Comparative analysis of early ontogeny in *Bursatella leachii* and *Aplysia californica*

Zer Vue, Bishoy S Kamel, Thomas R. Capo, Ana T. Bardales, Mónica Medina

Opisthobranch molluscs exhibit fascinating body plans associated with the evolution of shell loss in multiple lineages. Sea hares in particular are interesting because Aplysia californica is a well-studied model organism that offers a large suite of genetic tools. Bursatella leachii is a related tropical sea hare that lacks shell as an adult and therefore lends itself to comparative analysis with A. californica. We have established an enhanced culturing procedure for B. leachii in husbandry that enabled the study of shell formation and loss in this lineage with respect to A. californica life staging.

1 **Comparative analysis of early ontogeny in *Bursatella leachii***
2 **and *Aplysia californica***

3 Zer Vue[1,4,5], Bishoy Kamel[1,2], Thomas R. Capo[3], Ana T. Bardales[3], and Mónica Medina[1,2§]

4 [1] School of Natural Sciences, University of California, Merced, 5200 Lake Road, Merced,

5 California 95344, USA.

6 [2] Pennsylvania State University. Department of Biology. 208 Mueller Lab, University Park, PA

7 16802, USA.

8 [3] Rosenstiel School of Marine and Atmospheric Science, Division of Marine Biology and

9 Fisheries, University of Miami, 4600 Rickenbacker Causeway, Miami, Florida 33149, USA.

10 [4] Program in Developmental Biology, Baylor College of Medicine, Houston, Texas, USA

11 [5] Department of Genetics, University of Texas M.D. Anderson Cancer Center, Houston, Texas,

12 USA

13 [§]Corresponding author

Introduction

15   The Mollusca has been one of the most successful metazoan lineages in exploiting the

16   advantages of the hard, calcified shell (Lowenstam & Weiner 1989; Weiner S 2003). Yet there

17   are several molluscan groups that subsequently evolved to have a highly reduced shell, e.g. squid,

18   or have lost it completely, e.g. sea slugs (Kröger et al. 2011; Morton 1960). Shell reduction or

19   loss has also occurred in euthyneuran gastropods, i.e. marine and terrestrial slugs. Within the sea

20   slugs (Opisthobranchia), shell reduction or loss has occurred in members of the Cephalaspidea,

21   Anaspidea, Sacoglossa, Acochilidiacea, Nudibranchia, and Pleurobranchia among others (Wagele

22   & Klussmann-Kolb 2005). These events support the notion that shell reduction or loss is not an

23   isolated event and instead has evolved independently many times through parallel evolution

24   (Gosliner 1985; Gosliner 1991). Having a slug-like form may be well-suited for a borrowing or

25   swimming lifestyle, which is necessary for streamlining and reducing the weight of the organism

26   (in pelagic forms) (Vermeij 1993). Shell loss paved the way for extraordinary body plan

27   modifications observed in the different molluscan lineages that underwent this dramatic

28   anatomical change enabling them to occupy new niches such as plastic sequestration from

29   ingested macroalgae for photosymbiosis (Kempf 1984; Rumpho et al. 2000) in shell-less

30   gastropods (Vermeij 2013), camouflage (Rudman 1981; Rudman & Avern 1989) and mimicry of

31   unpalatable species (Kerstitch 1989), swimming to escape danger (Gillette & Jing 2001;

32   Lawrence & W. H. Watson 2002), and incorporation of defense mechanisms from their diet

33   organisms as their own, e.g. nematocysts (Conklin & Mariscal 1977; Greenwood & Mariscal

34   1984). Because these adaptations involve anatomical modifications that tend to take place during

35   early development, we consider that differential shell reduction and loss in sea hares provides an

36   excellent opportunity to investigate major transitions in gastropod body plan evolution.

37   Within the sea hares (Opisthobranchia: Anaspidea), shell reduction or loss has occurred at

38   least twice in adult individuals (Figure 1) but possibly more times.–The order Anaspidea is best

39   known for the work on *Aplysia californica* as a model system for the study of the cellular basis of

40   behavior (Kandel 1979) and molecular and genome resources are readily available (Heyland et al.

41   2011). Transcriptome profiling combined with whole mount in situ hybridization, has identified

42   differentially expressed genes during shell formation in early developmental stages of *A.*

43   *californica* (Heyland et al. 2011) providing a list of candidate genes involved in the process of

44   shell formation that can now be analysed in other anaspidean taxa.

45   Although the phylogeny of the Anaspidea is still partly unresolved (summarized in Figure

46   1), the monophyly of the group is well supported by several morphological synapomorphies, i.e.

47   reproductive system, defensive glands, radula, gizzard and nervous system (Ghiselin 1965;

48   Klussmann-Kolb & Dinapoli 2006; Mikkelsen 1996; Morton & Holme 1955), as well as

49   molecular phylogenies (Grande et al. 2008; Klussmann-Kolb & Dinapoli 2006; Medina & Walsh

50   2000; Thollesson 1999). The current understanding of phylogenetic relationships also enables us

51   to map the evolution of shell reduction and loss within the sea hares. While adults of the genus

52   *Aplysia* exhibit a reduced shell, the genus *Bursatella* represents the derived character state of

53   crown anaspidean taxa where adults lack a shell altogether. Thus the ragged sea hare, *Bursatella*

54   *leachii¸* exhibits some developmental differences relative to *A. californica* providing a good

55   comparative system to study shell evolution in this gastropod lineage. Both species undergo two

56   distinct periods of shell growth separated by cessation during the metamorphic process.

