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Authors

Lyons, Deirdre C

Henry, Jonathan Q

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Slipper snail tales: How *Crepidula fornicata* and *Crepidula atrasolea* became model molluscs

Deirdre C. Lyons[a,*], **Jonathan Q. Henry**[b]

[a]U.C. San Diego, Scripps Institution of Oceanography, La Jolla, CA, United States

[b]Marine Biological Laboratory, Marine Resources Center, Woods Hole, MA, United States

Abstract

Despite the great abundance and diversity of molluscs, only a few have attained "model research organism" status. One of those species is the slipper snail *Crepidula fornicata*. Its embryos were first used for classical lineage tracing studies in the late 19th century, and over a 100 years later they were "re-discovered" by our labs and used for modern fate mapping, gene perturbation, in vivo imaging, transcriptomics, and the first application of CRISPR/Cas9-mediated genome editing among the Spiralia/Lophotrochozoa. Simultaneously, other labs made extensive examinations of taxonomy, phylogeny, ecology, life-history, mode of development, larval feeding behavior, and responses to the environment in members of the family Calyptraeidae, which includes the genus *Crepidula*. Recently, we developed tools, resources, and husbandry protocols for another, direct-developing species, *Crepidula atrasolea*. This species is an ideal "lab rat" among molluscs. Together these species will be valuable for probing the cellular and molecular mechanisms underlying molluscan biology and evolution.

1. Introduction

Molluscs are abundant, diverse, and commercially valuable to humans, yet none of the more than 90,000 extant species serves as a *singular* molluscan model for biomedical research. Instead, several model species have been studied, with each possessing unique qualities useful for research (Davison & Neiman, 2021; Lesoway & Henry, 2019b; Wanninger & Wollesen, 2018). In one prime example, marine snails in the genus *Crepidula* have been used to study a wide variety of questions ranging from molecular and cell biology to ecology (Henry, Collin, & Perry, 2010; Henry & Lyons, 2016; Lesoway & Henry, 2019b). For example, *C. fornicata* development was first studied in the late 1800s for classical lineage tracing (Conklin, 1897). Over 100years later it was found to be an excellent species for cellular and molecular studies, especially because reagents for modern fate-mapping and gene perturbation could be delivered to their embryos, and the subsequent phenotypes readily studied during development (Henry, Collin, et al., 2010; Henry, Perry, & Martindale, 2010; Henry & Lyons, 2016; Henry, Lesoway, et al., 2017). *Crepidula* have also been the subject of a wide array of "eco-evo-devo" studies over the last 50+ years due to the diversity of their life history strategies and modes of development (Henry, Collin, et al., 2010). In

*Corresponding author: d1lyons@ucsd.edu.

this review we first introduce members of this group of snails and discuss how and why two species in particular were selected for more intensive study. We highlight recent insights into molluscan biology that came from studies with various *Crepidula* spp., and conclude by proposing future areas for research including biomineralization, sex determination, lineage tracing in adults, and transgenesis.

2. Natural history of the genus *Crepidula*

At least 53 *Crepidula* species have been recognized, although reassignments of previously classified *Crepidula* species to new genera like *Bostrycapulus* and *Crepipatella* have occurred since the first molecular phylogenetic analyses by Rachel Collin (Smithsonian Tropical Research Institute) (Collin, 2003a, 2005; Collin, Chaparro, Winkler, & Véliz, 2007). The motivation of Rachel's work was to understand the evolution of mode of development in marine invertebrates using a group for which inter-specific variation in development was already well-documented. Calyptraeid gastropods were selected, as K. Elaine Hoagland had revised the taxonomy of *Crepidula* in the 1970s (Hoagland, 1977) and had subsequently documented significant diversity in development across the group (Hoagland, 1978, 1984, 1986).

Crepidula snails are members of the subclass Caenogastropoda, belonging to the family Calyptraeidae, and are colloquially referred to as "slipper snails" because of the shoe-like shape of their shells (Fig. 1). Other common names include: slipper limpet, lady's slipper, baby's boats, boat shell, and quarter-deck shell. The shells of *Crepidula* are relatively simple and flattened, and possess an internal shelf or "deck" that separates the foot from the viscera (Fig. 1A, B, E; Henry, Collin, et al., 2010). In some species, individuals live together in curved stacks (Fig. 1A, D), while other species remain unstacked and firmly attached to a substrate by their foot (Fig. 1A and B). Taxonomy in this group can be challenging because of their relatively simple, plastic (influenced by environmental conditions) shells and the existence of cryptic species, though other characteristics like developmental mode and molecular signatures have been used to distinguish species (e.g., Collin, 2000).

