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Evolution of Shell Loss in Opisthobranch Gastropods: Sea Hares (Opisthobranchia, Anaspidea) as a Model System

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Quantitative and Systems Biology

by

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To my Father and Mother

Nou Vue and Chong Xiong

I'm not always the best with words, but I would like you to know that I am thankful for everything that you have done for me.

To my Family

Neng Vue, Kia Vue, Teng Vue, Kao Vue, Lee Jay Vue,

Tue Ker Vue, Mein Vue, Mone Vue, MouaJeng Vue

Thanks for the various support from everyone. I would not have been able to do it without you guys.

To Larry Vang

Thanks for catching me every time I fell.

To Monica Medina

with love

Thank you for the great opportunity to work with you.

I'll always have a special place for you in my heart.

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Evolution of Shell Loss in Opisthobranch Gastropods: Sea Hares (Opisthobranchia, Anaspidea) as a Model System

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SHELL DEVELOPMENT IN SEA HARES: COMPARATIVE ANALYSIS OF EARLY ONTOGENY IN *BURSATELLA LEACHII* AND *APLYSIA CALIFORNICA*

Due to the advantages of the hard, calcifying shell, the Mollusca are one of the most successful animal phyla. The shell forms during embryonic and larval

development; however, many molluscan groups have a highly reduced shell or have lost it completely as development and maturation proceeds. These major developmental transitions in shell morphology frequently correlate with ecological transitions (e.g. diet change/change from planktonic to bethic existence pre- and post-metamorphosis, respectively). While shell loss may leave an organism vulnerable to predation, many have evolved alternative means to deter predators. Here we compare and contrast the post-hatching larval development and shell growth through the use of the life cycle staging of *Bursatella leachii* and *Aplysia californica* in laboratory settings. The larval developmental sequence of *B. leachii* is indistinguishable to other previously described plankotrophic aplysiids. However, the growth rate and size of *B. leachii* larvae differ from *A. californica* larvae substantially, growing relatively faster and larger by an average of 10 µm. We also describe the life cycle of *B. leachii* in context of the development of the larval shell and its subsequent loss in post-metamorphic stages. Comparison of the Stage 6 shells, both whole and cross-sections, of *A. californica* and *B. leachii* through the use of SEM showed little difference in morphology. These data indicate that we have established a reliable culturing technique for *B. leachii* in the laboratory which makes this species can be easily amendable to experimentation at all developmental stages. Metamorphosis and shell loss/reduction in *A. californica* and *B. leachii* highlight the differences of the developmental program of both species, which reflects its complexity at a molecular, cellular and organismal level. The comparison of sea hares is an ideal evolutionary comparative model system for the loss of acquired features.

CHARACTERIZATION OF MANTLE SECRETING GENES THROUGHOUT LARVAL DEVELOPMENT IN *APLYSIA CALIFORNICA*

Molluscan biomineralization has been of broad scientific interest ranging from paleontological (molluscan shells provide one of the best fossil records for a metazoan phylum), to material science (perl and nacre formation) research. Although the properties (i.e. evolutionary origins, construction, patterning, physical) of the molluscan shell have been studied for decades, the underlying molecular and cellular mechanisms of how shell formation occurs are just recently surfacing with the identification of a handful of shell forming proteins. It is now known that one of the main components involved in the control of shell synthesis are the proteinaceous constituents of the shell matrix with in different kinds of functions (i.e. cell signaling, enzymatic activities), which are contributing to the diversity of different shell types in gastropod, bivalve and scaphopod molluscs. However, the differential gene expression and regulation within the mantle still remains unknown. Here we relate the developmental expression of eleven genes present in the mantle, the organ responsible for the secretion of the shell, in the sea hare *Aplysia californica* (Opisthobranchia, Anaspidea). Six genes that show very little changes in expression levels (Cluster 1). Three genes shows increased levels of expression during trochophore and veliger stages which then decrease in metamorphic stages (Cluster 3). Two genes had peptide-like profiles, genes that low expression during early development but have high expression levels late in development (Cluster 4). All eleven genes display dynamic spatial and temporal expression profiles within the larval shell field and mantle for the construction of the larval shell. The expression data from these eleven genes reflect the regulatory complexity that underlies the molluscan shell construction during larval stages. While the fabrication of the shell is taking place, the incorporation of both ancient and novel genes during also suggest

that there is a core set of mantle-secreting genes for shell construction was provided by a shared metazoan ancestor to produce the range of molluscan shell types we see today.

INTRODUCTION

Homology and Homoplasy

In comparative studies of all different types of two or more biological units (i.e. genes, cells, tissues, organs, structures, behavior or individuals) homology is the fundamental foundation of all biology. The popularity of homology among many disciplines has resulted in a large scientific debate regarding compiled definition of homology, currently a compiled list of 19 definitions are available [1-3]. In spite of this, homology can be referred to "as the continuous occurrence of the same feature in two organisms whose common ancestor also possessed the feature" [4]. However, clarifying the classification of common ancestry may not always be an easy task due the appearance of similar features as a result of independent evolution, a phenomenon also known as homoplasy [5]. Homoplasy has been categorized into three separate classes: convergence, parallelism and reversals/atavisms, rudiments/vestiges. Hall [6] concluded that features of parallel descent would inhibit similar developmental pathways, convergent features will use divergent or different pathways and reversal etc. will retain and use ancestral developmental pathways, which contradicts previous beliefs of homoplasy. These observations lead to theoretical questions that dealt with a consensus definition for homology and homoplasy.

Shell loss in Sea Hares (Opisthobranchia, Anaspidea) as a case study

The Mollusca are particularly well-known for their great diversity in shell morphology and are one of the most successful animal phyla in exploiting the advantages of the hard, calcified shell, an innovation that brought about protection and support to the organism [7]. Yet there are several molluscan groups, such as

ethyneuran gastropods (i.e. marine and terrestrial slugs), either have a highly reduced shell or have lost it completely [8]. In particular within the sea slugs (Euthyneura: Opisthobranchia), shell reduction/loss has occurred remarkably often in members of the Cephalaspidea, Anaspidea, Sacoglossa, Acochilidiacea, Nudibranchia, and Pleurobranchia among others [9]. These events support the notion that shell reduction/loss is not an isolated event and instead has evolved independently many times through parallel evolution [10, 11].

Within the Anaspidea (sea hares), a relatively small opisthobranch order, shell reduction/loss has occurred at least twice. The order Anaspidea is best known for the work on *Aplysia californica* as a model system for the study of the cellular basis of behaviour [12]. Although shell loss may seem like a disadvantage for these creatures, it is quite the contrary. Without a shell, they have evolved a variety of alternatives to deter predators, suggesting defensive strategies [9]. We hope to broaden the use of this model organism into the study of evolution of shell loss in opisthobranchs. *Bursatella leachii*¸ the ragged sea hare, exhibits some developmental and ecological differences relative to *A. californica* providing a good comparative system.

In particular, the mechanisms of shell development in these two species are similar in early embryonic stages but diverge as juvenile development takes place. Both species undergo 2 distinct periods of shell growth separated by cessation during the metamorphic process. The first growth occurs during the planktotrophic larval phase, where the shell grows spirally, and second just post metamorphosis at stage 10, when the shell changes from a spiral to a planar shell. *A. californica* has an internalized shell in adulthood, whereas *B. leachii* loses its shell soon after metamorphosis, after undergoing post-metamorphic shell growth [13].

We want to describe the most apparent differences in the developmental program of *A. californica* and *B. leachii*, with emphasis on metamorphic stages and

shell reduction/loss respectively. We aim to develop these sea hares into an evolutionary comparative model system for the loss of acquired features. To better understand the underlying molecular and cellular mechanism of shell reduction/loss, we have chosen to investigate with *in situ* hybridization expression patterns of a number of biomineralization candidate genes that were identified in a recent screen in Heyland et al. [14] from *A. californica*.

SHELL DEVELOPMENT IN SEA HARES: COMPARATIVE ANALYSIS OF EARLY ONTOGENY IN *BURSATELLA LEACHII* AND *APLYSIA CALIFORNICA*

INTRODUCTION

The “explosion” of hard shelled animals in the fossil record approximately 500 million years ago clearly suggests that an exoskeletal structure was an important innovation during animal evolution that brought about adaptive advantages such as protection, support and cellular processes [7]. The Mollusca is one of the most successful animal phyla in exploiting the advantages of the hard, calcified shell. Yet there are several molluscan groups that either have a highly reduced shell or have lost it completely [8]. For instance, shell reduction is found in mobile organisms like cephalopods [9]. Shell reduction/loss has also occurred in euthyneuran gastropods (i.e. marine and terrestrial slugs). In particular within the sea slugs (Euthyneura: Opisthobranchia), shell reduction/loss has occurred remarkably often in members of the Cephalaspidea, Anaspidea, Sacoglossa, Acochilidiacea, Nudibranchia, and Pleurobranchia among others [9]. These events support the notion that shell reduction/loss is not an isolated event and instead has evolved independently many times through parallel evolution [10, 11]. Having a slug-like form may be well-suited for a borrowing or swimming lifestyle, which is necessary for streamlining and reducing the weight of the organism (in pelagic forms). Some sea slugs have the advantage of harbouring entire colonies of symbiotic algae (zooxanthellae) or absorbing the chloroplast by eating the algae. Instead of covering the body with a shell, exposing the body to sunlight allows them to utilize photosynthetic energy [15].

Although shell loss may leave sea slugs vulnerable to predation, many have evolved alternative means to deter predators. Toxin secretion (may it be through natural metabolites or sequestered from the diet) [16, 17], mimicking unpalatable

species [18], becoming well-camouflaged [19, 20], being nocturnal and hide in crevices during the day (which would be harder if a shell was present) [21], swimming rapidly to escape danger [22, 23] and being able to absorb and incorporate defences from other organisms (i.e. nematocyst) from their diet into their own defences [24, 25], have helped many molluscs gain protection. In some species of sea slugs, since the mantle cavity and the original gills (ctenidia) that housed it were lost with the shell, they have evolved secondary gills as outgrowths from the skin [26].

Within the Anaspidea (sea hares), a relatively small opisthobranch order, shell reduction/loss has occurred at least twice (Figure 1). The order Anaspidea is best known for the work on *Aplysia californica* as a model system for the study of the cellular basis of behaviour [12]. We hope to broaden the use of this model organism into the study of evolution of shell loss in opisthobranchs. The genus *Aplysia* has been well described in major reviews [12, 27, 28]). *Bursatella leachii*¸ the ragged sea hare, exhibits some developmental and ecological differences relative to *A. californica* providing a good comparative system. In particular, the mechanisms of shell development in these two species are similar in early embryonic stages but diverge as juvenile development takes place. Both species undergo 2 distinct periods of shell growth separated by cessation during the metamorphic process. The first growth occurs during the planktotrophic larval phase, where the shell grows spirally, and second just post metamorphosis at stage 10, when the shell changes from a spiral to a planar shell. *A. californica* has an internalized shell in adulthood, whereas *B. leachii* loses its shell soon after metamorphosis, after undergoing post-metamorphic shell growth [13].

To date, the phylogeny of the Anaspidea is still partly unresolved (Figure 1), although the monophyly of the group is well supported by several morphological synapomorphies (i.e. reproductive system, defensive glands, radula, gizzard and

nervous system) [29-32], as well as molecular phylogenies [29, 33-35]. Some authors have presented evidence in support of Cephalaspidea as the sister group to sea hares, [9, 30, 35-37], while others have suggested that the most likely sister group is the pelagic pteropods (orders Thecosomata and Gymnosomata) [29, 38, 39]). A consensus anaspidean phylogeny is presented in Figure 1 based on published data [29, 34]. Although *A. californica* and *B. leachii* are closely related species, their natural lifestyles and behaviours are different. While *A. californica* is found in temperate waters, *B. leachii* is confined to tropical waters [27, 40]. *B. leachii* tends to stay in shallow subtidal waters and avoids intertidal habitats, whereas *A. californica* shows the opposite distribution [27, 40, 41]. With respect to their diet, *A. californica* is usually found grazing on red algae, primarily of the genus *Laurencia* [41], while *B. leachii* feeds on a microalgal layer, usually unicellular blue-green or cyanobacterial in origin, that covers a sandy substrate [13, 40]. This algal mat extends shoreward but never extends to the intertidal zone, which is likely linked to the distribution of *B. leachii* [40].

A. californica is one of a few invertebrate species with long-lived planktotrophic larvae yet it can be successfully cultured in the lab [27, 42]. Today, after optimized short generation times and life cycle inducers, a large number of *A. californica* can be grown in the laboratory under controlled hatchery conditions. High fecundity and quick growth provide abundant experimental stock of multiple life stages [43]. With the success of *A. californica* cultures year-round, having additional hatchery populations of other anaspidean species is now an attainable goal. In this study we aim to better characterize the *B. leachii* life cycle and the optimal culture conditions for this species. In addition, we want to describe the most apparent differences in the developmental program of *A. californica* and *B. leachii*, with emphasis on metamorphic stages and shell reduction/loss respectively. We aim to

develop these sea hares into an evolutionary comparative model system for the loss of acquired features.

METHODS AND MATERIALS

Broodstock and eggs/larval rearing

Aplysia californica adults were collected by staff from Santa Barbara Marine Biologicals, and *Bursatella leachii* adults were collected by biologists at the University of Miami's Rosenstiel School of Marine and Atmospheric Science (RSMAS) during the summer along the coast of Key Biscayne, Florida. All organisms were housed in the flow-through seawater system at the National *Aplysia* Resource Facility as described by Capo et al. [43, 44]. Eggs and larvae rearing conditions are described by Capo et al. [36]. Weekly shell length (SL) of 25 larvae were measured and the larval stage for both *A. californica* and *B. leachii* were determined through Kriegstein's staging scheme for *A. californica* [42]. Larvae were placed in filtered seawater (0.22 µm) containing 340mM of magnesium chloride. Once animals were narcotized, photographs were taken with an Olympus BX51 microscope or a Leica MZ16F stereoscope.

Scanning Electron Microscopy

Intact and broken pieces of shells from Stage 6 larvae of *B. leachii* and *A. californica* were collected from the National *Aplysia* Resource Facility [44] and placed in 100 percent ethanol and stored at 4˚C. Soft tissues were removed and the shells were cleaned with 1% NaOCl for 1 hour. Shells were placed in 100% acetone for 10 minutes, rinsed twice, placed in 100% ethanol, and placed onto filter paper to dry. Shells were placed on a stub and coated in gold with a Polaron SEM sputter-coater (model #E5000). Samples were viewed and photographs were taken on a FEI Quanta 200 ESEM (Hillsboro, OR), an environmental scanning electron microscope with a tungsten filament, under full vacuum with an accelerating voltage of 10kV and

a spot size of 4.0 (Figure 4A, Figure 4D); voltage of 12.5 and a spot size of 3.0 (Figure 4B); voltage of 10.0 and a spot size of 3.0 (Figure 4C).