57   Following the *A. californica* life cycle staging (Kriegstein 1977a), characteristic veliger spiral

58   shell growth commences during the encapsulated embryonic phase and continues to the end of

59   the planktotrophic larval phase, stage 6. Growth resumes post metamorphosis at stage 10, when

60   the shell changes from a spiral to a planar shell growth pattern. *A. californica* has an internalized

61   shell in adulthood, whereas *B. leachii* undergoes post-metamorphic shell growth followed by

62   shell loss soon after metamorphosis (Paige 1988).

63   *Aplysia californica* is one of a few invertebrate species with long-lived planktotrophic

64   larvae that can be successfully cultured in the lab (Carefoot 1987; Kriegstein 1977a). Today,

65   after optimized short generation times and developmental inducers, a large number of *A.*

66   *californica* can be grown in the laboratory under controlled hatchery conditions. High fecundity

67   and quick growth provide abundant experimental stock of multiple life stages (Capo et al. 2009).

68   With the success of *A. californica* cultures year-round, having additional hatchery populations of

69   other anaspidean species is an attainable goal given our understanding of the ecology and

70   evolution of related taxa (Carefoot, 1987; Eales, 1921). Habitat and dietary preferences in *B.*

71   *leachii* are now well-known, facilitating animal husbandry. *B. leachii* lives in tropical subtidal

72   waters (Ramos et al. 1995) feeding on cyanobacterial biofilms found on sandy substrates (Paige

73   1988; Ramos et al. 1995).

74   In this study we report a more detailed description of the *B. leachii* life cycle than

75   previously available normalized to the *A. californica* hatchery culturing procedures currently in

76   place at the National *Aplysia* Resource facility (Capo et al. 2009). We also report new optimal

77   culture conditions for *B. leachii*. We conclude by describing the most apparent differences in the

78   developmental program of both species, with emphasis on metamorphic stages during which shell

79   reduction and loss take place with discussion of potential biomineralization proteins involved in

80   shell formation in sea hares.

81   ## Methods
82   ### Broodstock and eggs/larval rearing

83   *Aplysia californica* adults were collected by Santa Barbara Marine Biologicals in 2006.

84   *Bursatella leachii* adults were collected along the coast of Key Biscayne, Florida during the

85   summer of 2006. All organisms were housed in the flow-through seawater system at the National

86   *Aplysia* Resource Facility at the University of Miami's Rosenstiel School of Marine and

87   Atmospheric Science (RSMAS) as previously described (Capo et al. 2009; Capo et al. 2002).

88   The animals were fed a daily ration of the following laboratory-cultured seaweeds: *Gracilaria*

89   *ferox* (for *A. californica*) and a mixture of blue-green algae and epiphytes (for *B. leachii*). The

90   light cycle of both species was maintained at 12 hrs light: 12 hrs dark. The seawater temperature

91   was 13-15ºC for *A. californica* and 22-26ºC for *B. leachii*. In year 1 of the study cultures were

92   maintained at the same temperature (22ºC) but the *B. leachii* cultures died. In the subsequent trial,

93   parallel cultures were maintained at 22ºC and 25ºC for *A. californica* and at 25ºC for *B. leachii*.

94   Mating pairs were monitored throughout the day for active egg-laying. During oviposition, a 10

95   cm portion of egg strand was collected, rinsed immediately with 0.45µm filtered seawater, placed

96   in a 2l flask to which Na_2EDTA (0.25mg/l) was added to bind heavy metals in the natural

97   seawater that may deleteriously affect development (Capo, 2002). The eggs and seawater were

98   vigorously aerated until one day prior to hatching in a temperature-controlled incubator at 22º C

99   and 25 º C for *A. californica* and 25 º C B. *leachii* in the last trial of the culturing experiments.

100   Hatching occurred 7-8 days after the eggs were deposited and the cordon (egg strand) was

101   inspected under a dissecting microscope at six days post-oviposition to validate normal and

102   synchronized development of embryos. Strands not meeting these standards were discarded.

103   The number of larvae/mm of cordon was estimated by cutting three portions of known

104   length, using an ocular micrometer. Each segment was dissolved in 2% sodium hypochlorite and

105   the shells were counted. Day 0 shell length (SL) for both species was determined by measuring

106   25 individuals from each portion of the cordon using an ocular micrometer at 50X magnification.

107   The appropriate initial larvae density was provided by aseptically cutting the appropriate cordon

108   length, immediately rinsing with 2µm filtered seawater, and directly transferring it into the larvae

109   culture vessel.

110   Seawater was collected from Bear Cut, Virginia Key, FL and prepared by prefiltration

111   through a 15 μm glass media filter. The salinity was adjusted to 32 ppt with deionized water, and

112   aerated with chloramphenicol (2.5 mg/l), Na_2EDTA (0.25 mg/l). Eighteen to 24 hr later the

113   seawater was vacuum filtered through a 2 μm prefilter (Millipore AP2504700) (Kriegstein et al.

114   1974; Nadeau et al. 1989). The desired concentration of microalgae and estimated length of egg

115   mass were added to filtered seawater in 2 L roller bottles (Corning). The vessel was sealed with

116   Parafilm[®] and plastic wrap to eliminate the air-water interface (Capo et al. 1987; Paige 1986;

117   Tamse et al. 1990). The cultures were incubated on a continuously rotating (1 rpm) roller bottle

118   apparatus (Wheaton), with a 24 hr fluorescent light regime (~0.001 $\mu E/cm^2/s$) at a constant

119   temperature of 22°C (Kriegstein et al. 1974) , (Nadeau et al. 1989) and (Tamse et al. 1990).