Like other calyptraeids, adult slipper snails are sedentary filter feeders. They lift themselves slightly off the surface of the adjacent snail or other hard substrate beneath them, and use their gills to create currents that trap phytoplankton, which are transferred to the mouth and ingested. *Crepidula* are protandrous hermaphrodites, changing from male to female as the animal grows (Hoagland, 1977; Lesoway & Henry, 2019a). They live in temperate and tropical climates around the world in intertidal and shallow subtidal zones; perhaps because of their accessibility and wide distribution, various researchers work on a wide range of "local" species. Animals that settle and grow on the hulls of shipping vessels, or those that grow directly on oysters that are being transported for human consumption, have been introduced outside of their endemic ranges, making them excellent models for studying invasiveness (Moulin et al., 2007; Pechenik, Diederich, Browman, & Jelmert, 2017). Introduced populations can impact native species of *Crepidula* and damage local fisheries (e.g., oyster beds) by competing for habitat (Blanchard, 1997).

3. *Crepidula* are well-suited for studying the links between ecology, evolution, and development

Calyptraeid phylogeny is well-resolved and has been used to infer the direction of dispersal around the globe and the nature of speciation events (Collin, 2003a), as well as the evolution of modes of development (Collin, 2000, 2001, 2003a, 2003b, 2003c, 2004, 2019; Collin et al., 2007; Veliz, Winkler, Guisado, & Collin, 2012). The clade includes species with indirect development with planktonic feeding larvae, as well as direct developers, and also species that exhibit adelphophagy (cannibalism of one embryo by another) and poecilogony (intraspecific variation in developmental mode; McDonald, Collin, & Lesoway, 2014). This variation can be examined in light of the group's solid phylogeny. For example, the evolutionary loss of planktonic feeding larvae is generally thought to be irreversible, but mapping the modes of development onto the calyptraeid phylogeny suggested instead that there were at least three instances of re-gaining feeding larvae (Collin, 2004; Collin et al., 2007).

Within this framework, studies have also examined the (1) variation of feeding behaviors and structures (Collin, 2003; Collin & Moran, 2018; Collin, Shishido, Cornejo, & Lesoway, 2021), (2) the molecular differences that correlated with embryos that cannibalize sibling embryos vs those that are cannibalized (Lesoway, Abouheif, & Collin, 2016; Lesoway, Collin, & Abouheif, 2017), and (3) the relationship between life history variation and environmental factors (Carrillo-Baltodano & Collin, 2015; Collin & Salazar, 2010; Ly & Collin, 2021; Mérot & Collin, 2012). Other studies examined the way *Crepidula* species respond to stressors like low pH and nutritional reduction, to predict how marine species might respond to global climate change (Bogan, McMahon, Pechenik, & Pires, 2019; Maboloc & Chan, 2021; Maboloc & Chan, 2017; Reyes-Giler et al., 2021). Those studies measured the effects of such stressors on the rates of metamorphosis and larval growth in the F0 population, as well as measured the effects on the F1 generation (when the F0 have been exposed to stressful conditions) to identify multigenerational consequences of stress. The effects of microplastics on larval settlement and growth have also been examined (Lo & Chan, 2018).

4. Why did we choose *C. fornicata*?

We began using *C. fornicata* because of the advantages of working with their embryos, and because of the historical knowledge about their embryology that was gleaned over 125years ago from the work of Edward Grant Conklin (1863–1952) at the Marine Biological Laboratory, Woods Hole, MA. Conklin was an American developmental biologist who studied cytological details of marine invertebrate embryos to understand developmental proximal causes–roughly akin to what we now call "mechanisms." Along with contemporaries like E.B. Wilson, F.R. Lillie, and T.H. Morgan, Conklin worked at a pivotal time in American biological research when natural history and morphological explanations of biology were unseated by approaches that used experimentation. As a graduate student with W. K. Brooks at Johns Hopkins University, Conklin traveled to the Marine Biological Laboratory to initially study siphonophores, but finding none there,