RESULTS

Post-hatching larval development and shell growth

The embryonic development of *B. leachii* is similar to that described by Bebbington [45] and Paige [13] and will not be further discussed here. The incubation temperature of the egg masses was 25˚C. The life cycle staging of *Bursatella leachii* mentioned here is equivalent to the staging scheme that was described for *Aplysia californica* by Kriegstein [42] and cultured at the UM/National Resource for *Aplysia* . Stage 1 (which is determined by a newly hatched veliger containing a Type 1 shell [46] *B. leachii* larvae have a maximum shell diameter of 141.1 ± 6.9 µm (N=25). The veliger's shell grows rapidly -- an average of 21 µm per day (Fig. 1). Stage 2, defined by the appearance of the eyes, is reached within 4 days post-hatching. By day 5, the shell length is 264.6 ± 13.9 µm (N=25) with the presence of 1.5 whorls. After 6 days post-hatching, the larval heart appears (Stage 3).* By day 7, the maximum shell size (Stage 4) is reached at 284.2 ± 19.0 µm (N=25). Simultaneously on day 7, the foot expands to form a well-developed propodium (Stage 5). On day 9, the larvae settle and reach competency (Stage 6) when exposed to a substratum. A morphological pigmented spot on the shell, similar to *A. californica* [42], is also present in *B. leachii*. Pigmentation is a clear indicator of competency to metamorphose, and can be reached as early as 9 days post-hatching.

Metamorphic larvae development of *Bursatella leachii*

Metamorphic development and post-larval development of *Bursatella leachii* is similar to other previously described aplysiids [13, 42, 47, 48] (Fig. 2). At Stage 5, the propodium forms, an essential structure needed for settlement and crawling after settlement. At Stage 6 (Fig. 3A), metamorphic competence occurs, along with the

appearance of other morphological traits, such as a pigmented spot on the right side of the perivisceral membrane underneath the shell [42]. Once the larva has settled, in the presence of an environmental cue [13, 49], it will attach itself permanently and shed its velar lobes (Stage 7) (Fig 3B). The metamorphic transition occurs when the two halves of the velar lobe rudiments fuse together and the larval heart stops beating, which is also an indicator of Stage 8. The post-metamorphic shell growth in *A. californica* and *B. leachii* (Stage 9) are characterized by an elongation of the larval shell (Fig 3C). Stage 10 is reached when the shell reaches its maximum size and flattens prior to being discarded (Fig3D). The shell is discarded at Stage 11, when the juvenile begins to show adult characteristics. Fig 3E shows a late Stage 11 juvenile, approximately 2 mm long, with a discarded shell. The juvenile takes on adult characteristics, such as the appearance of small bumps all over the body and rudiments of the fleshy villae. The rhinophores are already elongated and tubular and the oral tentacles are expanding laterally. The body is pigmented with large, white granular patches. At Stage 12, *Bursatella leachii* (Fig 3F) is approximately 8 mm long. The villae cover the entire body, will multiply and become branched later in adulthood.

Scanning electron microscopy

Whole shells of Stage 6 veligers of *A. californica* (Figure 4A) and *B. leachii* (Figure 4C) have several smooth surfaces on the curved external area of the shell with a few small visible knobs near the whorl. Extending from the whorl are conspicuous growth lines that are present throughout the shell, following the pattern of the shell, in both species. Cross sections of *A. californica* (Figure 4B) and *B. leachii* (Figure 4D) show three layers: an outer layer (periostracum), an inner layer, and a middle layer.

DISCUSSION

Due to the well-characterized life cycle of *Aplysia californica*, the life cycle of *Bursatella leachii* was relatively easily to characterize. Having access to the complete life cycle of a second anaspidean species enables us to carry out experimental comparative developmental studies within the sea hare clade. In the present study we describe the life cycle of *B. leachii* in the context of the development of the larval shell and its subsequent loss in the post-metamorphic stages.

Bursatella leachii development

The larval developmental sequence of *B. leachii* is indistinguishable from previously described planktotrophic aplysiids [47] -- a hatched veliger with a sinistral coiled shell, a reddish tint, and bilobed velum. *B. leachii* larvae differ both in size and growth rate relative to *A. californica*, being larger in average by 10 µm and faster growing, though the larval development that follows the staging sequence devised by Kriegstein (1977;[13]). Initial stages of post-metamorphic development of aplysiids with a planktonic larval form are also similar [47]. Table 1 summarizes the larval development of *B. leachii* [13] relative to *A. californica* [42, 43]. Recent advances in larval culture techniques provide the tools for life cycle comparisons. The need for readily available developmental stages is important for experimental approaches in developmental biology studies such as metamorphic transitions. In the particular case of sea hares, hatchery populations provide an ideal supply of samples for the study of larval shell loss at the organismal and molecular levels.

Differences after metamorphosis occurs at Day 40 during Stage 9 when *A. califonica* juveniles acquire pink pigmentation due to the red algal diet, while *B. leachii* juveniles become white with dark bands on the head [13]. Despite this post-metamorphic physical difference, their developmental programs remain highly similar to each other up until this point. A major difference in *B. leachii* post-metamorphic

development happens at Stage 11 when the shell is discarded. At this stage in *A. californica*, the shell becomes internalized. Given that both species follow a similar developmental program through metamorphosis, it seems quite plausible that the underlying mechanism of larval shell formation is also quite similar, only differing post-metamorphosis/settlement. Now that we have more clearly established that larval shell formation is homologous in these two species, it will be feasible to characterize this process at the molecular level, examining the spatial and temporal gene expression of genes involved in the formation of the shell in these species in comparison to fully shelled gastropods.

We have established a reliable culturing technique for *B. leachii* that makes this species amenable to experimentation at all developmental stages [43]. Additionally, with access to microarray gene expression data [14] and whole mount *in situ* hybridization protocols recently established for *A. californica* [14, 50], this goal of experimentation is more attainable. Finally, with the sequencing of the *Aplysia californica* genome near completion, we can likely gain more insight into gene regulatory networks of different developmental processes such as biomineralization not only in sea hares but molluscs as a whole.

Larval shell morphology

SEM of larval shells from both species did not reveal major differences. Given the small size of the shells, however, there was limited resolution to resolve mineral composition on cross-sections. The shells of *A. californica* and *B. leachii* Stage 6 larvae are morphologically similar; with three distinct layers: An outer (periostracum) layer; an inner layer; with a clear section in-between where there are multiple layers of minerals building on top of each other. The physical characteristics of *Aplysia punctata* and *A. fasciata* larval shell that were described by Jaklin et al. [51] through

SEM are also consistent with the larval shell characteristics from *A. californica* and *B. leachii*.

Previous studies [52] have shown that layers of calcium carbonate are separated by organic layers in the adult shell. The shell of *Aplysia punctata* was described by Tonejc et al. [53] and showed that aragonite minerals are the first to be deposited in the pre-hatching, developing larvae [51]. This observation indicates that biochemical pathways that structure the shell appear to be the same. However, the shell in *A. californica* gets internalized after metamorphosis while in *B. leachii,* the shell is lost after metamorphosis. It could be possible that genes involved in the biomineralization process are either turned off in post-metamorphic *B. leachii*, or are co-opted for other developmental pathways.

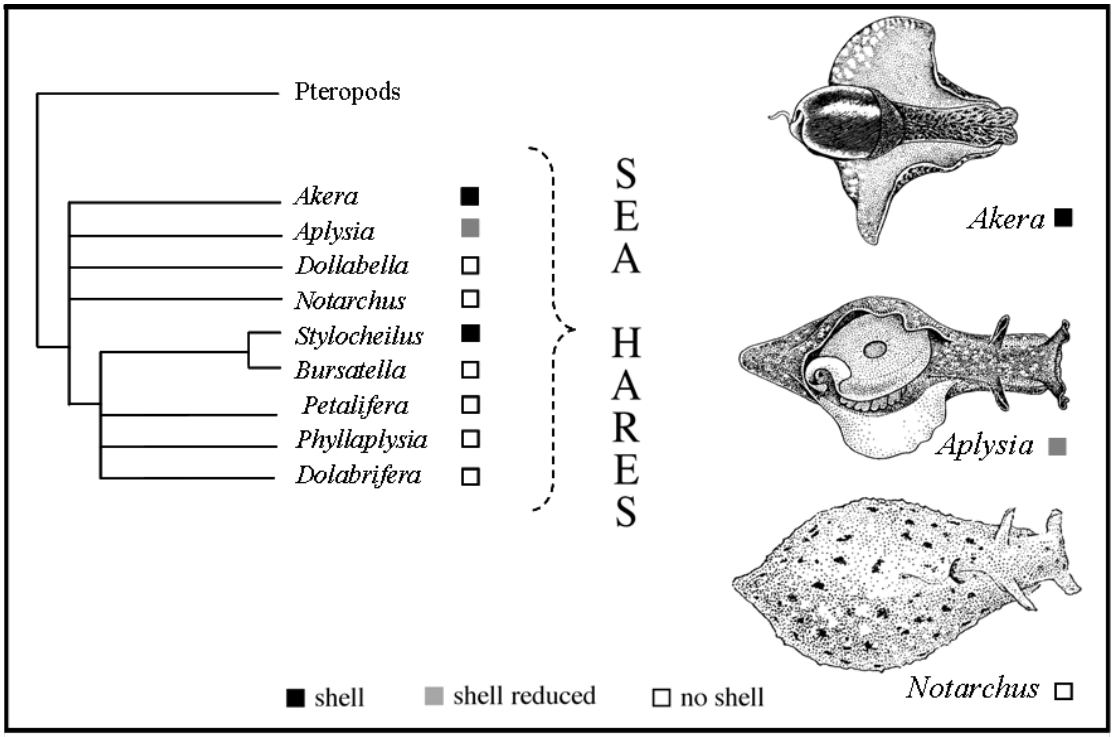

Figure 1. **Phylogenetic trees depicting gastropod relationships.** Consensus phylogeny of sea hares (Anaspidea) based on published partial mitochondrial rDNA data of Medina and Walsh (2002) and Klussmann-Kolb and Dinapoli (2006). Shell character states are depicted by boxes on terminal nodes

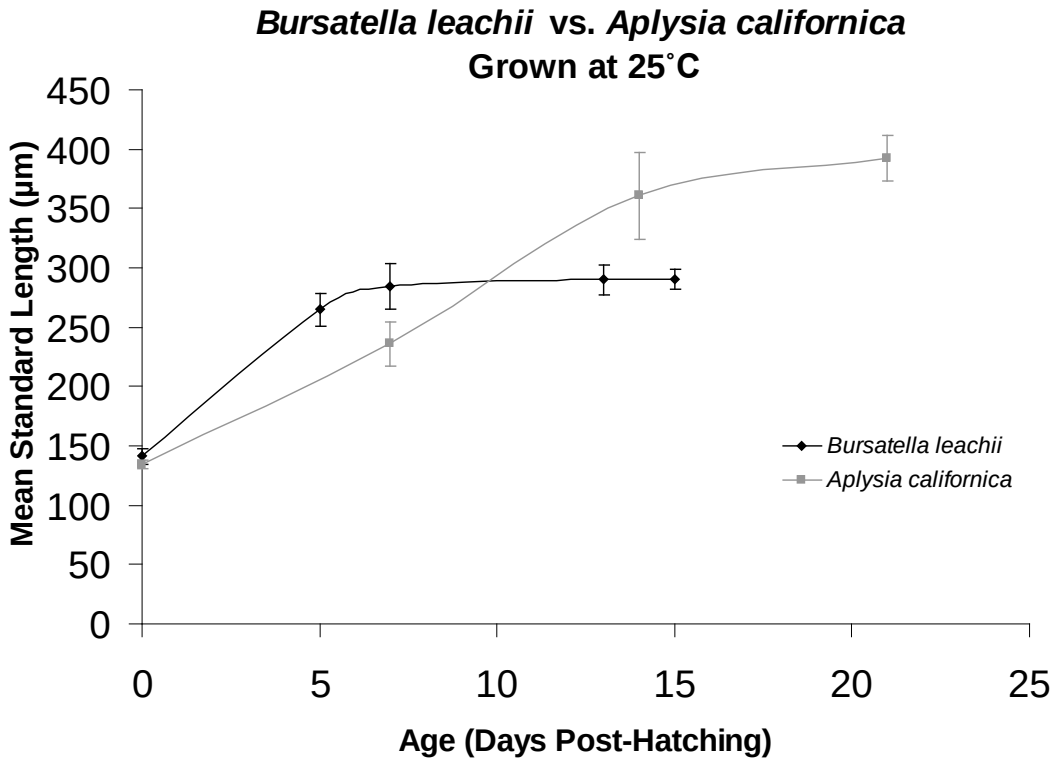

Figure 2. **Larval and juvenile growth of *Bursatella leachii* and *Aplysia californica* in laboratory settings.** Veliger shell length trajectories of *A. californica* and *B. leachii* larvae were grown at 25˚C. Shell length was measured weekly from day of hatching until 80% competency, error bars represent ± 1 standard deviation.

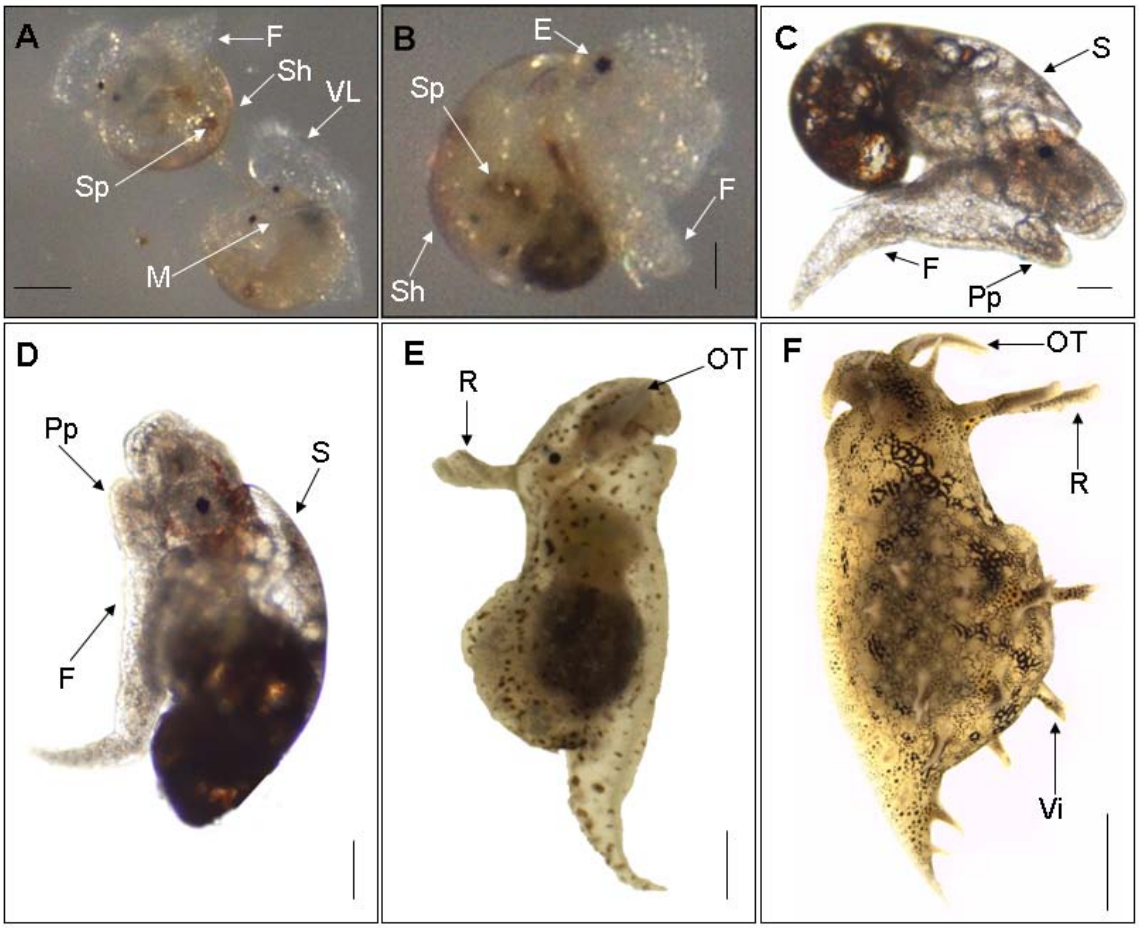

Figure 3. **Metamorphic development of *Bursatella leachii*.** Metamorphic competence of the veliger larvae (stage 6, A) correlates with many morphological characteristics (i.e.: red spots, propodium, etc.). Once settled, the larvae will attach and shed their velum lobes, becoming benthic (stage 7, B). Stage 8 (not shown) marks the end of metamorphosis, characterized by the fusion of the two halves of the velum lobe and the loss of the larval heart beat. Stages 9 – 10 marks the development of specific morphological structures of juveniles, such as the elongation of the juvenile or post metamorphic shell (stage 9, C; stage 10, D). Adult characteristics, such as the complete shedding of the shell, rhinophores, villae and oral tentacles, will start to appear in late stage 11 (E) and the adult (F). VL: Velar Lobe, Sh: Shell, Sp: Spot; M: Mouth; F: Foot; E: Eye, Pp: Propodium; R: Rhinophores; OT: Oral Tentacles; Vi: Villae. Scale bar in A: 100 µm, in B: 67 µm, in C: 108 µm, in D: 134 µm, in E: 254 µm, in F: 1mm.

