal or right lateral mantle cavity wall under the shell near the anus" of in the veligers of many opisthobranchs. These organs were previously called larval kidneys, and the name change reflects the idea that these organs are no longer believed to be excretory in function (Bonar, 1978a; Robertson, 1985). Schaefer (1996) argued that the term 'anal gland' should be used for this structure because many other opisthobranchs possess similar

glandular structures whose secretions are colourless. However, in most of the recent literature, the term PMO has remained in use.

In early larvae of *B. californica,* the PMO included two distinct tissue types: a and b (Fig. 17A). Tissue types a and b each consisted of large, vacuolated cells containing dense products that are likely secretory in nature. These glandular tissues appeared to be similar to the tissues observed in the PMOs of other opisthobranchs such as *Corambe [Doridella] steinbergae* (Bickell and Chia, 1979b), *Melibe leonina* (Bickell and Kempf, 1983), and *Phestilla sibogae* (Bonar and Hadfield, 1974).

Older larvae and juveniles possessed a third tissue type, c, which did not resemble any structure in larval heterobranchs reported in the literature (Figs.17B, 17C). Based on its structure and position in the anterior-dorsal region of the visceral cavity, I postulated that tissue c might represent a developing blood gland. Histologically, the blood gland was described as possessing cells 10-20 μm in diameter with small nuclei, loosely linked via connective tissue threads (Wägele, 1998). However Wägele (pers. comm., 2006; University of Bonn, Germany), an expert in blood gland histology, indicated that this was unlikely to be a blood gland because the darkly stained granules observed in tissue c appear to consist of a storage or secretory product, which was not consistent with the blood gland material she has described. Thus, the identity and function of tissue c in the PMO of *B. californica* larvae currently remains a mystery.

4.2 Use of Developmental Characters for Phylogenetic Reconstruction

While it seems logical that studies of evolutionary relationships between organisms should take into account all stages of an organism's life, within the

Heterobranchia, historically larval characteristics have rarely been considered. However, just as with phylogenetic data from adult morphology or molecular sources, there are both advantages and disadvantages to using developmental characters for phylogenetic reconstruction.

Potential reasons for not including developmental characters in phylogenetic analyses include the small size and difficulties encountered in rearing larvae, lack of knowledge of the relationships between ecological factors and developmental differences, and the differing developmental constraints and selection pressures on larval and adult stages (Wägele and Willan, 2000; Willmer, 1990). Indeed it has been shown in echinoderms that using only larval characters for phylogenetic reconstruction can result in cladograms very different from those constructed using adult morphology and molecular data (Wray, 1996).

Despite these reservations, characters from larval morphology may be useful because larvae are smaller, less complex, typically have fewer functions, and may indicate relationships with other groups more clearly than more complex adult stages (Willmer, 1990). Ponder and Lindberg (1997) successfully used a number of larval characters in their seminal phylogeny of gastropods. Additionally, Wray (1996) found that it was possible to distinguish between robustly supported clades and false ones produced by suites of parallel morphological transformations by comparing statistics such as tree length and consistency indices. Since these methods are widely used to determine which morphological characters in adults are phylogenetically important, there is no reason they cannot be used to evaluate larval characters as well. Finally, Willmer (1990) suggested that "only the tighter relationships indicated from embryology" be accepted,

"where functional reasons for convergence are not apparent". This same advice could equally be applied to any other source of phylogenetic data.

While it would be incorrect to take the Haeckelian view that "ontogeny recapitulates phylogeny" (Haeckel, 1868), it would be equally erroneous to take the opposing view that no useful phylogenetic information can be found by studying larval forms. The compromise of course is found somewhere between these two extremes, where developmental characters are used with the same careful awareness of potential functional convergence and divergence that should be used when evaluating other sources of comparative information (Willmer, 1990).

4.3 Phylogenetic Implications of Larval Characters

Recent phylogenetic hypotheses regarding the relationship between the Pleurobranchoidea and nudibranchs differ substantially depending on whether they are based on adult morphological characters or molecular data. Recent phylogenies based on adult morphology place the Pleurobranchoidea as sister group to the Nudibranchia (Fig. 19A) (Salvini-Plawen, 1991; Salvini-Plawen and Steiner, 1996; Schmekel, 1985; Wägele and Willan, 2000). A phylogeny based on mitochondrial gene sequence data divided the nudibranchs into two groups, Cladobranchia and Anthobranchia, and placed the Pleurobranchoidea as sister to the anthobranchs, forming the Pleuroanthobranchea (Fig. 19B) (Grande et al., 2004b).

Morphological characters of larvae, including dissolution of internal shell whorls, formation of the notum by reflexion of inner mantle fold epithelium, the presence of five ampullary neurons in the ASO, the absence of larval opercula, and the absence of larval

osphradia were mapped onto these existing phylogenies. This mapping process showed a close relationship between the Pleurobranchoidea and nudibranchs, and tentatively supported the clade Pleuroanthobranchea (Figs. 19A, 19B).