he looked for other embryonic material and settled on *C. fornicata* and related species (Maienschein, 2015). Abundant on the east coast of North America, *C. fornicata* (Linnaeus, 1758) lives in communal stacks that form characteristic arches (Fig. 1D), from which their species name is derived; *fornicata* comes from the Latin fornix, for arch. Conklin found that *C. fornicata* produced large broods (Fig. 1E and F) of synchronously-dividing embryos, making it easy to follow early cell divisions; nuclei and mitotic spindles were observable in fixed, stained, and sectioned specimens (Conklin, 1897; Steinert, 2016). The abundant material allowed Conklin to construct a developmental series and early embryonic cell lineage. Similar work in multiple mollusc and annelid species allowed Conklin, Wilson, Lillie and others the means to identify specific developmental cellular homologies and evolutionary relationships among animals with divergent body plans.

Conklin's meticulous studies of the early embryo led to his identification of a single cell that gave rise to mesoderm. That cell, which is born at the 25-cell stage, is now referred to as the 4d cell. Conklin recognized that it forms both endoderm and mesoderm, and that it is the first lineage in the embryo to exhibit bilateral symmetry. This finding was significant because Conklin's goal was to relate the early cell division of the egg to the formation of germ layers and axial properties of fully formed organs. Conklin made other important observations and contributions; though he nearly overlooked this, he reported that *C. fornicata* possesses a very small polar lobe, a transient cytoplasmic protrusion characteristic of some annelid and mollusc embryos, identifying another important similarity between the groups. His most enduring contribution may be his beautiful and detailed camera lucida drawings of developmental stages, and his improved spiralian nomenclature system that is still used in lineage studies today (Figs. 2 and 4).

5. How did evo-devo research with *C. fornicata* start up again?

After Conklin, no further lineage tracing studies used *C. fornicata* over the next century until Jonathan Henry's and Mark Martindale's work in the summer of 2004. They started working on *C. fornicata* rather accidentally–as Conklin had previously–when the animals they had planned to work with that summer in the Embryology course (taught at the Marine Biological Laboratory) were not gravid. Starting several years before that, Jon and Mark began a collaboration to apply modern lineage tracing techniques to less-studied invertebrate groups. Having first met in 1981 in graduate school at the University of Texas, Austin in the lab of Gary Freeman, they began work on experimental embryology and lineage tracing; this endeavor eventually turned into a collaboration that took-off during their respective postdoctoral years and continued after they received faculty positions. They promoted the use of a wide range of animals, including spiralians/lophotrochozoans (Henry, 2014), as important subjects for understanding the evolution of cell fate specification and axial patterning in metazoans (Henry & Martindale, 1998, 1999; Martindale & Henry, 1998).

Together they presented key findings about the regulative ability and fate map of nemerteans (Henry & Martindale, 1994, 1995, 1996; Martindale & Henry, 1995a), the cell lineage of acoels and polyclad turbellarians (Boyer, Henry, & Martindale, 1996; Henry, Martindale, & Boyer, 2000), the cell lineage of hemichordates (Henry, Tagawa, & Martindale, 2001), and the nature of inductive interactions, regulative development, regeneration, and the unique

fate map of ctenophores (Martindale & Henry, 1995b, 1997), among others. Along with contemporaneous lineage tracing studies carried out on other spirally-cleaving organisms [including *Platynereis* (Ackermann, Dorresteijn, & Fischer, 2005), leeches (Stuart, Blair, & Weisblat, 1987; Weisblat & Shankland, 1985), and gastropod molluscs *Patella* and *Tritia* (*Ilyanassa*) (Damen & Dictus, 1994; Lambert, 2001; Render, 1991)] their work helped to revive an interest in using comparative lineage tracing to answer evo-devo questions in spiralians. Some of these fundamental questions remained the same as in Conklin's era, including: How are the organs and axial properties of larvae and adults related to the organization of the egg and early embryonic cells? How plastic (regulative) or fixed (mosaic) are cell lineages? How are cell lineages modified over the course of evolution to build disparate body-plans? On the other hand, the tools and intellectual framework had changed considerably; for example Conklin did not have antibiotics at his disposal and could not raise embryos outside of their capsules for very long, making experimentation difficult. Mark and Jon also had at their disposal high resolution microscopes, fluorescently tagged intra-cellular lineage tracers, and phylogenetic trees based on molecular characters, upon which one could map developmental character states and their transitions. As an undergraduate at Mount Holyoke College (MA), Dede Lyons read their papers, which sparked her interest in leveraging spiralians' homologous cell lineages to elucidate the mechanisms of developmental evolution (Lyons & Weisblat, 2009).