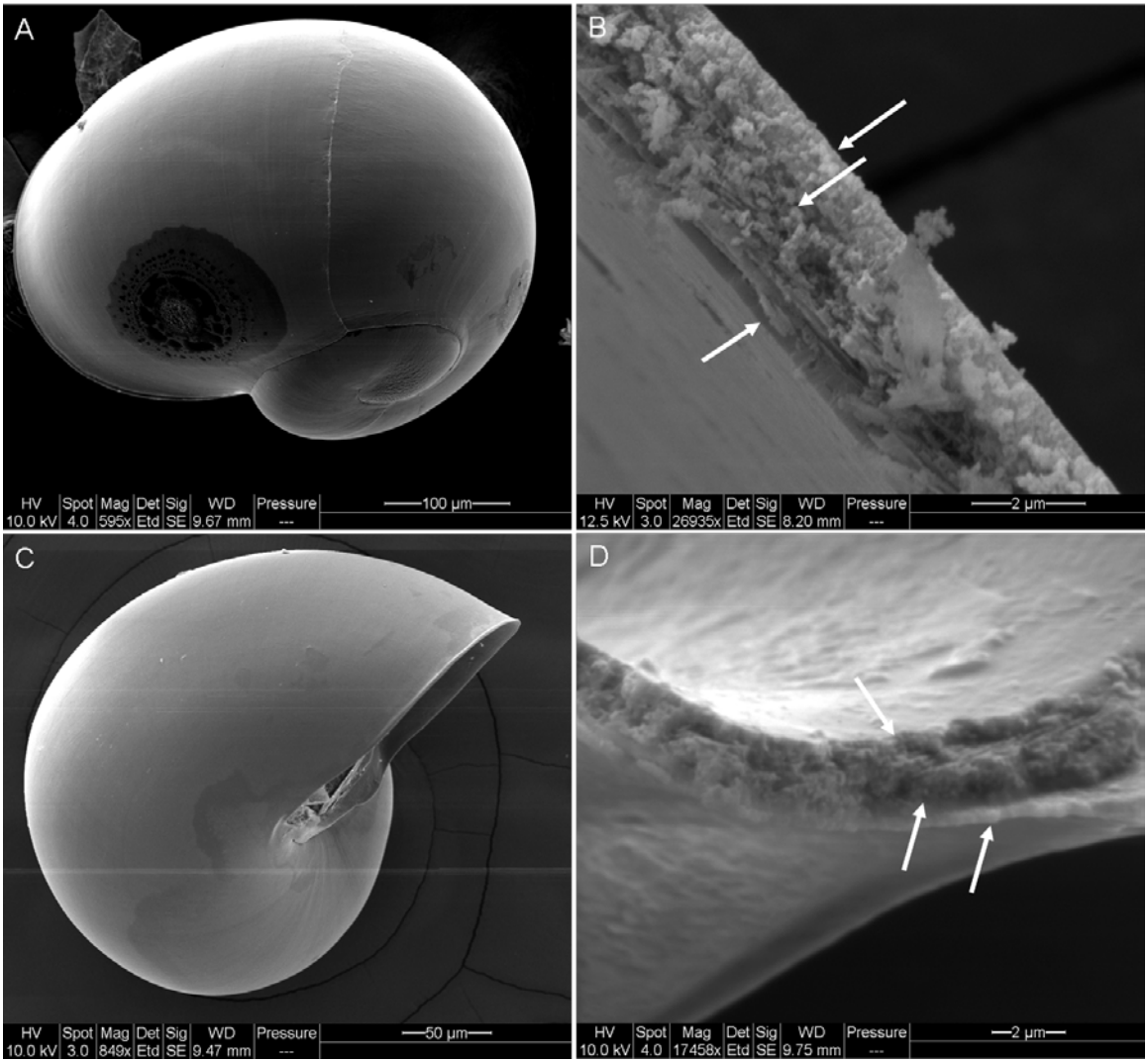

Figure 4. **SEM of *Aplysia californica* and *Bursatella leachii* larval shells.** Whole shell of Stage 6 veligers of *Aplysia californica* (Fig. 4A) and *Bursatella leachii* (Fig. 4C) and cross sections of *Aplysia californica* (Fig. 4B) and *Bursatella leachii* (Fig. 4D).

Table 1. **Comparison of developmental schedules of *Aplysia californica* and *Bursatella leachii.*** Comparison of morphological development schedules of *A. californica* larvae as reported by Kriegstein (1977) compared to Capo et al. (2009) and comparison of *B. leachii* larvae as reported by Paige (1989) compared to the present study. Values are the number of days post-hatching until the specified developmental stage was observed.

Stage	Description	Bursatella*	Bursatella□	Aplysia**	Aplysia***
2	Eyes	6	4	14	7
3	Larval Heart	12	6^	21	14^
4	Maximum Shell Size	15	7	28	17
5	Propodium	17	7	30	19
6	Competency	19	9	32	22
6	Red Spots	None	1 large spot	Present	Present
7	Metamorphosis	20	12	34	24

* Paige, 1988 [21]
**Kriegstein, 1977 [35]
***Capo et al., 2009 [36]
^ 50 beats/minute not taken into consideration
□Present Study

CHARACTERIZATION OF MANTLE SECRETING GENES THROUGHOUT LARVAL DEVELOPMENT IN *APLYSIA CALIFORNICA*

iNTRODUCTION

The wide variety and construction of mineralized structures, whether it may be a rigid skeleton or a nonskeletal mineralization [7], is a specialized function that persists in the majority of taxa in the metazoan world and have contributed to their evolutionary success, helping in a wide range of functions: tissues support, UV protection, shelter against predation, nutrition, reproduction, gravity, light or magnetic field perceptions, storage of mineral ions [54, 55]. Among these groups, calcium carbonate biomineralization is the most common form of crystalline discovered. The study of molluscan biomineralization, the formation of a rigid exoskeleton – the shell, has sparked the interest of many interdisciplinary fields, ranging from paleontology to material science [7] and is well known for their great diversity in shell morphology (Figure 1).

Although the evolutionary origins, construction, patterning and physical properties of the molluscan shell have been studied for decades, the underlying molecular and cellular mechanisms of how shell formation occurs are just recently surfacing [54]. The development of the shell is thought to be an organo-mineral construction, where the organic component, macromolecules such as proteins, glycoproteins, lipids and polysacharrides, only consists of less than 5% (by dry weight) [56, 57]. As quantitatively minor as this constituent may seem to be, it is the driving factor controls different aspects of the shell formation processes such as the synthesis of transient amorphous minerals and evolution to crystalline phases, the choice of the calcium carbonate polymorph (calcite vs aragonite), and organization of crystallites in complex shell textures (microstructures) [54, 58]. It is now known that the secretion of shell proteins along the mantle, the organ responsible for shell

formation, is the underlying factor that contributes to the diversity of different shell types in gastropod, bivalve and scaphopod molluscs [7]. However, the differential gene expression and regulation within the mantle still remains unknown.

The EST work of Jackson et al. (2006) on the abalone, *Haliotis asininia,* during shell calcification generated a significant amount of sequences that encoded secreting proteins and contributed to the discovery of the shell "secretome," giving us more insights into the complexity of the mantle transcriptome. Interestingly, 85% of the secreted proteins are unknown and only 19% of the *H. asinina* secreted proteins had homologues in the genome of the patellogastropod, *Lottia scutum*. One of the major conclusions of this study is that the specific structure and construction of the shell is due to the unique features of a rapidly evolving secretome [59].

Haliotis asinina is a member of the basal lineage of gastropods, traditionally known as "prosobranch," that are shelled organisms, with a few exceptions to some secondarily shell-less species (Figure 1). In general, Gastropods are an extremely diverse group of organism with a remarkable variety of diverse shell morphology. The more derived euthyneuran gastropods, the opisthobranchs (sea slugs) and the pulmonates (land snails), has independently exhibit mulitiple reversals (homoplasy) such as shell reduction, shell loss and torsion. Particularly in Opisthobranchs, many species of each lineages: Cephalaspidea, Anaspidea, Sacoglossa, Acochilidiacea, Nudibranchia, and Pleurobranchia have either a rudiment of a lost/reduced shell [9], supporting the concept that it has evolved multiple times independently [10, 11]. To better understand the underlying molecular and cellular mechanism of shell reduction/loss through differences in the expression and set of biomineralization genes, we have utilized *Aplysia californica* (Opisthobranchia, Anaspidea) to further study these processes.

At the embryonic or larval stages of *A. californica*, it forms a completely normal shell. Soon after metamorphosis, *A. californica* internalizes its shell and even

though it continues to calcify in the adult, the “original” purpose of a shell (such as protection) is no longer relevant, whereas in prosobranchs, the shell and its purpose are kept with the organism throughout its whole livelihood.

For this reason, we have chosen to investigate with *in situ* hybridization expression patterns of a number of biomineralization candidate genes that were identified in a recent screen in Heyland et al. [14] Our data show that the genes show specific patterns that are indicative of a function in biomineralization. Furthermore, many of the genes go through a phase of spatial refinement of expression where the expression of each gene is highly diffused in early development. However, as development progresses, the spatial expression of the gene becomes more localized and specific to certain parts of the organ(s).

METHOD AND MATERIALS

Animal preparation

Aplysia californica adults were collected by Santa Barbara Marine Biologicals and were housed in the flow-through seawater system the Resource as at described by Capo et al. [44] at the Rosenstiel School of Marine and Atmospheric Science (RSMAS). Eggs and larvae rearing conditions were set identical to conditions described by Capo et al. [60]. Early embryonic stages were anesthetized by placing larvae into purified seawater (0.22 μm) containing 3.5 percent magnesium chloride. The larvae were incubated overnight at 4˚C in an in situ fixative containing 4% Paraformaldehyde in 0.1M 3-(N-morpholino) propane sulfonic acid pH 7.5 (MOPS), 2mM MgSO4, 1mM ethyleneglycoltetraacetic acid EGTA and 0.5M sodium chloride NaCl. Larvae in egg cases were removed using fine forceps and scissors. Fixed samples were then rinsed twice with PBS buffer plus 0.1% Tween 20, washed three times with 100% ethanol and stored at -20°C in 70% ethanol.

In Situ Hybridization

The spatial expression of transcripts in *A. californica* larvae was determined through *in situ hybridization*. The in situ hybridization protocol, which was modified from ones previously published protocols [14, 61, 62], utilizes a non-radioactive chromogenic in situ hybridization protocol based on immunological detection using alkaline phosphatase-conjugated antibodies. We utilize digoxygenin (DIG)-labeled antisense RNA probes with the Perkin Elmers Flouroscein TSA Plus System as the substrate for detection of single mRNA species. DIG-labeled uridine triphosphate (UTP) is incorporated into the antisense RNA probe when it is synthesized. After hybridization of the probe to whole-mount embryos, the preparations are incubated with alkaline phosphatase-conjugated anti-DIG antibodies. Visualization for the substrate is a fluorescent staining.

All procedures described below should be done at room temperature unless stated otherwise. Agitation during any of the incubations or washes is optional, and is usually done only during antibody incubations and washes. Particular attention should be paid to maintaining an RNase-free environment. To help avoid contamination, solutions should be made in small batches in disposable sterile 50 ml plastic centrifuge tubes (Corning Incorporated, NY, USA; Cat. No. 430921), and all incubations are done in disposable sterile 96-well cell culture plates (Corning Incorporated, NY, USA; Cat. No. 3524). Best results are achieved by transferring embryos into new wells for each solution change. We have concluded from experience that all the solutions are stable for up to three months if stored at 4 °C with the exception of the hybridization and detection buffers, both of which should either be prepared fresh before the experiment or stored in aliquots at −20 °C. Formation of precipitates may be observed in some washing solutions with high salinity such as 5× saline sodium citrate buffer (SSC) when stored at 4 °C; however, the solutions may be heated to room temperature or the incubation temperature and precipitates will re-dissolve without any ill affects. Chemicals were obtained from Sigma unless otherwise indicated.

Probe preparation

Digoxygenin-labeled antisense RNA probes are transcribed in vitro using SP6 or T7 polymerases from full-length cDNA clones, ligated into pCRII-TOPO vector (Invitrogen) from a PCR reaction with specific M13 primers. Full-length sense probes are used for negative controls. Five microliter of the PCR reaction should be run on a 1% agarose gel with ethidium bromide staining to check for quality of the reaction, one microliter should be used on the nanodrop for the concentration and the remainder is purified using a PCR purification kit (Qiagen).

Typically, 7 µl of the purified PCR product (approximately, 1 µg of plasmid) is used as a template in the probe synthesis reaction. Probe synthesis is carried out

using the DIG RNA Labeling Kit (SP6/T7) (Roche; Cat. No. 1175025) according to the manufacturer's directions (7 µl template, 1 µl NTP labeling mix, 1 µl 10× transcription buffer, 1 µl RNase inhibitor, 1 µl SP6 or T7 RNA polymerase, 37 °C for 3.5 h). After 3.5 hours, one microliter is added to the reaction and incubated at 37 °C for 15 minutes. The reactions are stopped with the addition of 1.5 µl 12 M LiCl2 and 38 µl of 100% Ethanol and stored in -20 °C overnight for RNA precipitation. The reaction is rehydrated in water.