120   Roller apparatus positions were randomly assigned to each culture vessel and remained fixed

121   throughout the experiment.

122   After hatching, larvae were measured and the culture media was changed every 7 days.

123   The larvae were collected on a 74 μm mesh screen, rinsed with filtered seawater (FSW) and

124   transferred to a sterile crystallizing dish. An iodine-based surfactant (Betadine Surgical Scrub)

125   was added to resuspend any larvae entrapped by the air-water interface. Larvae were treated with

126   1.25 ml of a solution of 2.5 mg/ml Poly (vinylpyrrolidone)–Iodine complex (Sigma) and 2.0

127   mg/ml pH 8.3 fish-grade Trizma (Sigma) solution for 5 minutes to inhibit bacterial growth. This

128   treatment also acted to suppress larval swimming behavior and provided a non-lethal method to

129   facilitate shell length measurements. Weekly SL of 25 larvae was measured and the larval stage

130   for both *A. californica* and *B. leachii* was determined through Kriegstein's staging scheme for *A.*

131   *californica* (Kriegstein 1977a). Once the exposure period ended, the iodine concentration was

132   reduced by incremental addition of a 0.4% sodium thiosulfate solution to the treatment bath until

133   the characteristic iodine color disappeared. The larvae were rinsed in FSW and transferred to a

134   clean, acid-washed roller bottle with FSW containing the appropriate amount of microalgae and

135   sealed (250×10^3 cells/mL, *Isochrysis sp.* – CCMP1324). The bottles were then returned to the

136   previously assigned roller bottle apparatus and position.

137   For imaging of each stage of *B. leachii*, the larvae were placed in filtered seawater (0.22

138   μm) containing 340 mM of magnesium chloride. Once animals were narcotized, photographs

139   were taken with an Olympus BX51 microscope or a Leica MZ16F stereoscope. Scanning

140   Electron Microscopy was performed on a limited numbers of larval shells from both species.

Results

Post-hatching larval development and shell growth

143   The life cycle staging of *B. leachii* mentioned here is equivalent to the staging scheme that was

144   described for *Aplysia californica* (Kriegstein 1977a) and currently in use at the University of

145   Miami's *Aplysia* hatchery (National Resource Facility for *Aplysia*. Available at

146   http://aplysia.miami.edu/ (accessed 4 December 2012). Stage 1 is characterized by a newly

147   hatched veliger containing a Type 1 shell (Thompson 1961). In *B. leachii*, Stage 1 larvae have a

148   maximum shell diameter of 141.1 ± 6.9 μm (N=25) and the veliger's shell grows rapidly – an

149   average of 21 μm per day (Figure 2). Stage 2, defined by the appearance of the eyes, and is

150   reached within 4 days post-hatching. By day 5, the shell length is 264.6 ± 13.9 μm (N=25) with

151   the presence of 1.5 whorls. After 6 days post-hatching, the larval heart appears (Stage 3). By day

152   7, the maximum shell size (Stage 4) is reached at 284.2 ± 19.0 μm (N=25) (Supplementary 1).

153   Almost at the same time the foot expands to form a well-developed propodium (Stage 5). On day

154   9, the larvae reach competency and settle (Stage 6) when exposed to a substratum. A

155   morphological pigmented spot on the shell, similar to *A. californica* (Kriegstein 1977a), is also

156   present in *B. leachii*. Paige (1988, 1986) failed to observe and report pre-metamorphic

157   pigmentation most likely due to the use of artificial seawater. Pigmentation is a clear indicator of

158 competency to metamorphose, and can be reached as early as 9 days post-hatching. *A.*

159 *californica* larvae grown at 22˚C and 25˚C showed that there was no difference in growth. A

160 two-way repeated measures ANOVA reflects that there was no difference in the size of *A.*

161 *californica* grown at 22˚C versus 25˚C (Supplementary 1 and 2). In 2006, total mean shell

162 length (n=25) for *A. californica* grown at 22˚C averaged 134.6 µm ($s = 3.7$ µm) for Stage 1,

163 227.6 µm ($s = 15.0$ µm) for Stage 2, 337.7 µm ($s = 20.8$ µm) for Stage 3 and 392.8 µm ($s = 10.0$

164 µm) for Stage 5. Total mean shell length (n=25) for *A. californica* grown at 25˚C averaged 134.6

165 µm ($s = 3.7$ µm) for Stage 1, 236.1 µm ($s = 18.6$ µm) for Stage 2, 360.6 ($s = 36.9$ µm) for Stage 3

166 and 392.3 µm ($s = 18.9$ µm) for Stage 5 (Supplementary 2).