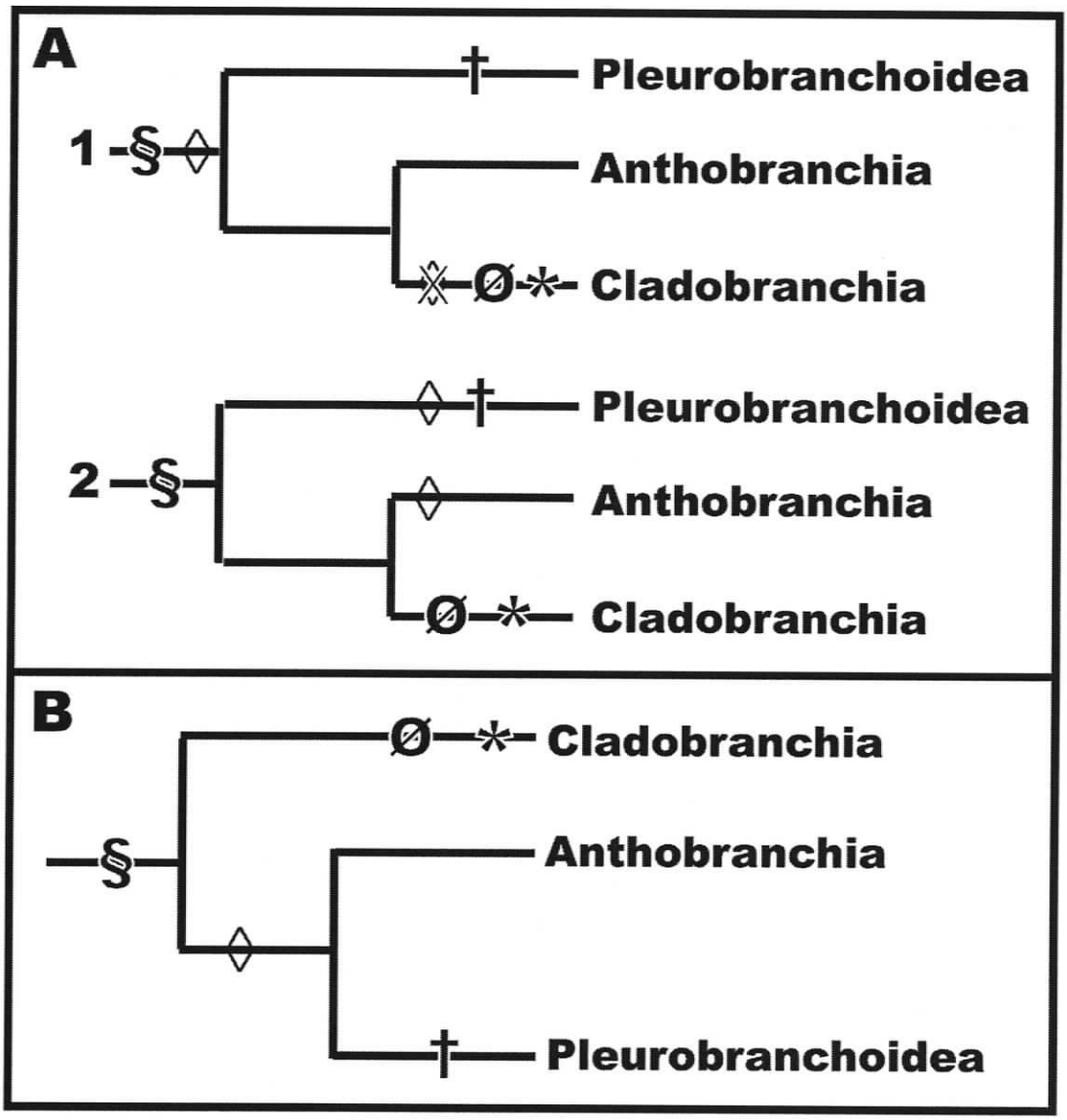

Figure 19. Competing hypotheses of pleurobranchoidean classification.

A: Cladogram based on adult morphology with the Pleurobranchoidea as sister to the Nudibranchia (Wägele and Willan, 2000) showing 2 scenarios of larval characters: Scenario 1 shows loss of mantle formation via reflexion (◊) in the Cladobranchia, while in scenario 2 that trait must evolve twice. **B:** Cladogram based on mitochondrial gene data (Grande et al., 2004a) in which the distribution of larval characters is more parsimonious. § indicates dissolution of internal shell whorls, ◊ indicates formation of the notum by reflexion of mantle fold tissue, * indicates the presence of 5 ampullary neurons in the ASO, † indicates the absence of opercula, Ø indicates the loss of osphradia in all life history stages.

The larval characters identified in the current study and used to support the hypothesis developed from molecular data require further investigation before the hypothesis can be robustly supported. The protoconchs of more species of heterobranchs should be examined to confirm that internal shell whorl dissolution occurs in all nudibranchs and pleurobranchoideans, and to confirm that dissolution does not occur in groups outside of the Nudipleura. Additionally, more sampling of ASO structure across the Heterobranchia is necessary to confirm whether or not the presence of four and five ampullary neurons is consistent throughout the Pleuroanthobranchea and Cladobranchia respectively, because this character may be variable even among species of the same genus in other heterobranch groups such as in the cephalaspidean genus *Haminoea* (Schaefer and Ruthensteiner, 2001). Thus, the number of ampullary neurons in the ASO is tentatively included here as a potentially phylogenetically informative character despite the need for further sampling because among the cladobranch and pleuroanthobranch species studied to date, no within-group variation in this character was observed.

I undertook this study because of the lack of information on the development of pleurobranchoideans and the discrepancies between phylogenetic hypotheses for pleurobranchoidean-nudibranch relationships based on molecular and morphological data. Since larval characteristics had not previously been incorporated into morphological data sets for phylogenetic analysis in this group, I examined larvae to identify characters that could be used to support any of the existing phylogenetic hypotheses. The five characters highlighted in Figure 19 represent an important first step towards using larval morphology for cladistic analysis in nudipleurans, and should be applicable across the entire heterobranch clade. The set of larval characters identified in this thesis may have

phylogenetic significance and prove useful for phylogenetic reconstruction, and thus further investigation into the states of these characters in larvae of other species of heterobranchs is warranted.

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