The NTP labeling mix contains either DIG-11-UTPs (DIG RNA Labeling Mix, Roche; Cat. No. 1277073) for synthesis of DIG-labeled probes. The quality of plasmids, restriction digests, and synthesized probes should be checked on a 1% agrose gel with ethidum bromide staining prior to use and all concentrations of can be accurately determined using an NanoDrop ND-1000 Spectrophotomerter (USA).

Larvae processing and probe hybridization

Fully entacted, fixed larvae are transferred to PBT (0.1% Tween 20, 1XPBS) for 10 min, placed in two 10 min incubations in ME (0.05M EGTA,pH 8, in Methanol) for shell permeablisation and dehydrated by sequential 5 min washes of 25, 50 and 75% PPE (4% Paraformaldehyde/1XPBS/0.05M EGTA) and 100 PPE for 20 min. Larvae are rehydrated by its placement in five sequential five minute washes of PBT on ice. Embryos are post-fixed in 4% formaldehyde in 0.025% PBT for 45 min at 4 °C to ensure the morpholgy of the larvae is still entact. Following post-fixation in 4% formaldehyde, the larvae are washed in five changes of PBT on ice.

Next, the larvae are transferred to hybridization buffer (HB: 50% formamide, 5 mM EDTA, 5× SSC (20× SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.0), 1× Denhardt solution (0.02% ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 0.1% Tween 20, 0.5 mg/ml yeast tRNA (GIBCO BRL)). Prehybridization incubation is then done immediately for 3 h at 65 °C. Next, 2–6 µl of each probe is added and hybridization allowed to proceed overnight for 12–14 h at 65 °C.

Immunological detection

Immunological detection is performed using anti-digoxygenin-AP Fab antibody fragments at a dilution of 1:1000 (Roche Diagnostics, Mannheim, Germany). After probe hybridization, the larvae are washed in the following solutions: 50% formamide/2× SSC/0.1% Tween 20 for 20 min at 65 °C, then 2× SSC/0.1% Tween 20 for 20 min at 65 °C, and then 0.2× SSC/0.1% Tween 20 at 65 °C for 30.

Larvae are transferred to three washes of TNT (0.1M TrisHCl/0.15M NaCl/0.1% Tween 20) for 10 min each and an additional wash for 1 h. The larvae are then placed in 5% goat serum in TNT (TNB) for 60 min. Afterwards, alkaline phosphatase-conjugated antibodies are then added and incubation allowed to proceed for 12–14 h at 4 °C.

Development using the Perkin Elmers Flourescein TSA Plus System

After incubation with antibody, the larvae are transferred to TNT and washed in five 5 min changes of TNT followed by one 1-hour wash of TSA Plus amplification diluent for equilibration of the tissue. Afterwards, a dilution of 1:100 of reconstituted fluorescent tyramide in the NEN plus amplification diluent is added for development.

Larvae are kept in the dark and monitored every 10–15 min for the development of staining. The developing ganglia should be viewed for only brief intervals to avoid excessive exposure to light. Development should be terminated after cell-specific labeling is clearly visible and before excessive background begins to appear.

Development is terminated by transferring the larvae to a single wash in TNT and two washes of PBT on ice for 5 min each. Larvae are dehydrated in a sequential wash of 30, 50, 70, 90 and 100% ethanol at 4 °C. Immediately following the final transfer to 100% ethanol, the larvae are transferred to Dapco 33-LV and is left to equilibrate overnight for 12–14 h at 4 °C. Samples are mounted on microscope slides in Dapco 33-LV.

Imaging

Images were acquired by using a Photometric CoolSnap EZ Turbo 1394 camera mounted onto a Nikon Eclipse TE2000-U confocal microscope.

RESULTS

Temporal and spatial expression of biomineralizing genes

We have isolated eleven biomineralization genes that are expressed in the mantle that are involved in the construction of the shell in *A. californica* during embryonic stages (Table 1). These genes were recruited from the set of biomineralization genes that were identified in Heyland et al. [14]. Overall, 196 potential biomineralization genes were identified that were mapped to four different clusters based on their expression in different developmental stages. For selection of the biomineralization genes in this study, we chose genes based on their putative function and their expression pattern through developmental time. The genes are separated into clusters, due to their expression patterns (Figure 3). Cluster 1 represents genes that show very little changes in expression levels, which contains genes CaM kinase II alpha [DA15], ribosomal protein rpl17 [DA21667], ribosomal protein rpl7a [DA22104], 40S ribosomal protein S15 [DA443], ribosomal protein rps30 [DA8011] and ribosomal protein S14 [DA834]. Cluster 2 represents genes that are highly (>20) up-regulated 60-hours post-metamorphosis. In this study, there are no genes that fall under this category. Cluster 3 shows increased levels of expression during trochophore and veliger stages which then decrease in metamorphic stages. There are three genes that assemble under Cluster 3 expression patterns, which are dermatopontin 2 [DA25779], calmodulin [DA36880] and CDGSH1 iron sulfur domain 1 [DA17224]. Cluster 4 shows genes that low expression during early development but has high expression levels late in development (i.e. peptide-like profiles); two genes in this study follows this expression pattern in this study, ferritin [DA20216] and an unknown, identified mantle-secreting gene with no annotation present at this time, but is thought to involved in biomineralization (ML8H1) [DA5095] (see Heyland et al. [14] for further information).

***Soma ferritin* [DA20216]**

Soma ferritin transcripts are highly diffused throughout the ectodermal cells throughout the trochophore embryo, including the shell field. (Figure 4A and Figure 4B). Expression within the early pre-hatching veligers has high expression within the mantle and multiple other cells throughout the larvae (Figure 4C and 4D). In the Stage 1 veliger, ferritin transcripts are present in the mantle, the kidney and the statocyst (Figure 4E and 4F).

***CDGSH iron sulfur domain 1* [DA17224]**

CDGSH iron sulfur domain 1 transcripts are diffused in the ectoderm tissue throughout the trochophore larvae (Figure 5A and 5B). Spatial expressions of these transcripts in the early pre-hatching veligers are present throughout the mantle tissue that is covering the digestive gland (at low concentrations) and the statocyst and pedal ganglia at high concentrations (Figure 5C and 5D). As development progresses, CDGSH1 iron sulfur domain 1 becomes concentrated at particular areas of the Stage 1 embryo: the mantle tissue underneath the shell (posterior) and a strip down the lateral sides of the shell, the velum lobes and the cerebral ganglion (Figure 5E and 5F).

***Ribosomal protein rpl7a* [DA22104]**

Transcripts of ribosomal protein rpl17a are diffused throughout the ectodermal cells of the trochophore larvae, where the shell field is present, the prototroch and the cells close towards the mouth (Figure 6A and 6B). During early pre-hatching veliger, there are very little transcripts present, only a small amount of expression in certain cells of the mantle and the statocyst organ (Figure 6C and 6D). . During Stage 1 veligers, ribosomal protein rpl7a transcripts are restricted within the mantle tissue at the

posterior edge and a small amount at certain cells at the dorsal mantle (Figure 6E and 6F).

Ribosomal protein rpl17 **[DA21667]**

Ribosomal protein rpl17 transcripts are diffused throughout the ectodermal cells during the trochophore larval stage (Figure 7A and 7B). At the early veliger stage (pre-hatching), there is specific expression of certain cells at the dorsal mantle, while there is strong expression in the kidney and statocyst and little expression in the velum lobes (Figure 7C and 7D). The Stage 1 veligers have some spatial expression at the dorsal mantle (at particular cells), the mantle tissue covering the stomach (ventral) and expression within the stomach itself and expression in the statocyst (Figure 7E and 7F).

Ribosomal protein rps30 **[DA8011]**

WM-FISH indicates that ribosomal protein rps30 is diffused and restricted to the ectodermal cells of the trochophore larvae, where shell gland and the growing shell will be present, but the expression never extends pass the prevelum and mouth (Figure 8A and 8B). Early pre-hatching veligers localized ribosomal protein rps30 at the statocyst, the velum lobes, and high expression in the kidney (Figure 8C and 8D). Ribosomal protein rps30 expression in the Stage 1 veliger is restricted to mantle tissue that is covering the hindgut at high concentration, the statocyst and the kidney (Figure 8E and 8F).

40s ribosomal protein S15 **[DA443]**

40s Ribosomal protein S15 transcripts were detected diffusely throughout the ectodermal cells in the trochophore embryo (Figure 9A and 9B). Expression in the early pre-hatching veliger is scattered in the mantle tissue laterally, but never

exceeds to the ventral end of the embryo. Expression is also present in the velum lobes and the statocyst, but at lower concentrations (Figure 9C and 9D). Stage 1 veligers show transcripts present near the subvelum, cells within the statocyst and the mantle tissue covering the stomach and expression within the stomach itself (Figure 9E and Figure 9F).

Ribosomal protein S14 **[DA834]**

Trochophores express ribosomal protein S14 transcripts throughout the ectodermal cells diffusely (Figure 10A and 10B). Spatial expressions in early veligers (pre-hatching) are present diffusely in the mantle, with some strong expression at certain mantle cells at the posterior, the velum lobes, the kidney and statocyst (Figure 10C and 10D). Stage 1 veligers expression is restricted in the mantle, only where the digestive gland is present, and in the ganglion (cerebral) (Figure 10E and 10F).

Unknown (ML8H1) **[DA5095]**

This is an unknown biomineralization gene. Unknown biomineralization gene transcripts are diffused throughout the ectodermal cells in the trochophore larvae (Figure 11A and 11B). Transcripts in the early pre-hatching veliger stages are present at high concentrations in particular cells in the dorsal and lateral mantle, the velum lobes and statocyst (Figure 11C and 11D). Unknown biomineralization gene transcripts are localized in the mantle tissue, only where the stomach is present (at high concentrations), the velum lobes, the statocyst and the cerebral ganglion at low concentrations (Figure 11E and 11F).

CaM kinase II alpha **[DA15]**

CaM kinase II alpha transcripts are localized in the ectoderm tissue of the trochophore larvae with relatively high expression at the posterior end of the embryo

(towards the mouth) and expression at the site of the growing shell gland (Figure 12A and 12B). Transcripts in the early pre-hatching veligers are diffused throughout the mantle at low expression with some specific cells at the dorsal, high expression in the statocyst and expression in the cerebral and pedal ganglions (Figure 12C and 12D). Transcripts in Stage 1 veligers were detected in the velum lobes (at low concentrations), in the cerebral ganglion and the mantle tissue covering the stomach only (Figure 12E and 12F).

***Calmodulin* [DA36880]**

Calmodulin transcripts in the trochophore were highly localized in the ectodermal tissue of the growing shell field and low expression near the mouth area (Figure 13A and 13B). Transcripts in the early pre-hatching veligers are proliferating in specific towards the dorsal and lateral parts of the mantle tissue, but never reaching to the ventral end of the embryo (Figure 13C and 13D). Stage 1 veligers reveal calmodulin transcripts to be present thoughout the mantle tissue at particular cells underneath the shell and other cells throughout the embryo, including at high concentrations in the statocyst (Figure 13E and 13F).

***Dermatopontin 2* [DA25779]**

Dermatopontin 2 was observed in the ectodermal cells, expressed diffusedly throughout the trochophore embryo and high concentration of dermatopontin2 transcripts at the growing shell field (Figure 14A and 14B). In early pre-hatching veligers, the transcripts are present throughout the thin mantle tissue surrounding the shell at low concentrations, at specific but high concentrations at the dorsal cells of the mantle, in the statocyst and low concentrations in the velum lobes (Figure 14C and 14D). In the Stage 1 veligers, dermatopontin2 transcripts are highly concentrated and expressed in the mantle tissue that is covering the hindgut, with

some small expression in the mantle tissue covering the stomach, in the statocyst and a faint expression in the velum lobes (Figure 14E and Figure 14F).

DISCUSSION

The larval shell of *Aplysia californica* is an ideal system to study the molecular and cellular events that occurs in shell construction and patterning. The spatial and temporal expression patterns of genes in development can offer important insights to address fundamental questions about how complex developmental programs functions, mechanisms linking phenotype with genotype.

The shell is generally composed of an outer organic layer, the periostracum, and inner calcified layers, primarily composed of calcium carbonate (in the crystallographic form of calcite and/or aragonite), embedded in an organic matrix [55, 63]. The shell lies outside the tissues and is secreted by many different regions of the outer mantle epithelium and other underlying calcium gland cells. The thickness of the shell highly varies due to the habitat [64, 65]. Once shells are formed, they are not immutable; parts of the shell can be modified and reworked in the interior and exterior surfaces of the shell [66].

Changes in shell structure and pattern coincides with *A. californica*'s environment

The life cycle of *Aplysia californica* includes a long pelagic veliger state with a quick transition to a benthic adult. *A. californica* undergo 2 distinct periods of shell growth separated by cessation during the metamorphic process. The first biomineralization event occurs during the planktotrophic larval phase; fabrication of the shell takes place inside the egg mass, 3-4 days after the egg mass is laid. Before the completion of gastrulation, the shell field appears as an enlargement of the cells in an area of the prospective dorsal side of the embryo, followed by a transitory invagination of the central area forming the shell gland. The shell gland will eventually evaginate and the shell field spreads becoming the mantle. This is the tissue that continues to secrete the shell in adult animals [67]. The pigmented

(usually brownish) shell grows spirally and allows the organism to retract into a protective environment [7]. As development proceeds, the shell will become stronger, bigger and pigmentation will become more evident. The presence of an environmental cue will induce metamorphosis, which will cause biomineralization of the larval shell to come to a halt. Metamorphic competence is accompanied with many morphological traits such as pigmentation of 6-8 red spot [42, 49, 68]. The second biomineralization event happens post-metamorphosis when the shell changes from a spiral to a planar shell. The shell will become internalized and continue to elongate throughout the adult life. Coincidentally, the internalization of the shell is also accompanied with the inking defence mechanism, chemicals/toxic products they produce to deter predators, which can be sequestered from a variety of secondary metabolites from their food [16, 17, 69].

Differential change of mantle genes reflect changes in shell structure

The spatial and temporal expression patterns in these eleven genes that we have investigated in this study have given us many insights into the complexities of constructing a shell. Several of these molluscan mantle-specific genes analyzed in this study may play a very important role in the process of shell formation (since we suspect that mantle specific genes are the main contributors in the physiology of calcification) as well as many other cellular processes in *Aplysia californica*. Although the expression of each gene is highly diffused in early developmental stages, as development proceeds and the shell becomes more less susceptible to breakage, there seems to be a spatial refinement of the genes, where the genes are focusing on only particular parts of the mantle (and other organs) to strengthen those particular parts of the shell.

The first set of genes falls into cluster 1, in that the transcripts show very little changes in expression levels. The genes in this study that follows this pattern of

expression are all the ribosomal genes {ribosomal protein rpl17 [DA21667]; ribosomal protein rpl7a [DA22104]; 40s ribosomal protein S15 [DA443]; ribosomal protein rps30 [DA8011]; ribosomal protein S14 [DA834] } and CaM kinase II alpha [DA15]. The expression of these genes are important in that they essential to the cell to maintains the cell's stability. Any modification to their expression can cause many pleiotropic effects on the organism that may be deleterious.