167 **Metamorphic larvae development of *Bursatella leachii***

168 Metamorphic development and post-larval development of *Bursatella leachii* is similar to other

169 previously described sea hares (Kriegstein 1977a; Paige 1988; Switzer-Dunlap 1978; Switzer-

170 Dunlap & Hadfield 1977). At Stage 5, the propodium forms, an essential structure needed for

171 settlement and crawling after settlement. At Stage 6 (Figure 3A), metamorphic competence

172 occurs, along with the appearance of other morphological traits, such as a pigmented spot on the

173 right side of the perivisceral membrane underneath the shell (Kriegstein 1977a). Once the larva

174 has settled, in the presence of an environmental cue (Heyland 2006; Paige 1988), it will attach

175 itself permanently and shed its velar lobes (Stage 7) (Figure 3B). The metamorphic transition

176 occurs when the two halves of the velar lobe rudiments fuse together and the larval heart stops

177 beating, which is also an indicator of Stage 8. Post-metamorphic shell growth in both *A.*

178 *californica* and *B. leachii* (Stage 9) is characterized by an elongation of the larval shell (Figure

179 3C). Stage 10 is reached when the shell reaches its maximum size and flattens prior to being

180 discarded (Figure 3D). The shell is discarded at Stage 11, when the juvenile begins to show adult

181   characteristics. Figure 3E shows a late Stage 11 juvenile, approximately 2 mm long, after

182   discarding its shell. The juvenile takes on adult characteristics, such as the appearance of small

183   bumps all over the body and rudiments of the fleshy villae. The rhinophores are elongated and

184   tubular and the oral tentacles expand laterally. The body is pigmented with large, white granular

185   patches. At Stage 12, *Bursatella leachii* (Figure 3F) is approximately 8 mm long. The villae

186   cover the entire body, multiply and become branched later in adulthood. Shell development is

187   similar in early embryonic stages but diverges as juvenile development takes place leading to

188   shell loss in *B. leachii*. We examined by SEM both whole shells and cross-sections of larval

189   shells (supplementary 3). Despite some noticeable similarities between the two species,

190   unfortunately due to the small size of the larval shells, we either did not have enough replicates

191   per stage or missed stages altogether to raise clear conclusions about larval shell shape and

192   internal structure.

193   ## Discussion

194   The life cycle of *Bursatella leachii* was characterized in reference to the well-known *A.*

195   *californica* life cycle. Having access to the complete life cycle of a second anaspidean species

196   enables comparative developmental studies within the sea hare clade. In the present study we

197   describe the life cycle of *B. leachii* in the context of the development of the larval shell and its

198   subsequent loss in the post-metamorphic stages.

199   ### *Bursatella leachii* development

200   The embryonic development of *Bursatella leachii* has been described previously (Bebbington

201   1969; Paige 1988) and will thus not be further discussed here. The larval developmental sequence

202   of *B. leachii* is similar to other sea hares (Switzer-Dunlap 1978) – a hatched veliger with a

203   hyperstrophically coiled shell, a reddish tint, and bilobed velum. *B. leachii* larvae differ both in

204   size and growth rate relative to *A. californica*, being both larger (approximately 10 μm) and faster

205   growing, though the larval development follows the staging sequence previously devised in the

206   literature (Kriegstein, 1977; Paige, 1988). Similar to Kriegstein (1977a), our study demonstrated

207   the presence of one prominent Stage 6 pigmentation spot in *B. leachii*.

208   Initial stages of post-metamorphic development of sea hares with a planktonic larval form

209   are also similar, Table 1 summarizes the larval development of *B. leachii* (Paige 1988) relative to

210   *A. californica* (Capo et al. 2009; Kriegstein 1977a). Recent advances in larval culture techniques

211   provide the tools for life cycle comparisons. The need for readily available developmental stages

212   is important for experimental developmental biology studies such as metamorphic transitions. In

213   the particular case of sea hares, hatchery populations provide an ideal supply of samples for the

214   study of larval shell loss.

215   Differences after metamorphosis occur at Day 40 during Stage 9 when *A. californica*

216   juveniles acquire pink pigmentation due to the red algal diet, while *B. leachii* juveniles become

217   white with dark bands on the head (Paige 1988). Despite this post-metamorphic physical

218   difference, their developmental programs remain highly similar to each other up until this point.

219   A major difference in *B. leachii* post-metamorphic development happens at Stage 11 when the

220   shell is discarded. At this stage in *A. californica*, the shell becomes overgrown by folds of the

221   mantle, causing the shell to be internalized. Given that both species follow a similar

222   developmental program through metamorphosis, it seems quite plausible that the underlying

223   mechanism of larval shell formation is also quite similar, only differing during settlement/post-

224   metamorphosis. We conclude that larval shell formation appears to be homologous in these two

225   species, which makes this process amenable to comparisons such as the examination of spatio-

226   temporal gene expression of genes involved in the formation of the shell in both species. It seems

227   plausible that the evolution of shell loss is the consequence of modifications to the regulatory

228   machinery of shell formation genes, as most molluscs have the ability to make shells at least in

229   the embryonic stages.

230 **Shell development in Anaspidea**

231 Shell building in molluscs is on the cellular level characterized by modifications to the

232 extracellular matrix (ECM) that create an environment conducive to crystal deposition in the

233 extrapallial space. Analysis of the shell "secretome," during calcification in the abalone, *Haliotis*

234 *asinina* by Jackson et al. (Jackson et al. 2006) yielded a significant number of transcripts. A direct

235 comparison of the transcriptomes of nacre-forming cells from *H. asinina* (gastropod) and

236 *Pinctata margaritifera* (bivalve) led to the conclusion that there are dramatic differences in the

237 gene sets used to build the nacreous layer of the shell (Jackson et al. 2010). These differences

238 also extend within the Gastropoda *(H. asinina vs. Lottia gigantea)*. A comparison of a single

239 biomineralizing gene family (shermatin) across three species of *Pinctata*, suggested that secreted

240 proteins with repetitive low-complexity domains (RLCDs) are an important feature in molluscan

241 evolution but are the consequence of evolutionary convergence (Jackson et al. 2010) thus

242 supporting the notion that the molluscan shell-secretome is rapidly evolving (Jackson et al. 2006).