Like Conklin before them, Henry and Martindale found that *C. fornicata* embryos were excellent for lineage studies (Fig. 2). The large clutches of synchronously-developing eggs were easily freed from their capsules (Fig. 1E and F) with tweezers, and the embryos were "naked" inside, free of any additional maternal investments like fertilization envelopes. Thus, the embryos were amenable for microinjection and experimental manipulation. The relatively large zygotes (180μm) proved easy to inject, as did the smaller daughter cells of the four basic embryonic quadrants A, B, C, D, and tiers of micromeres 1q through 4q (Figs. 1E and F, and 2). Jon and Mark, along with Andi Hejnol, leveraged these advantages to construct an initial modern cell lineage fate map for the major founding lineages in the embryo (Fig. 2; Hejnol, Martindale, & Henry, 2007; Henry, Hejnol, Perry, & Martindale, 2007). Jon and Mark also determined the developmental role of the small polar lobe that Conklin had first observed (Henry, Perry, & Martindale, 2006). *C. fornicata* produces two very small polar lobes during the first two rounds of cell division (Henry et al., 2006). They showed that the polar lobe usually fuses with the future D cell at the four-cell stage. Using a combination of lineage tracing, surgical removal of the polar lobe, blastomere isolations, and individual blastomere ablations, their experiments established that *C. fornicata* behaves like other "equal-cleaving" spiralian embryos and that the D-quadrant organizer, 4d (the cell that Conklin first identified as the mesentoblast), is conditionally specified from signals sent by the primary quartet micromeres. The "equal cleavage" nature of *Crepidula* development would prove to be an important factor for comparing developmental mechanisms with another well-studied caenogastropod, *Tritia* (*Ilyanassa*) *obsoleta*, which has larger polar lobes (Chan & Lambert, 2014; Lambert, Johnson, Hudson, & Chan, 2016; Rabinowitz, Chan, Kingsley, Duan, & Lambert, 2008). Jon and Mark continued working with *C. fornicata* embryos in subsequent summers, resulting in a detailed fate map, and in examinations of the developmental role of signaling pathways including

B-catenin (Henry, Perry, & Martindale, 2010). Inspired by the work in *Tritia* (*Ilyanassa*) (Lambert & Nagy, 2001, 2003), Jon and his longtime collaborator, Kim Perry, also studied the role of MAPK signaling in organizer specification (Henry, Collin, et al., 2010; Henry & Lyons, 2016; Henry & Perry, 2008).

6. Growing the *Crepidula* evo-devo community

While Jon and Mark carried out their work at the MBL and taught in the Embryology course, they helped train a generation of students on embryological manipulation, intracellular injection, and comparative development. In earlier years, injected material would often be fixed and brought home to their labs at the University of Illinois or the University of Hawaii for further examination and documentation. This strategy was successful for some studies (the bulk of the images in their first *C. fornicata* lineage paper were taken by Andi Hejnol from fixed samples); however, more detailed lineage tracing experiments needed to be documented closer to the time of injection, or preferably on live samples, because the tracer signals tended to fade over time. When Dede took the Embryology course in the summer of 2009, Jon and Mark invited her to image live specimens as part of a study to determine the fate map of 4d sublineages. Jon invited Dede back in subsequent summers as a teaching assistant for the Embryology course to help complete the 4d lineage fate map (Lyons, Perry, Lesoway, Henry, 2012). This project grew into a long-term collaboration resulting in an series of detailed sub-lineage fate maps and live-imaging studies of morphogenesis (Henry & Lyons, 2016; Lyons & Henry, 2014; Lyons, Perry, Batzel, & Henry, 2020; Lyons, Perry, Henry, 2015, 2017; Lyons et al., 2015, 2017), and other studies examining the expression patterns of regulatory genes (Perry et al., 2015).

Through interactions with other students and visitors to the MBL and to the Embryology course, Jon encouraged and supported a number of other junior researchers to use *C. fornicata*, including Cristina Grande (Universidad Autónoma de Madrid, Spain) and her student Marta Truchado-Garcia (U.C. Berkeley, CA), Maryna Lesoway (U.C. San Diego, CA), Victoria Sleight (University of Aberdeen, Scotland), Nicole Webster (University of Saskatchewan, Canada), and others. This community continues to grow. Summaries of work through 2016 can be found in Henry and Lyons (2016). In the next sections we highlight several recent studies using *C. fornicata*.