Although the structure of the ribosome has been well worked out for many decades, it has become apparent in recent studies that many ribosomal proteins have multifunctional roles and are involved in many processes as disparate as DNA repair and iron-binding [70]. As for the ribosomal genes in this study, they are all actively involved in protein biosynthesis as well as other cellular functions within the organism. Ribosomal protein rpl7a [DA22104] is directly involved in L13a-mediated translational silencing the expression of Ceruloplasmin (caeruloplasmin); the major copper-carrying protein in the blood and plays an additional role in iron metabolism (officially known as ferroxidase or iron(II):oxygen oxidoreductase). 40s ribosomal protein S15 [DA443] binds directly to 16S rRNA to help the nucleate assembly of the platform of the 30S subunit by binding and bridging several RNA helices of the 16S rRNA. Ribosomal protein rps30 [DA8011] has been found to be involved in growth, development, positive regulation of growth rate and translation within the cell. Cellular roles of ribosomal protein S14 [DA834] have been linked to erythrocyte differentiation, negative regulation of transcription from RNA polymerase II promoter and emetine resistance. The identification of a limited amount of ribosomal proteins in this study will help provide a basis for an in-depth analysis of their function and roles in biomineralization.

CaM kinase II alpha [DA15] (Ca^{2+}/calmodulin‐dependent protein kinase) is key regulator of plasticity in synaptic physiology and behaviour. Alterations of any kind in its activity can produce pleiotropic effects that include synaptic transmission

and development, as well as various aspects of behaviour [71]. In *A. californica*, expression of CaM kinase II alpha [DA15] transcripts is present in the statocyst organ and the cerebral and pedal ganglions (Figure 11C-F). In biomineralization, CaM kinase II alpha [DA15] has been shown to be involved in calcium metabolism during nacre/pearl formation in *Pinctada fucata* [72]. Since nacre/pearl formation does not occur within *A. californica* shell production and this gene is expressed in the mantle at the early pre-hatching veliger and Stage 1 veliger stages, we hypothesize that this gene that affects the growth of aragonite crystals.

Expression profiles of genes that fall under cluster 3 (genes that shows increased levels of expression during trochophore and veliger stages but decrease during metamorphic stages) include dermatopontin2 [DA25779], calmodulin [DA36880] and CDGSH1 iron-sulfur domain 1 [DA17224]. The differential regulations of these genes are important and required for the construction of the shell at this particular life cycle stage, the time period where the fabrication of the shell takes place (from trochophores larvae to Stage 1 veligers).

The dermatopontin gene encodes a shell matrix protein (associated with aragonite cross – lamellar crystallites) that was identified in the derived gastropod, *Biomphalaria glabrata* [73]. Since then, many homologues of the *B. glabrata* dermatopontin gene have been obtained in seven other freshwater and land snails through the use of degenerate primers based on the dermatopontin amino acid sequence, many being incomplete sequences [74]. The analysis of the transcript expression patterns showed that some dermatopontins are ubiquitous, whereas others are only expressed in the mantle tissue. However, it is hypothesized that the second category of dermatopontins may be real shell proteins, but this can only be validated at the protein level. In Sarashina et al. [74], they report that Pulmonate snails have two or three types of dermatopontins; however, only Type-1

dermatopontin (Derm1 or dermatopontin1) is considered to be a shell matrix protein [74, 75]. In *A. californica*, we were able to extract only one dermatopontin gene. Dermatopontin2 [DA25779] (Figure) is expressed at low concentrations throughout of the mantle in the outer edges during the pre-hatching veliger stage, while it is highly expressed in cells of the outer layer of the mantle in Stage 1 veligers. This goes to show that this gene may have a specialized role in the shell matrix, as well as other general functions. We hypothesize that this gene's presence in that particular area of the mantle is where aragonite cross – lamellar deposition may occur in *A. californica*. However, the precise function of these dermatopontin genes between Pulmonates and Opisthobranchs may well be different between each lineage.

Not only is dermatopontin involved in the proteinaceous extracellular matrix, it is also have roles in mediating adhesion by cell surface integrin binding and may serve as a communication link between the dermal fibroblast cell surface and its extracellular matrix environment. Its presence also enhances TGFB1 activity, inhibits cell proliferation and organizes collagen fibrils, by accelerating collagen fibril formation and stabilizing collagen fibrils against low-temperature dissociation.

Calmodulin is a well-known gene involved in the transport and secretion of calcium in biomineralization [76] and has been shown to mediate the control of a large number of enzymes and other proteins through the use of Ca^{2+}. The expression pattern of calmodulin in the *A. californica* trochophore larva seems to be involved in various roles, other than biomineralization, which is reflective of the well-studied mammalian systems [77, 78]. However, in the tropical abalone *Haliotis asininas* (Vetigagastropoda) [79] and bivalves (*Pinctada fucata*) [76, 80], calmodulin transcripts are not present in the mantle until the veliger larva attains competence. In juvenile animals of both organisms, calmodulin is expressed within the gills and mantle. In contrast, in *A. californica*, the expression calmodulin within the mantle

starts as early as a pre-hatching veliger and is present in the Stage 1 veliger. However, since *in situ* hybridizations were not done on competent veligers or juvenile *A. californica* organisms, we are unsure if calmodulin is present within the gills, which does not emerge until Stage 10 [42].

CDGSH1 iron-sulfur domain 1 (also known as mitoNEET) is an integral membrane protein located in the outer mitochondrial membrane and may be involved in transporting iron into the mitochondria [81]. Iron is required for many mitochondrial enzymes to function and iron-sulfur proteins are essential in vital processes that involve energy homeostasis and metabolism, even for the simplest organisms [82, 83]. Therefore, it would not be surprising that this protein may have many different roles other than biomineralization in the cell.

In this study, there are 2 genes, ferritin [DA20216] and an unknown, identified mantle-secreting gene that may be involved in biomineralization (ML8H1) [DA5095], are the only genes that show the same expression pattern as Cluster 4, which are genes that has peptide-like profiles (low expression during early development but high expression later in development). Ferritin is an evolutionarily conserved protein that is involved in cellular iron ion homeostatsis, oxidoreductase activity and oxidation reduction. In biomineralization, it is involved in iron incorporation into the shell. In this study, it is diffused throughout the trochophore larva, including the shell field. However, in early pre-hatching veligers and in Stage 1 veligers, it has high expression within the mantle, signifying that ferritin is most likely to be involved in intracellular events that are essential for shell deposition. These expression patterns are also similar to those found in other mollucan species, considering calcification processes [79, 84].

The unknown gene [DA5095] was identified as one a homologue to the mantle-secreting gene ML8H1 in the EST work on *Haliotis asininia* during shell

calcification, or the “secretome” [59]. According to the WM-FISH data that we have, it seems to have a role in constructing the shell due to its expression in the mantle in the veliger stages. However, there seems to be expression in other parts of the embryo, implying that there must be other roles that this gene must be involved in.

In this study, we did not have any biomineralization genes that fell under Cluster 2, which were genes that were highly (>20) up-regulated at 60-hours post-metamorphosis. Even from the 196 unique contiqs that were identified to be coding for potential biomineralization genes, only 3 genes fell under this category [14]. At this particular time period of development (Stage 9), there is no calcium carbonate biomineralization occuring due to the metamorphosis, where growth of the larval shell halts. The growth of the shell does not continue until Stage 10. However, biomineralization genes are present and highly up-regulated during metamorphosis, which is where the larval shell has come to a complete halt. This can be explained through anticipatory development. The presence of biomineralization proteins at Stage 6 helps set up the next developmetal stage, to help induce metamorphosis with the presence of an environmental cue [79, 85].

The expression patterns of these eleven genes give us some more insight into how biomineralization is controlled in *Aplysia californica*. However, it also highlights the complex regulatory mechanisms that underlies molluscan shell construction during larval stages. Not only are ancient genes present in this study, but also novel genes. This suggests that both ancient and novel proteins are needed to construct the larval shell of *A. californica*. This also indicates that there is a core set of mantle-secreting genes needed for shell fabrication that was provided by a shared metazoan ancestor. In addition, this goes to show that these novel proteins

have a role in the synthesis of the diversity and distinctive molluscan shell forms that we observe today.

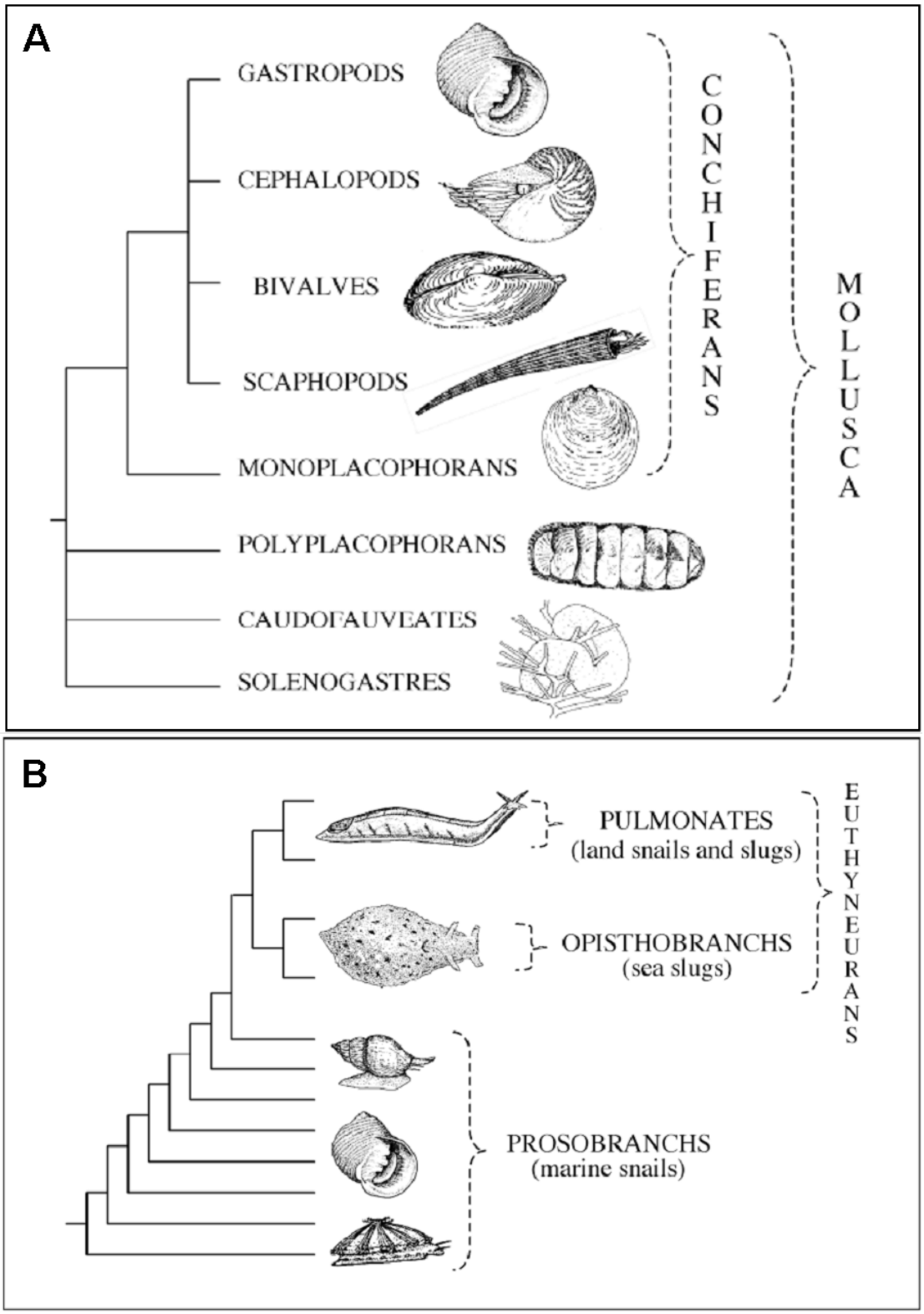

Figure 1. **Consensus Phylogeny of Molluscs and Gastropods.** Consensus tree of the molluscan phylogeny with its eight major clades depicted. Examples of the

diversity of shell morphology are presented within each major clade (1A). Depiction of the Gastropod tree with examples of prosobranch snails, basal lineage of gastropods. Crown gastropods (Euthyneura, encompassing Pulmonates and Opisthobranchs) often exhibit multiple reversals (homplasy) such as shell loss and slug body plans while prosobrachs are shelled organisms, with the exception of a few species secondarily shell-less (1B).

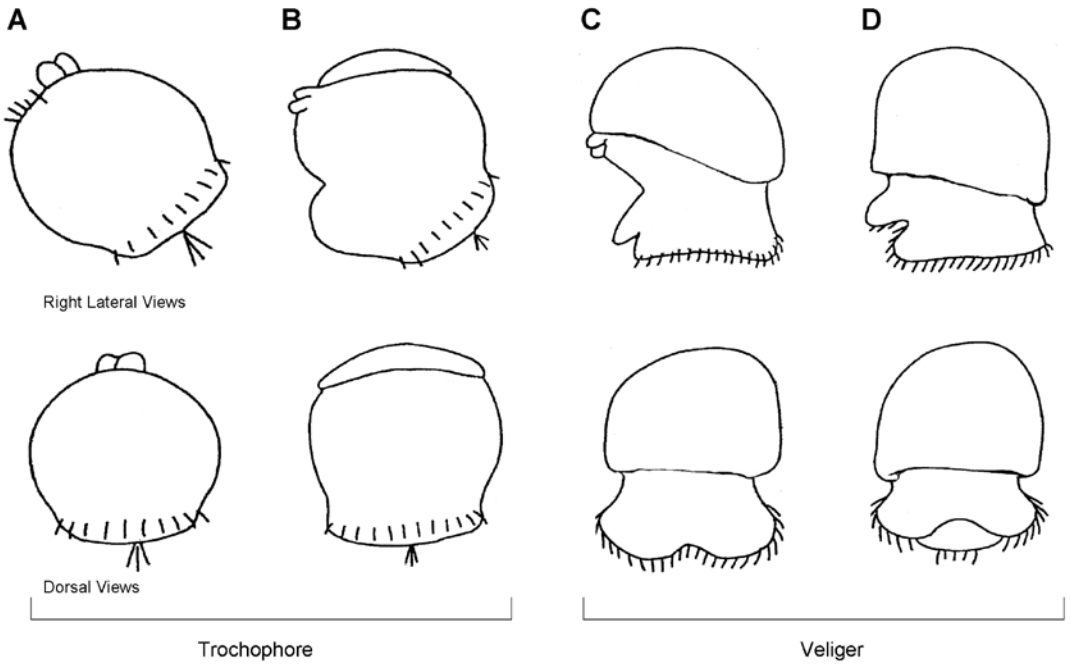

Figure 2. **Schematic representation of embryonic stages of *Aplysia californica.*** Schematic representation of a trochophore larva (2A) as it develops and proceeds to become a shelled veliger (2B). Schematic representation of an early pre-hatching veliger (2C). Schematic representation of a post-hatching Stage 1 veliger (2D). [All images presented in this figure were modified from Dickinson and Croll (2001) [86]].