243 The rapid evolution scenario complicates questions of functional homology across species as

244 many of the biomineralization proteins provide multiple other functions such as immune response

245 (Sarashina et al. 2006). Work on early developmental stages where the shell is starting to form is

246 of relevance to this study. Heyland et al. (Heyland et al. 2011) detected 196 different transcripts

247 that appear to be related to biomineralization in a developmental transcriptome time course in

248 *Aplysia californica*. These 196 transcripts were present during the whole course of development

249 and although not unique to the veliger stage, they were slightly overexpressed during the

250 veliger/trochophore stage and several are well known biomineralization proteins reported for

251 other molluscs such N66, Perlucin, Pearlin and Nacerin (Heyland et al. 2011). Reported gene

252 expression throughout the entire course of development hints at the fact that larval shell

253 development in *Aplysia* is primarily executed via regulatory mechanisms. The majority of the

254 detected transcripts lack annotation highlighting the importance of functional studies for the

255   discovery of new biomineralization-related proteins. The ability to transfer this information into

256   *B.leachii* would enable us to test multiple hypothesis about how conserved are the mechanisms of

257   shell building during early development in sea hares, a crucial step in increasing our

258   understanding of the fascinating phenomenon of biomineralization and evolution of shell loss in

259   opisthobranchs.

260   **B. leachii husbandry**

261   We present an improved strategy to culture *B. leachii* in larger numbers than previously

262   reported. We attempted to rear both species under similar conditions but *A. californica* is a

263   temperate species from the Western North America, where coastal upwelling is prevalent and

264   water temperatures low relative to tropical waters where *B. leachii* is common. Therefore we

265   decided to use a slightly higher temperature (25°C) for the second year the cultures were

266   established in the lab. The primary goal of this study was to produce individuals from comparable

267   stages, however, despite small sample sizes and limited controls, our efforts have lead to an

268   improved culturing method for *B. leachii* with larger larval yields than previously reported (Paige

269   1988).

270   **Conclusion**

271   We have established a reliable culturing technique for *B. leachii* that makes this species

272   amenable to experimentation at all developmental stages (Capo et al. 2009). Transcriptome data

273   and whole mount *in situ* hybridization available for *A. californica* (Heyland 2006) have enabled

274   developmental genetics research (Heyland et al. 2011) in anaspideans. While comparative studies

275   of biomineralization genes in sea hares are in their infancy, with developmental homology clearly

276   established and an improved cultivation protocol, we are primed to shed light on how the genetic

277   toolkit that controls shell formation and subsequent reduction or loss differs between *A.*

278   *californica* and *B. leachii*.

Acknowledgements

We thank Phillip Gillette for his efforts in culturing B. *leechii*; Benoît Dayrat for help with Figure

1; Alice Hudder for training. We also acknowledge the support of Michael R. Dunlap and the

Imaging and Microscopy Facility (IMF) at the University of California, Merced. This manuscript

was prepared by Zer Vue in partial fulfilment of requirements for the master's program in

Quantitative Systems Biology at UC Merced. We thank Chris Voolstra, Michael DeSalvo and

Shini Sunagawa for providing feedback on an earlier version of this manuscript.

References

Bebbington A. 1969. Bursatella leachii guineensis subsp. nov. (Gastropoda, Opistobranchia) from Ghana. *Proceedings of the Malacological Society of London* 38:323-341.

Capo T, Perritt S, and Paige J. 1987. The mass culture of *Aplysia californica*. Fifty-third annual meeting of the American Malacological Union. p 18.

Capo TR, Bardales AT, Gillette PR, Lara MR, Schmale MC, and Serafy JE. 2009. Larval growth, development, and survival of laboratory-reared Aplysia californica: Effects of diet and veliger density. *Comp Biochem Physiol C Toxicol Pharmacol* 149:215-223.

Capo TR, Fieber LA, Stommes DL, and Walsh PJ. 2002. The effect of stocking density on growth rate and maturation time in laboratory-reared california sea hares. *Contemp Top Lab Anim Sci* 41:18-23.

Carefoot T. 1987. Aplysia: its biology and ecology. In: Barnes M, ed. *Oceanography and Marine Biology Anual Review*: Aberdeen University Press, 167-284.

Conklin EJ, and Mariscal RN. 1977. Feeding behaviour, Ceras structure, and nematocyst storage in the aeolid Spurilla neapolitana (Mollusca). *Bulletin of Marine Science* 27:658-667.

Ghiselin MT. 1965. Reproductive function and the phylogeny of opisthobranch gastropods. *Malacologia* 3:327–378.

Gillette R, and Jing J. 2001. The Role of the Escape Swim Motor Network in the Organization of Behavioral Hierarchy and Arousal in Pleurobranchaea. *American Zoologist* 41:983-992.

Gosliner TM. 1985. Parallelism, parsimony and the testing of phylogenetic hypothesis: The case of opistobranch gastropods. In: Vrba ES, ed. *Species and Speciation*. Pretoria: Museum Monograph No. 4, Transvall Museum, 105–107.

Gosliner TM. 1991. Morphological parallelism in opistobranch gastropods. *Malacologia* 32:313–327.

Grande C, Templado J, and Zardoya R. 2008. Evolution of gastropod mitochondrial genome arrangements. *BMC Evol Biol* 8:61.

Greenwood PG, and Mariscal RN. 1984. The utilization of cnidarian nematocysts by aeolid nudibranchs: nematocyst maintenance and release in Spurilla. *Tissue and Cell* 16:719-730.