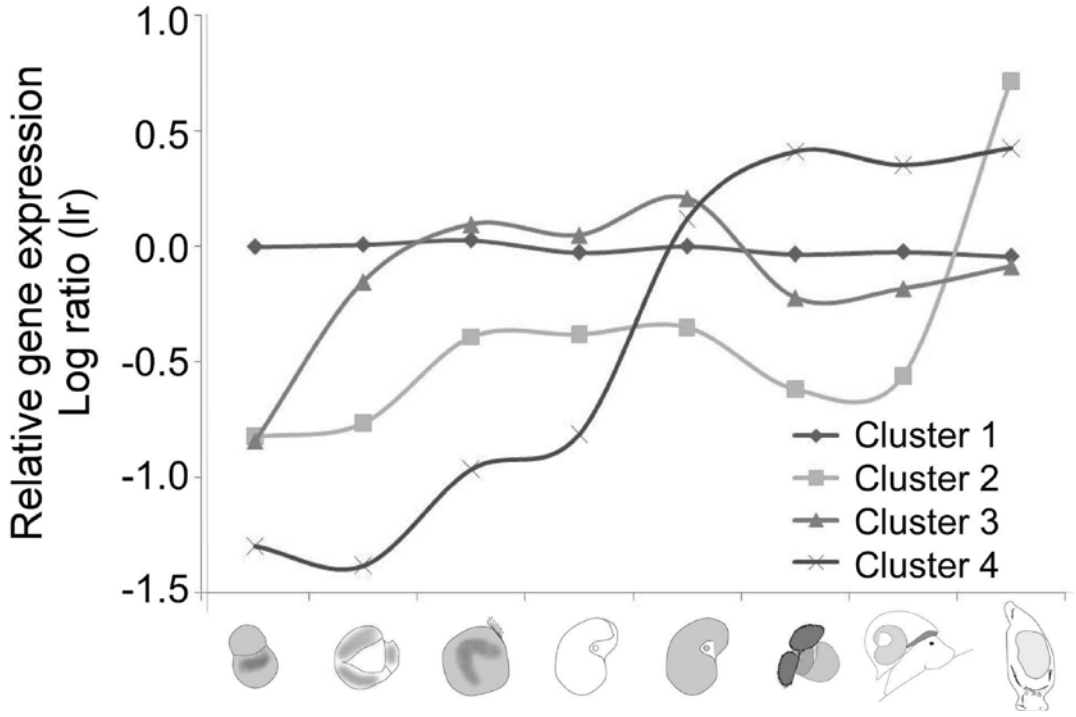

Figure 3. **Clusters of biomineralization genes by developmental expression.** Potential biomineralization genes were identified and each was analyzed for expression profiles using k-means cluster analysis. The series of cluster analyses helped us identify clustering patterns of developmental stages relative to each other as well as clustering of specific gene expression profiles. Cluster 1 transcripts show very little changes in expression levels. Cluster 2 represents genes that are highly (>20 fold) up-regulated 60 hours post-metamorphosis. Cluster 3 consists of genes that show elevated expression levels during trochophore and veliger stages with the exception of metamorphic stages. Finally, cluster 4 contains genes that show peptide-like expression profiles (i.e. very low expression levels during early development and very high expression levels late in development).

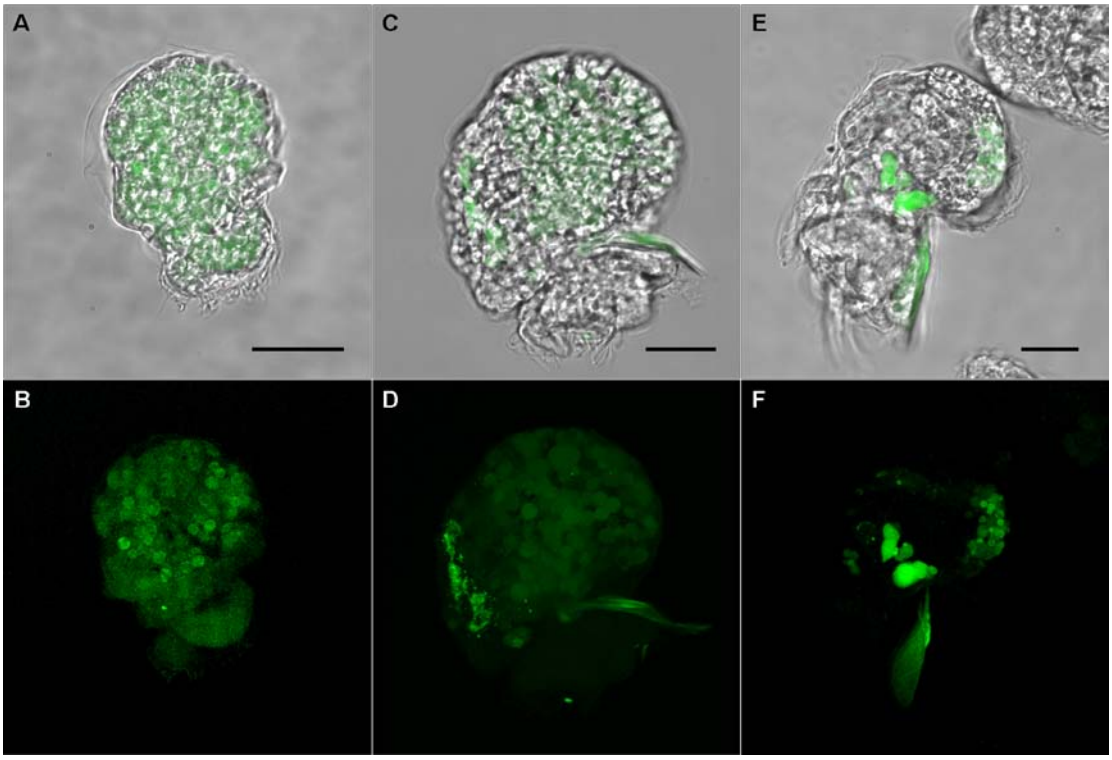

Figure 4. **WM-FISH of Soma Ferritin [DA20216] in *Aplysia californica.*** Soma ferritin transcripts are highly diffused throughout the ectodermal cells throughout the trochophore embryo, including the shell field. (4A and 4B), within the mantle and multiple other cells throughout the embryo in early pre-hatching veligers (4C and 4D) and in the mantle, the kidney and the statocyst in Stage 1 veligers (Figure 4E and 4F). Scale bar in A: 35 µm, in C: 37 µm, in E: 26 µm.

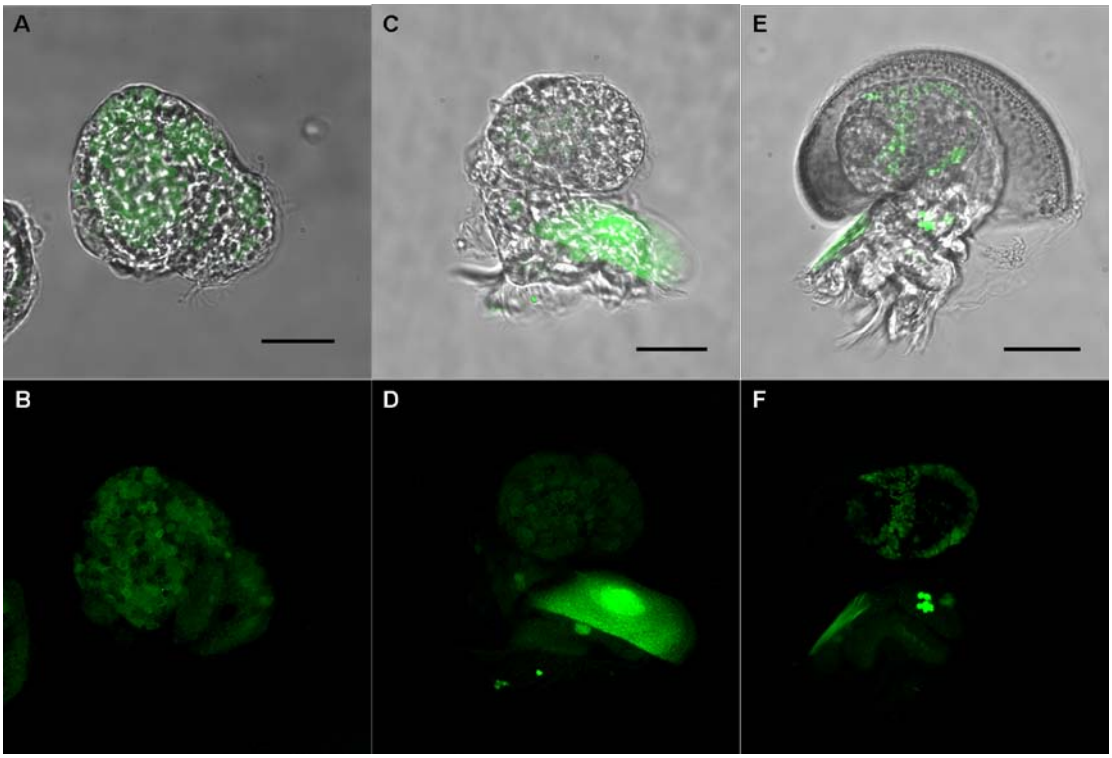

Figure 5. **WM-FISH of CDGSH iron sulfur domain 1 [DA17224] in *Aplysia californica.*** CDGSH1 iron sulfur domain 1 transcripts are diffused in the ectoderm tissue throughout the trochophore larvae (5A and 5B). In the early pre-hatching veligers are present throughout the mantle tissue that is covering the digestive gland (at low concentrations) and the statocyst and pedal ganglia at high concentrations (5C and 5D) and becomes concentrated at particular areas of the Stage 1 embryo: the mantle tissue underneath the shell (posterior) and a strip down the lateral sides of the shell, the velum lobes and the cerebral ganglion (5E and 5F). Scale bar in A: 35 μm, in C: 37 μm, in E: 38 μm.

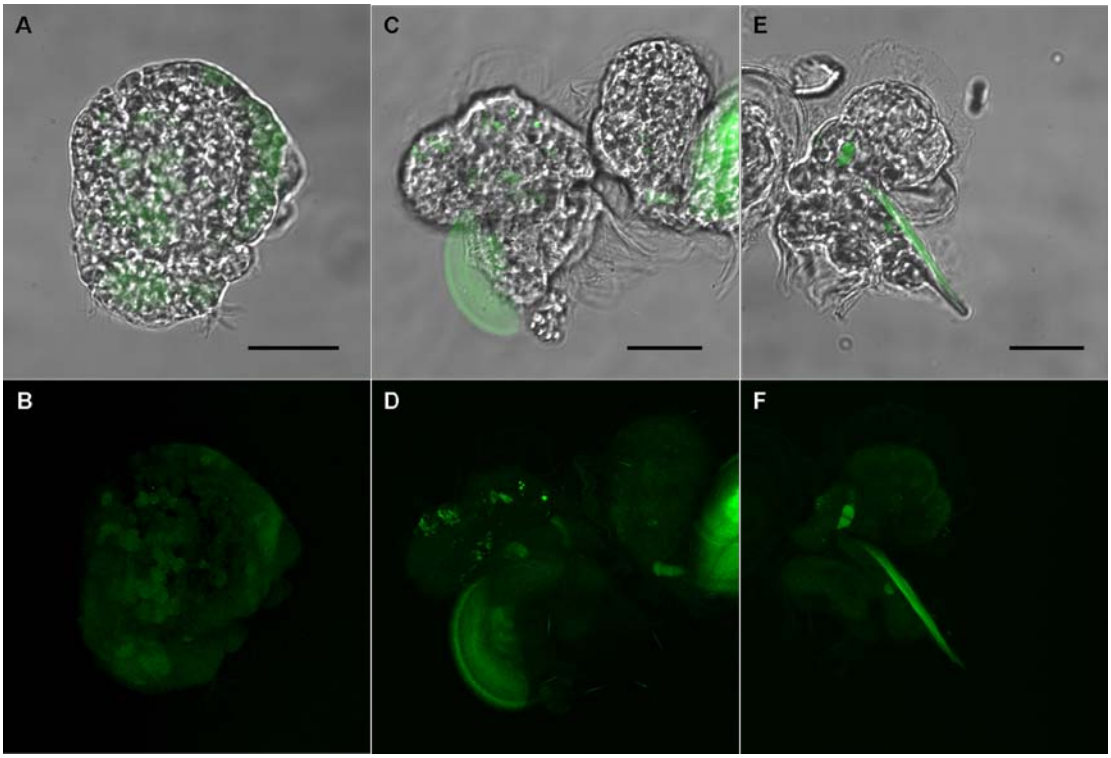

Figure 6. **WM-FISH of ribosomal protein rpl7a [DA22104] in *Aplysia californica.*** Transcripts of ribosomal protein rpl17a are diffused throughout the ectodermal cells of the trochophore larvae, where the shell field is present, the prototroch and the cells close towards the mouth (6A and 6B). There are very little transcripts present, only a small amount of expression in certain cells of the mantle and the statocyst organ in the pre-hatching veliger (6C and 6D) within the mantle tissue at the posterior edge and a small amount at certain cells at the dorsal mantle in Stage 1 veligers (Figure 6E and 6F). Scale bar in A: 35 μm, in C: 34 μm, in E: 38 μm.

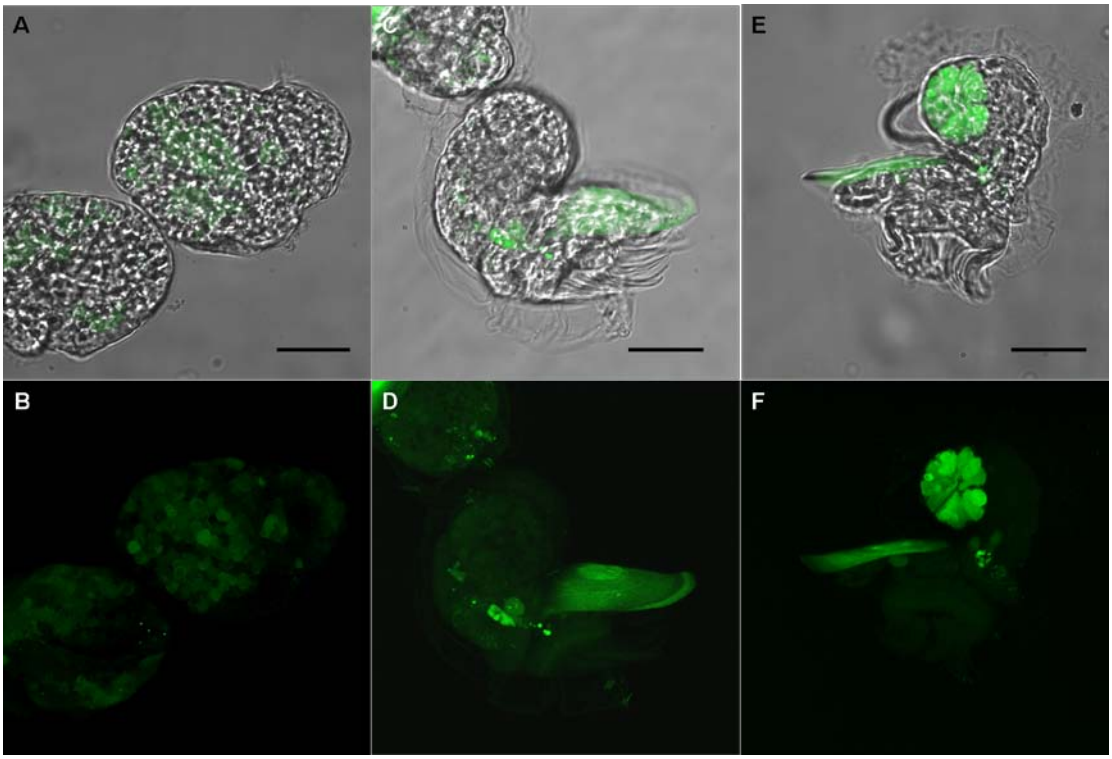

Figure 7. **WM-FISH of ribosomal protein rpl17 [DA21667] in *Aplysia californica.*** Ribosomal protein rpl17 transcripts are diffused throughout the ectodermal cells during the trochophore larval stage (7A and 7B). At the early veliger stage (pre-hatching), there is specific expression at the dorsal mantle, the kidney, the statocyst in the velum lobes (7C and 7D). Expression is present at the dorsal and ventral mantle tissue, the stomach and the statocyst in Stage 1 veligers (7E and 7F). Scale bar in A: 33 μm, in C: 34 μm, in E: 38 μm.

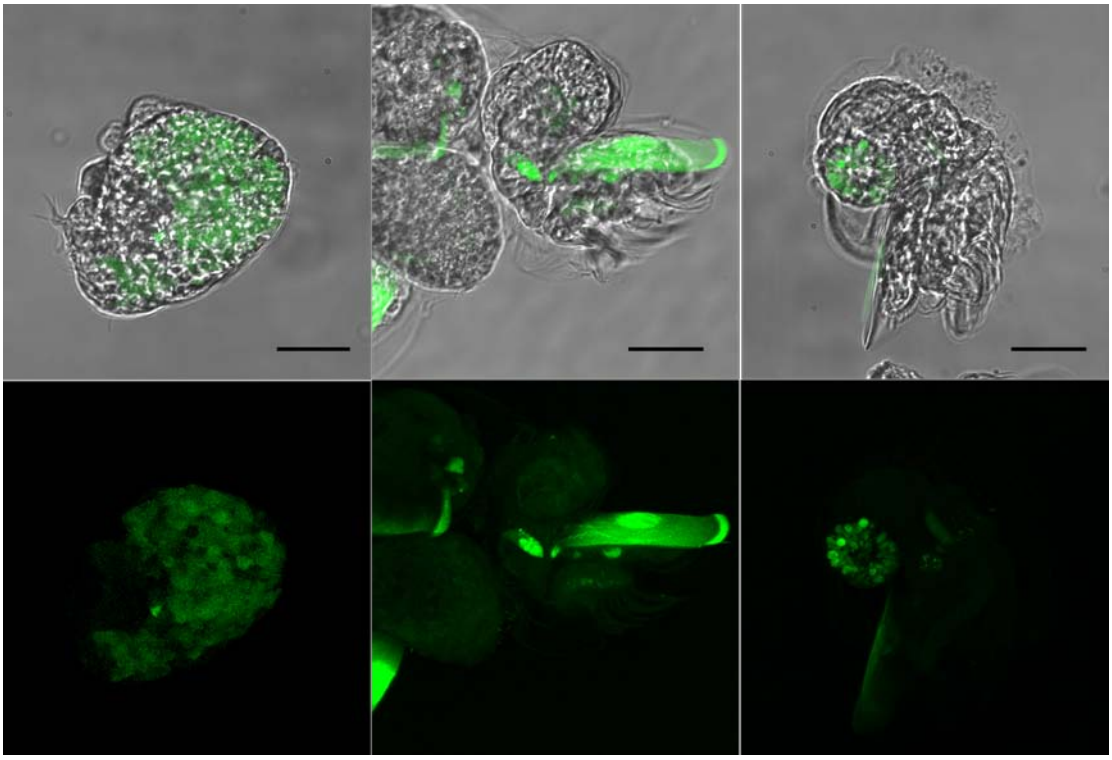

Figure 8. **WM-FISH of ribosomal protein rps30 [DA8011] in *Aplysia californica.*** Ribosomal protein rps30 is diffused and restricted to the ectodermal cells of the trochophore larvae and at the shell gland, but t never extends pass the prevelum and mouth (8A and 8B). In early pre-hatching veligers, localization is in the statocyst, velum lobes and kidney (8C and 8D). In the Stage 1 veliger, expression present in the ventral mantle tissue, statocyst and kidney (8E and 8F). Scale bar in A: 33 µm, in C: 34 µm, in E: 38 µm.

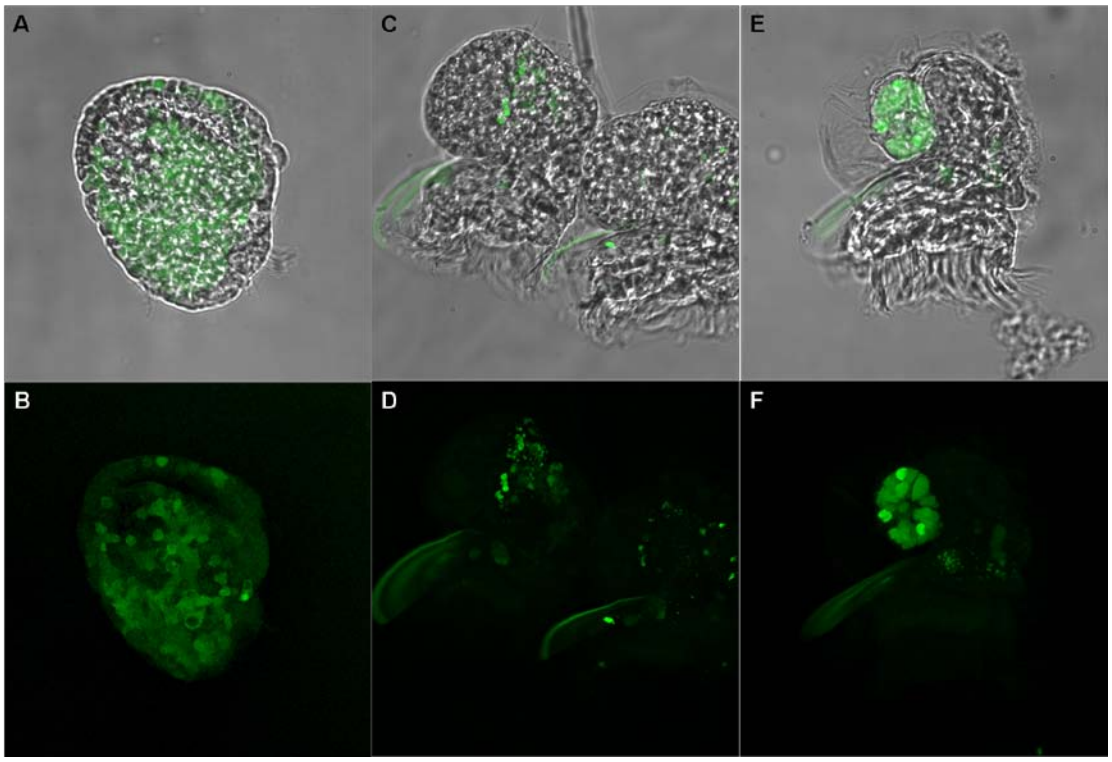

Figure 9. **WM-FISH of 40s ribosomal protein S15 [DA443] in *Aplysia californica.*** 40s ribosomal protein S15 transcripts were detected throughout the ectodermal cells in the trochophore embryo (9A and 9B). In the early pre-hatching veliger is scattered in the lateral mantle tissue (never exceeds to the ventral embryo), the velum lobes and the statocyst (9C and 9D). The subvelum, cells within the statocyst, the ventral mantle tissue covering the stomach and the stomach are expressed in Stage 1 veligers (Figure 9E and Figure 9F). Scale bar in A: 33 µm, in C: 34 µm, in E: 38 µm.

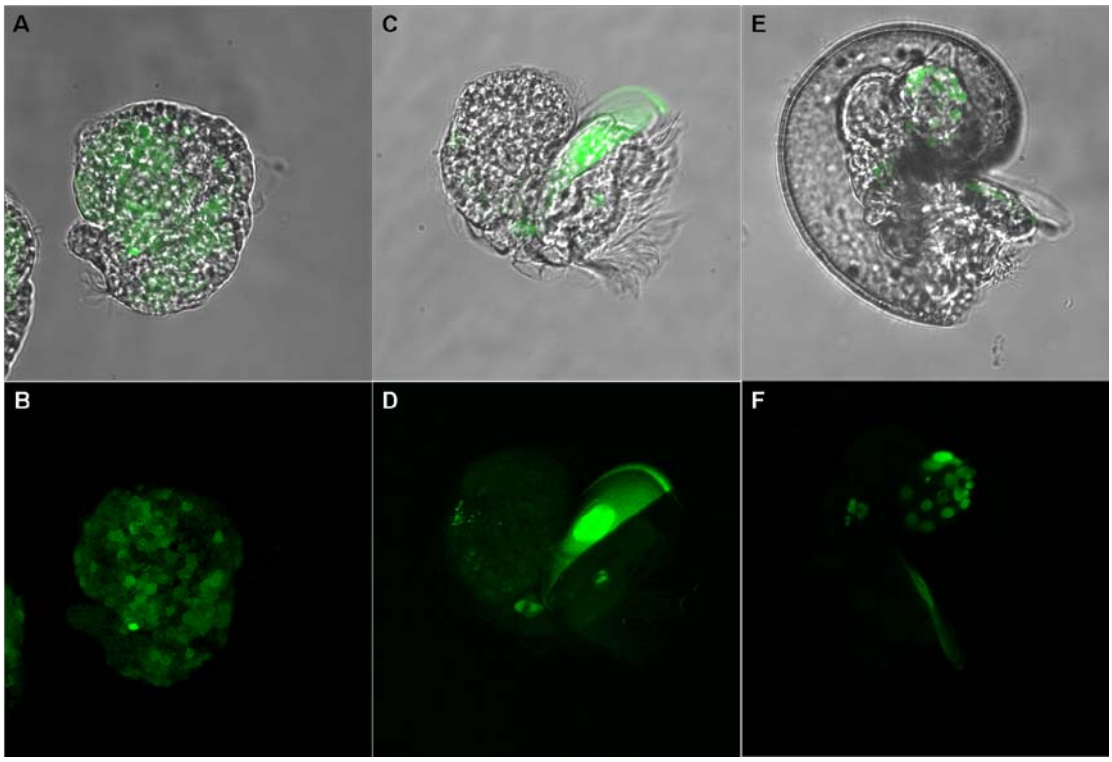

Figure 10. **WM-FISH of ribosomal protein S14 [DA834] in *Aplysia californica.*** Ribosomal protein S14 transcripts throughout the ectodermal cells diffusely in trochophore embryos (10A and 10B); it is also present in the mantle (strong posterior labeling), the velum lobes, the kidney and statocyst in the early pre-hatching veliger (10C and 10D). In Stage 1 veligers, expression is in the mantle and in the cerebral ganglion (10E and 10F). A: 35 µm, in C: 37 µm, in E: 38 µm.

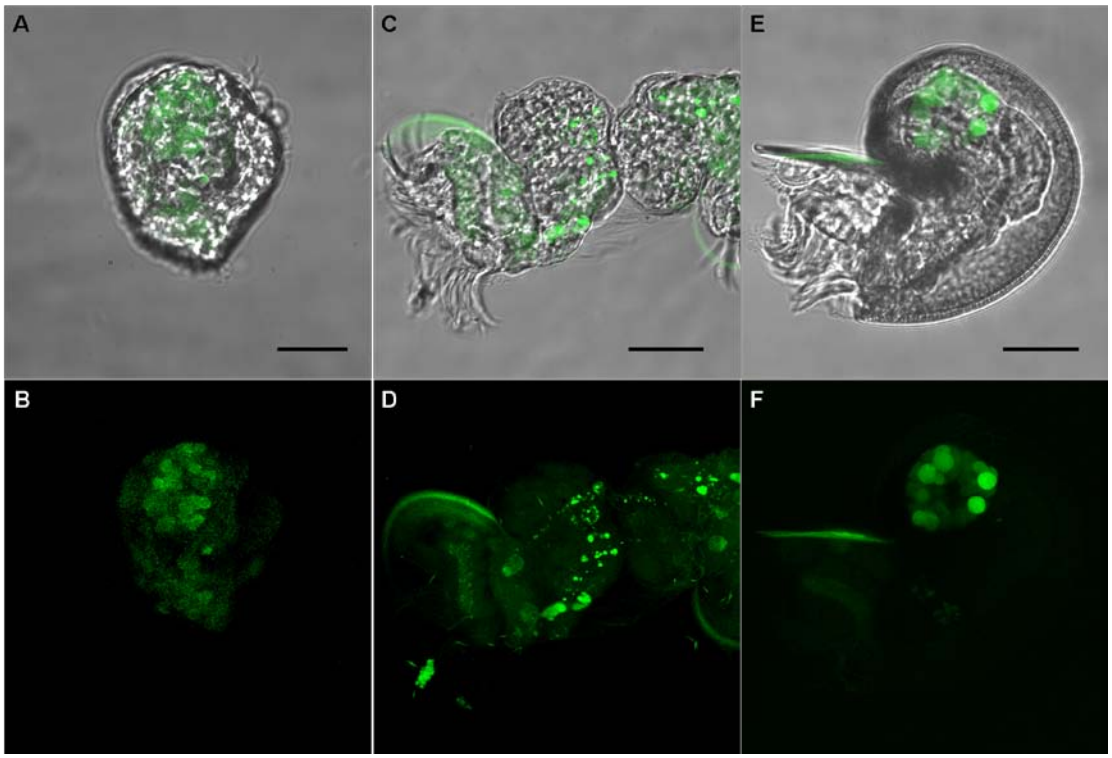

Figure 11. **WM-FISH of Unknown (ML8H1) [DA5095] in *Aplysia californica.*** In the trochophore larvae, the unknown biomineralization gene transcripts are diffused throughout the ectodermal cells (11A and 11B). In the early pre-hatching veliger and Stage 1 veliger stages, it is present in the dorsal an\d lateral mantle, the velum lobes and statocyst (11C-11F). It is also present in the cerebral ganglion at Stage 1 veligers (11E and 11F). A: 35 µm, in C: 37 µm, in E: 38 µm.