Heyland A. 2006. Signaling mechanisms underlying metamorphic transitions in animals. *Integrative and Comparative Biology* 46:743-759.

Heyland A, Vue Z, Voolstra CR, Medina M, and Moroz LL. 2011. Developmental transcriptome of Aplysia californica'. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution* 316:113-134.

Jackson DJ, McDougall C, Green K, Simpson F, Worheide G, and Degnan BM. 2006. A rapidly evolving secretome builds and patterns a sea shell. *BMC Biol* 4:40.

Jackson DJ, McDougall C, Woodcroft B, Moase P, Rose RA, Kube M, Reinhardt R, Rokhsar DS, Montagnani C, Joubert C, Piquemal D, and Degnan BM. 2010. Parallel Evolution of Nacre Building Gene Sets in Molluscs. *Mol Biol Evol* 27:591-608.

Kandel ER. 1979. *Behaviorial biology of Aplysia*. San Francisco W. H. Freeman and Company.

Kempf SC. 1984. Symbiosis between the zooxanthellae Symbiodinium (=Gymnodinium) microadriaticum (Freudenthal) and four species of nudibranchs. *Biological Bulletin* 166.

Kerstitch A, Sea Challengers, Monterey, California 1989. 1989. *Sea of Cortez Marine Invertebrates, A Guide for the Pacific Coast, Mexico to Ecuador*. Monterey, California: Sea Challengers.

331 Klussmann-Kolb A, and Dinapoli A. 2006. Systematic position of the pelagic Thecosomata and
332   Gymnosomata within Opisthobranchia (Mollusca, Gastropoda) – revival of the Pteropoda.
333   *Journal of Zoological Systematics and Evolutionary Research* 44:118-129.
334 Kriegstein AR. 1977a. Stages in the post-hatching development of Aplysia californica. *J Exp*
335   *Zool* 199:275-288.
336 Kriegstein AR. 1977b. Stages in the post-hatching development of Aplysia californica. *Journal of*
337   *Experimental Zoology* 199:275-288.
338 Kriegstein AR, Castellucci V, and Kandel ER. 1974. Metamorphosis of Aplysia californica in
339   laboratory culture. *Proceedings of the National Academy of Sciences* 71:3654-3658.
340 Kröger B, Vinther J, and Fuchs D. 2011. Cephalopod origin and evolution: a congruent picture
341   emerging from fossils, development and molecules. *Bioessays* 33:602-613.
342 Lawrence KA, and W. H. Watson I. 2002. Swimming Behavior of the Nudibranch Melibe
343   leonina. *Biol Bull* 203:144-151.
344 Lowenstam HA, and Weiner S. 1989. *On Biomineralization*. New York Oxford University Press.
345 Medina M, and Walsh PJ. 2000. Molecular systematics of the order Anaspidea based on
346   mitochondrial DNA sequence (12S, 16S, and COI). *Mol Phylogenet Evol* 15:41–58.
347 Mikkelsen PM. 1996. The evolutionary relationships of Cephalaspidea s. l. (Gastropoda:
348   Opisthobranchia): a phylogenetic analysis. *Malacologia* 37:375–442.
349 Morton JE. 1960. *Molluscs -An Introduction to Their Form and Function* Harper & Brothers.
350 Morton JE, and Holme NA. 1955. The occurrence at Plymouth of the opisthobranch Akera
351   bullata with notes on its habits and relationships. *J Mar Biol Assoc UK* 34:101–112.
352 Nadeau L, Paige J, Starczak V, Capo T, Lafler J, and Bidwell J. 1989. Metamorphic competence
353   in *aplysia californica* cooper. *Journal of Experimental Marine Biology and Ecology*
354   131:171-193.
355 Paige JA. 1986. THE LABORATORY CULTURE OF 2 APLYSIIDS, APLYSIA-BRASILIANA
356   RANG, 1828, AND BURSATELLA-LEACHII-PLEI (RANG, 1828) (GASTROPODA,
357   OPISTHOBRANCHIA) IN ARTIFICIAL SEAWATER. *Veliger* 29:64-69.
358 Paige JA. 1988. Biology, metamorphosis and postlarval development of *Bursatella leachii plei*
359   rang (Gastropoda: Opistobrachia). *Bulletin of Marine Science* 42:65-75.
360 Ramos LJ, Lopez Rocafort JL, and Miller MW. 1995. Behavior patterns of the aplysiid gastropod
361   Bursatella leachii in its natural habitat and in the laboratory. *Neurobiol Learn Mem*
362   63:246-259.
363 Rudman WB. 1981. Polyp mimicry in a new species of aeolid nudibranch mollusc. *Journal of*
364   *Zoology* 193:421-427.
365 Rudman WB, and Avern G. 1989. The genus Rostanga (Nudibranchia: Dorididae) in the Indo-
366   West Pacific *Zoological Journal of the Linnean Society* 96:281-338.
367 Rumpho ME, Summer EJ, and Manhart JR. 2000. Solar-powered sea slugs. Mollusc/algal
368   chloroplast symbiosis. *Plant physiology* 123:29-38.
369 Sarashina I, Yamaguchi H, Haga T, Iijima M, Chiba S, and Endo K. 2006. Molecular evolution
370   and functionally important structures of molluscan dermatopontin: Implications for the
371   origins of molluscan shell matrix proteins. *Journal of molecular evolution* 62:307-318.
372 Switzer-Dunlap M. 1978. Larval biology and metamorphosis of aplysiid gastropods. In: F-S C,
373   and ME R, eds. *Settlement and metamorphosis of marine envertebrate larvae* New York:
374   Elsevier, 197-206.
375 Switzer-Dunlap M, and Hadfield MG. 1977. Observations on development, larval growth and
376   metamorphosis of four species of Aplysiidae (Gastropoda: Opistobranchia) in laboratory
377   culture. . *Journal of Experimental Marine Biology and Ecology* 29:245-261.
378 Tamse C, Kuzirian A, and Capo T. 1990. Roller culture system: The fitness machine for
379   *Hermissenda crassicornis* larvae. *Amer Malacol Union Abstr 56th Ann Mtg*:61.

380   Thollesson M. 1999. Phylogenetic analysis of Euthyneura (Gastropoda) by means of the 16s
381   rRNA gene: use of a fast gene for higher-level phylogenies. *Proc R Soc Lond B* 266:75–
382   83.
383   Thompson TE. 1961. The importance of the larval shell in the classification of the Sacoglossa
384   and the Acoela (Gastropoda: Opisthobranchia). *Proc Malac Soc London* 34:233-258.
385   Vermeij GJ. 1993. *Evolution and Escalation: An Ecological History of Life*: Princeton University
386   Press.
387   Vermeij GJ. 2013. On Escalation. *Annual Review of Earth and Planetary Sciences* 41:1-19.
388   Wagele H, and Klussmann-Kolb A. 2005. Opisthobranchia (Mollusca, Gastropoda) – more than
389   just slimy slugs. Shell reduction and its implications on defence and foraging. *Frontiers*
390   *in Zoology* 2.
391   Weiner S DP. 2003. An overview of biomineralization processes and the problem of the vital
392   effect. *Biomineralization*. Washington, DC 1-29.

Comparison of developmental schedules of *Aplysia californica* and *Bursatella leachii.*

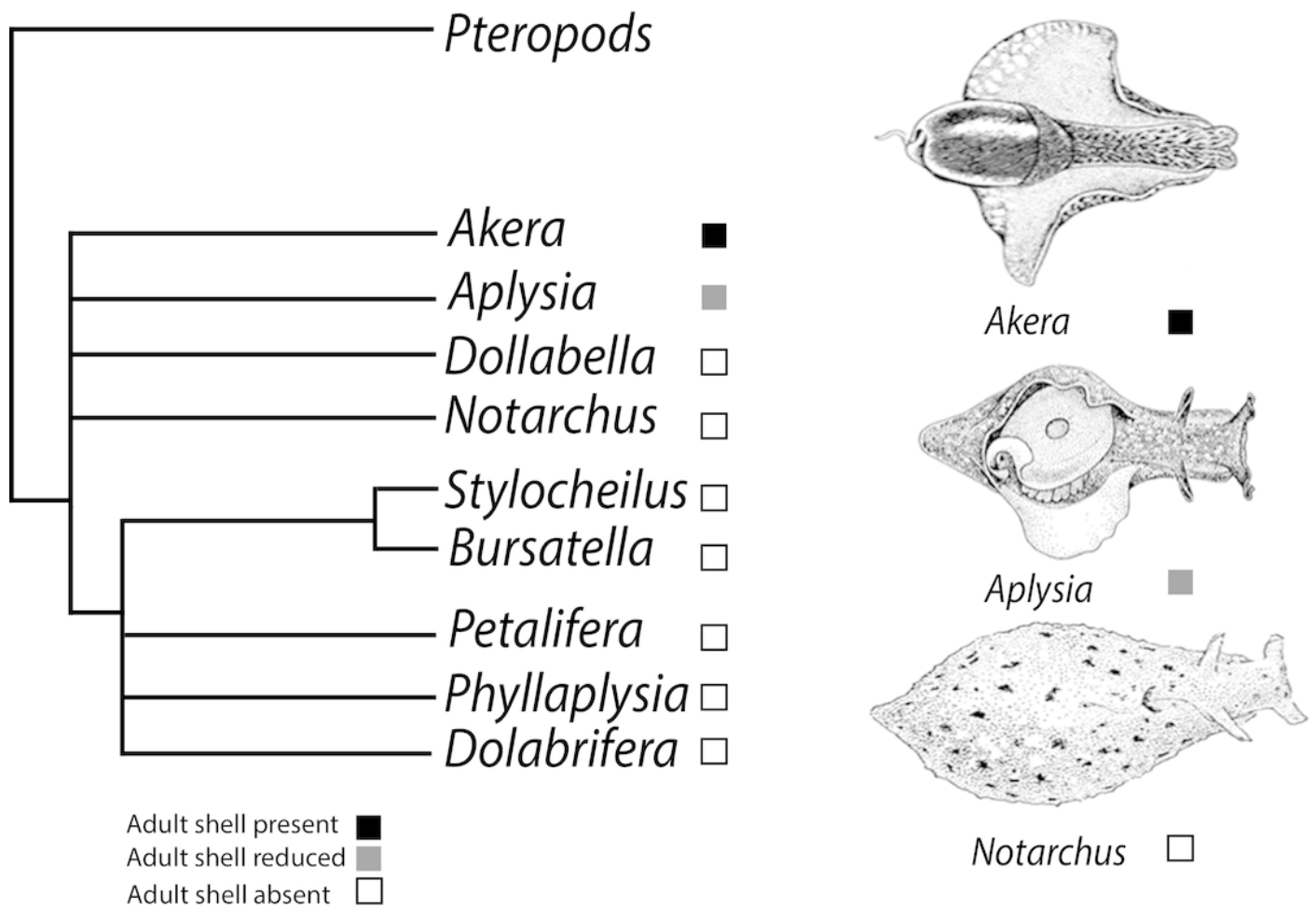
Comparison of morphological development schedules of *A. californica* larvae as reported by (Kriegstein, 1977b) compared to (Capo et al., 2009) and comparison of*B. leachii* larvae as reported by (Paige, 1988) compared to the present study. Values are the number of days post-hatching until the specified developmental stage was observed.