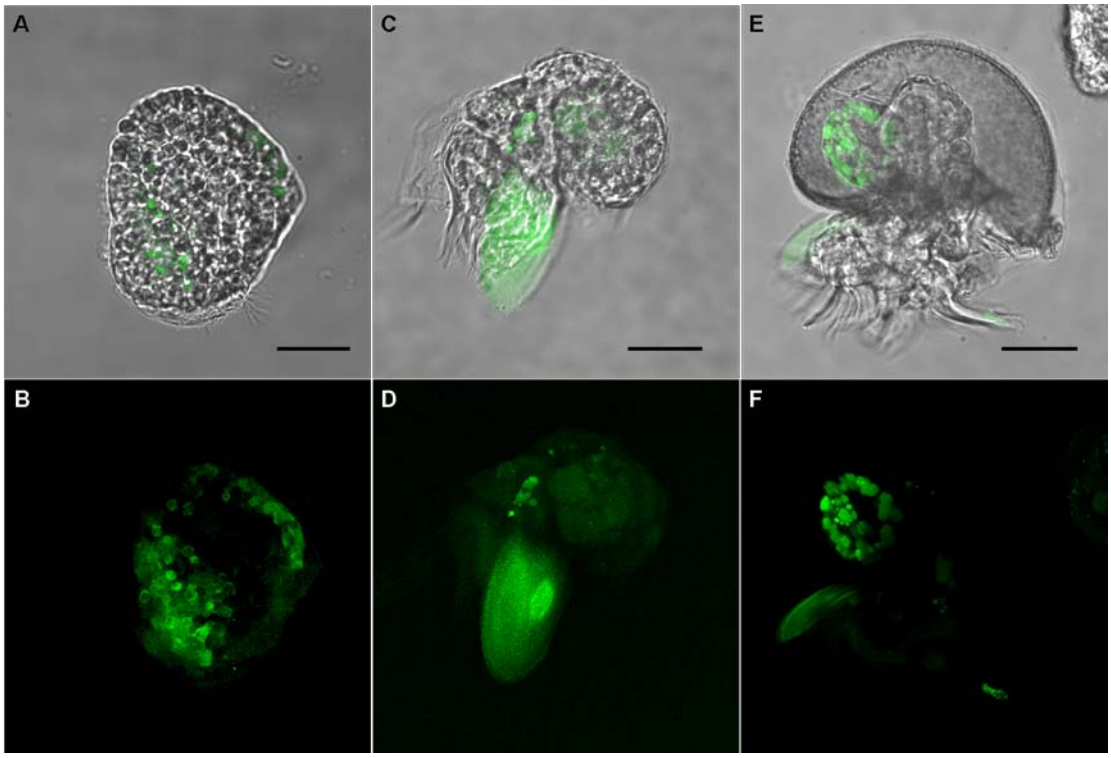

Figure 12. **WM-FISH of CaM kinase II alpha [DA15] in *Aplysia californica.***

CaM kinase II alpha transcripts are localized at the site of the growing shell gland and the posterior end of the embryo in the ectoderm tissue of the trochophore larvae (12A and 12B). In early pre-hatching veligers, expression is diffused throughout the mantle with some specific cells at the dorsal mantle, in the statocyst and in the cerebral and pedal ganglions (12C and 12D). Transcripts in Stage 1 veligers were detected in the velum lobes, the cerebral ganglion and the ventral mantle tissue (12E and 12F). A: 35 µm, in C: 37 µm, in E: 38 µm.

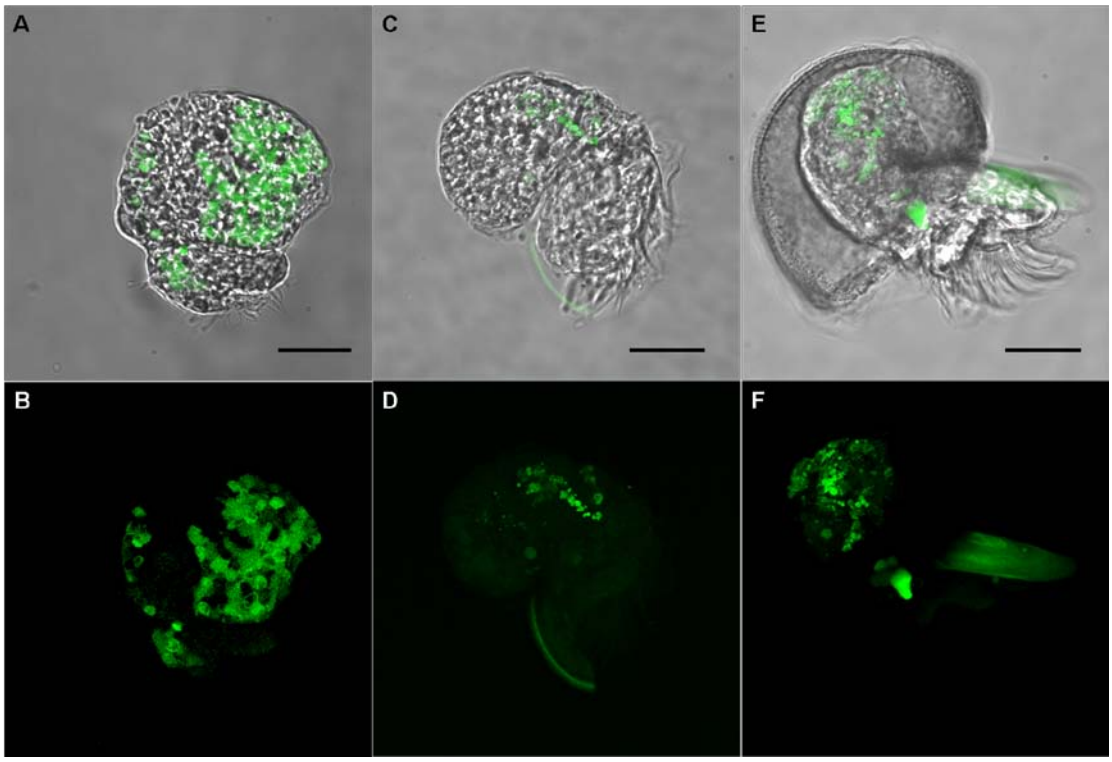

Figure 13. **WM-FISH of Calmodulin [DA36880] in *Aplysia californica.*** Calmodulin transcripts in the trochophore were highly localized in the ectodermal tissue of the growing shell field and some near the mouth area (13A and 13B). Early pre-hatching veligers shows specific staining at the dorsal and lateral mantle tissue (13C and 13D). Stage 1 veligers show expression in the mantle tissue at particular cells underneath the shell and other cells throughout the embryo, including the statocyst (13E and 13F). A: 35 µm, in C: 37 µm, in E: 38 µm.

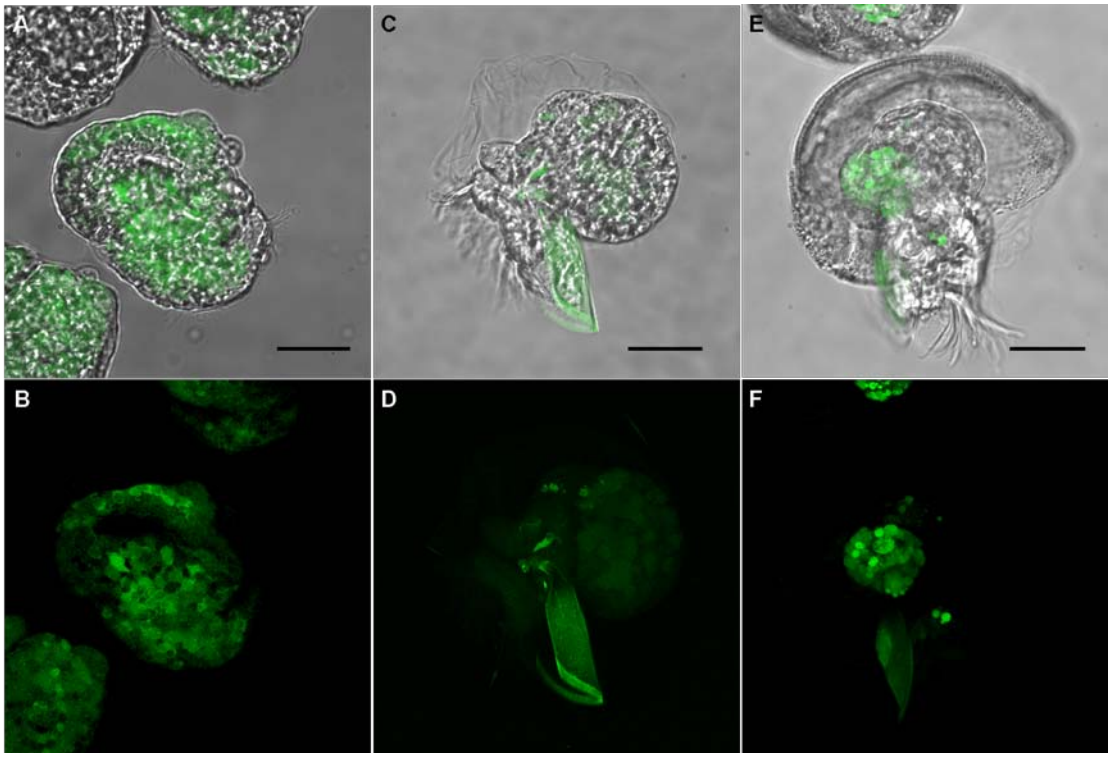

Figure 14. **WM-FISH of Dermatopontin 2 [DA25779] in *Aplysia californica.*** Dermatopontin 2 was observed in the ectodermal cells throughout the trochophore embryo and at the growing shell field (14A and 14B). Early pre-hatching veligers showed expression throughout the mantle tissue with specific staining to the dorsal mantle, in the statocyst and in the velum lobes (14C and 14D). Stage 1 veligers show expression in the ventral mantle tissue, the statocyst and in the velum lobes (14E and 14F). A: 24 µm, in C: 37 µm, in E: 38 µm.

Table 1. **Mantle-secreting biomineralization genes in A. californica.** This table shows the eleven isolated biomineralization genes that were examined in this study. The complete dataset with IDs, details of its annotations and its relative expression levels can be found here.

				LOG2-RATIOS - Developmental Reference							
ID	Manual Annotation	New Annotation	Biomin Cluster	cleavage	gastrula	trochophore	veliger	hatching	Stage6	Stage7	pm60
DA15	KPSH1 *[Pinctada fucata]*	CaM kinase II alpha *[Aplysia californica]*	1	-0.088572175	0.060379234	0.069898152	0.170094174	0.142466231	0.21473891	0.242217309	0.225954277
DA21667	ML1G3 *[Haliotis asinina]*	ribosomal protein rpl17 *[Eurythoe complanata]*	1	0.057965685	0.089846991	0.214782731	0.039373218	0.206423665	-0.164709766	-0.121760684	-0.107916551
DA22104	ML3E6 *[Haliotis asinina]*	ribosomal protein rpl7a *[Arenicola marina]*	1	0.187609341	0.070073003	0.180061465	-0.004783986	0.163824973	-0.120667155	-0.079998159	-0.134109168
DA25779	Dermatopontin2 (Fragment) *[Mandarina aureola]*	dermatopontin 2 *[Haliotis diversicolor supertexta]*	3	-0.879506508	-0.465353059	0.611336232	0.188843666	-0.354838305	-0.993085192	-0.910362499	-0.423964472
DA36880	Calmodulin *[Pinctada fucata]*	calmodulin, isoform A *[Drosophila melanogaster]*	3	-0.563863363	0.097837995	0.376648894	0.134570014	-0.014423499	-0.338059487	-0.290082504	-0.025047645
DA443	ML3F11 *[Haliotis asinina]*	40S ribosomal protein S15 *[Omithoctonus huwena]*	1	0.024172083	0.143747084	0.138925609	0.083362081	0.04076564	-0.191051772	-0.111784239	-0.162062527
DA5095	ML8H1 *[Haliotis asinina]*	Unknown	4	-0.496826446	-0.097940554	-0.228133668	-0.145940538	-0.065721953	0.264795051	0.448465956	-0.285332511
DA8011	ML1A7 *[Haliotis asinina]*	ribosomal protein rps30 *[Arenicola marina]*	1	0.055696378	0.080690873	0.176714405	-0.007040222	0.188436492	-0.117119553	-0.088877561	-0.030100763
DA834	ML5B1 *[Haliotis asinina]*	ribosomal protein S14 *[Haliotis discus discus]*	1	0.087779187	0.077517266	0.060123184	-0.032251423	0.131486245	-0.083882857	-0.045081927	-0.113714635
DA20216	ferritin-likeprotein *[Pinctada fucata]*	Soma ferritin [Aplysia californica]	4	-1.446626208	-1.36538254	-1.501315719	-1.297425787	0.259842728	0.339446407	0.459385504	0.475729667
DA17224	PREDICTED:similartoProteinC10orf70 *[Strongylocentrotus purpuratus]*	CDGSH1 iron-sulfur domain 1 *[Ixodes scapularis]*	3	-0.962093001	0.061723242	0.446898839	0.244914984	0.097397013	-0.445746557	-0.506984812	-0.165741169

CONCLUSION

Aplysia californica has been a model system for the study of the cellular basis of behaviour for decades [12]. *Bursatella leachii*¸ the ragged sea hare, exhibits some developmental and ecological differences relative to *Aplysia californica*; therefore, providing a good comparative system to broaden the use of this model organism into the study of evolution of shell loss in opisthobranchs. To be able to do this, we described the complete life cycle *Bursatella leachii* so that we would be able to carry out experimental comparative developmental studies within the sea hare clade.

As of today, *A. californica* can be grown in the laboratory under controlled hatchery conditions with optimized short generation times and life cycle inducers. . With the success of *A. californica* cultures year-round with high fecundity and quick growth (providing us with an abundant experimental stock of animals at multiple life stages), we have also established a reliable culturing technique for *B. leachii* that will be able to make this species amenable to experimentation at all developmental stages [43].

Additionally, with access to microarray gene expression data [14] and whole mount *in situ* hybridization protocols recently established for *A. californica* [14, 50], this goal of experimentation in both species is more attainable.

Sea hares (Opisthobranchia, Anaspidea) would be a good comparative system to understand and provide new insights to how the differential expression profile of these conserved molluscan biomineralization genes, the spatial and temporal developmental programs and the processes of calcification of more derived gastropods have evolved, compared to basal gastropods.

This study underlines the importance of a temporal and spatially characterization of genes involved in biomineralization. In general, large-scale expression studies will help to identify and select gene candidates on which a more

refined spatial expression analysis is worthwhile to be conducted in order to assess function and evolution of biological processes.

Finally, with the sequencing of the *Aplysia californica* genome near completion, we can likely gain more insight into gene regulatory networks of different developmental processes such as biomineralization not only in sea hares but molluscs as a whole.

ACKNOWLEDGEMENTS

SHELL DEVELOPMENT IN SEA HARES: COMPARATIVE ANALYSIS OF EARLY ONTOGENY IN *BURSATELLA LEACHII* AND *APLYSIA CALIFORNICA*

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CHARACTERIZATION OF MANTLE SECRETING GENES THROUGHOUT LARVAL DEVELOPMENT IN *APLYSIA CALIFORNICA*

Funding for this research was provided by NSF award 0542330 to Monica Medina. The rearing of *Aplysia calfornica* was supported by a grant from the NIH NCCR to the UM National Resource for *Aplysia* (RR10294). We wish to thank Christian R. Voolstra for the bioinformatics of the biominerlization genes; Andreas Heyland and Leonid L. Moroz for the help of optimizing the *in situ* hybridization protocol and for the use of the microarray data; Thomas R. Capo for the endless supply of *Aplysia californica*; the Benoît Dayrat for his contribution on the phylogenetic trees; Alice Hudder for training; and Mónica Medina for conceiving the study, coordinating its design, and in helping with preparations.

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