* Paige, 1988 **Kriegstein, 1977 ***Capo et al., 2009 ^ 50 beats/minute not taken into consideration ˇ Present Study

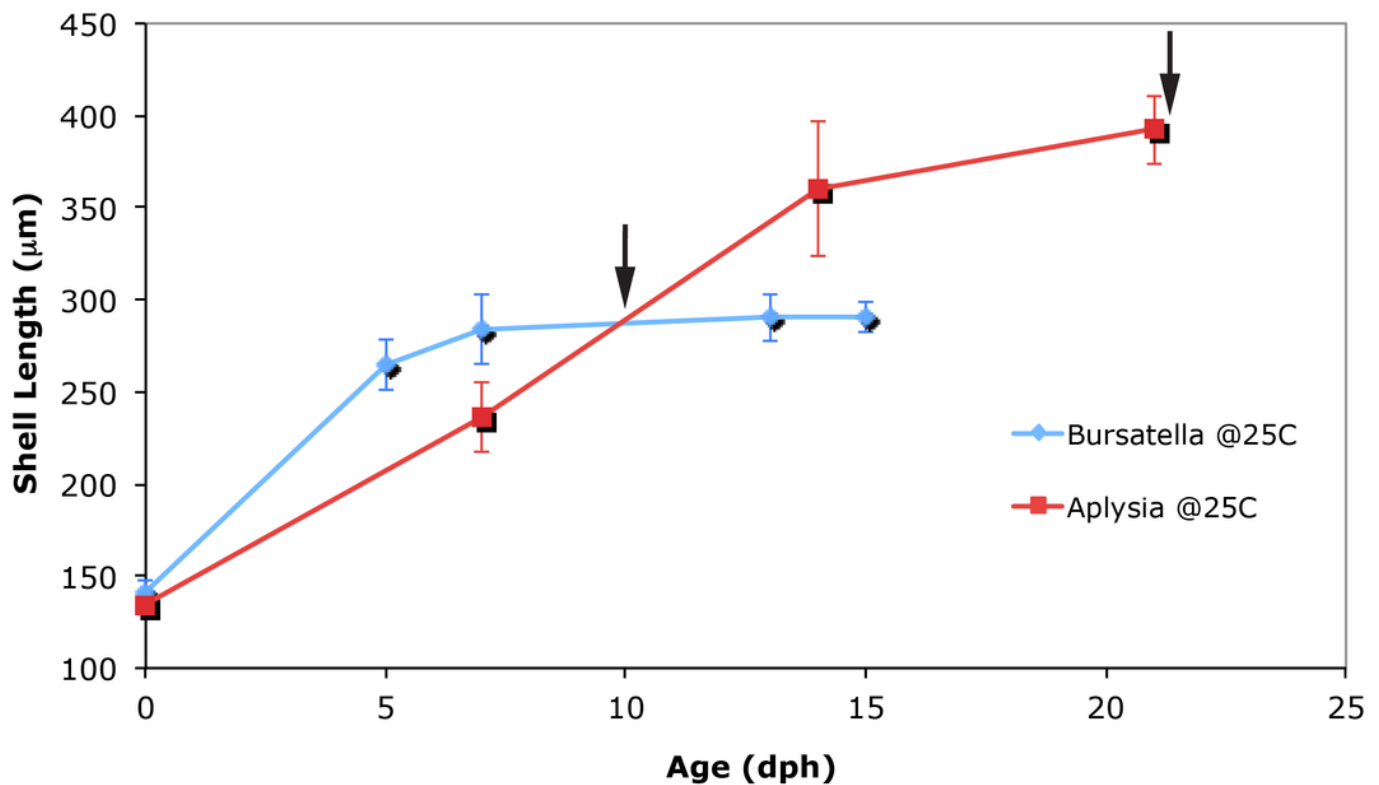
Stage	Description	Bursatella*	Bursatella[x]	Aplysia**	Aplysia***
2	Eyes	6	4	14	7
3	Larval Heart	12	6^	21	14^
4	Maximum Shell Size	15	7	28	17
5	Propodium	17	7	30	19
6	Competency	19	9	32	22
6	Red Spots	None	1 large spot	Present	Present
7	Metamorphosis	20	12	34	24

1

Phylogenetic tree depicting relationships of Anaspidea

Consensus phylogeny of sea hares (Anaspidea) compiled from Medina and Walsh (2002) and Klussmann-Kolb and Dinapoli (2006). Shell character states are depicted by boxes on terminal nodes.

2

Larval and juvenile growth of *Bursatella leachii* and *Aplysia californica* in laboratory settings.

Veliger shell length of *A. californica* and *B. leachii* larvae grown at 25˚C in 2006. Shell length was measured weekly from day of hatching until 80% competency, error bars represent ± 1 standard deviation. Arrow indicates timing of competency: 9 days post-hatching in *B. leachii* and 22 days post-hatching in *A. californica.* Previous attempts to culture *B. leachii* larvae at 22˚C were unsuccessful (not shown).

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 velar 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 heartbeat. 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.