7. Evolution of the spiralian embryonic organizer

Spiralians are an excellent group for examining the evolution of embryonic axial patterning (Lambert, 2008, 2010). The spiral cleavage program is conserved enough that individual cells can be compared between species, yet there is also enough variation in cleavage patterns and developmental mechanisms that one can use them to understand how axial patterning evolved (Hejnol, 2010; Henry, 2014; Lambert, 2010; Martín-Durán & Marlétaz, 2020). For example, experimental embryology studies revealed that the embryonic organizer is a different D-quadrant cell in *C. fornicata* (4d; Henry, Lyons, Perry, & Osborne, 2017; Henry, Lesoway, et al., 2017; Henry et al., 2006), mudsnail *Tritia* (*Illyanasa*) *obsoleta* (3D; Lambert, 2001; Lambert & Nagy, 2001), and the polychaete annelid *Capitella teleta* (2d; Amiel, Henry, & Seaver, 2013). The fact that organizer activity resides in cells of

different birth order in these species provides a unique opportunity for comparative studies of developmental mechanisms.

As noted above, even though *C. fornicata* produces a small polar lobe, the specification of the 4d organizer is more similar to that of equal-cleaving species (e.g., *Lymnaea*, Martindale, 1986; Martindale, Doe, & Morrill, 1985) than to unequal-cleaving species (examples of the latter include *Tritia obsoleta* and *Capitella teleta*). Similar to the results found in experiments with equal-cleaving spiralian embryos (Martindale, 1986; Martindale et al., 1985; van den Biggelaar, 1977; van den Biggelaar & Guerrier, 1979), cell ablation experiments in *C. fornicata* revealed that the first-quartet micromeres (cells 1q and daughters 1q1) are necessary for organizer specification (Henry, Lyons, et al., 2017; Henry, Lesoway, et al., 2017). If these cells are deleted prior to the birth of the organizer (4d), the resulting embryos lack a dorsal-ventral axis, indicating misspecification of the D quadrant. Therefore, the D quadrant is specified by an inductive signal from these micromeres sometime between 5th (20-cell stage) and 6th (24-cell stage) cleavage (Henry, Lyons, et al., 2017, Henry, Lesoway, et al., 2017). Furthermore, there is evidence that the micromeres must themselves be primed, by even earlier signaling (Henry & Perry, 2008). This knowledge about the timing of D quadrant specification, and of organizer cell birth, makes *Crepidula* a valuable species to better understand and dissect these early developmental processes among spiralians, at single-cell resolution.

8. Axial patterning and the primary quartet micromeres

We carried out other recent studies focused on the morphogenesis and molecular specification of the primary quartet micromeres (1q or 1a-1d; Fig. 2). The primary quartet micromeres were the last major cell lineages of the embryo that we fate-mapped in detail (Lyons et al., 2017). The classical spiralian lineage tracing literature assumes that 1q2 (1a2–1d2) daughter cells of the primary quartet micromeres universally give rise to the prototroch (larval feeding organ). On the contrary, we found that the 1q2 cells in fact never contribute to the prototroch, and instead actually make a provisional epithelium that might allow the embryo to undergo morphogenesis—a sort of “bending” movement—during gastrulation stages. This work made several additional seminal contributions to the spiralian development field, including proposing a new model for how the presumptive mouth cells become displaced to the animal territory during development and how the anterior neural tissues rearrange during embryonic stages.

In another study, where we examined the role of BMP signaling in early *C. fornicata* development, we found that instead of having an obvious role in specification of the organizer itself, the BMP pathway mediates the proper “relay” of organizer signaling to specify cells within the 1q1 sublineages, which give rise to the eyes and the central nervous system (Lyons et al., 2020). The fact that we found BMP signaling to be dispensable for organizer specification and initial organizer signaling is significant: a series of recent papers examining the role of BMP in organizer function in *Tritia* (Lambert et al., 2016), *Capitella* (Lanza & Seaver, 2018; Webster, Corbet, Sur, & Meyer, 2021), and *Lottia* (Tan, Huan, & Liu, 2022) have found widely differing results, despite the highly conserved spiral cleavage

program. It will be interesting to examine the mechanisms of BMP signaling in more depth in *C. fornicata*, as well as to identify the molecular nature of the organizer.

9. Unique sources of mesoderm

One interesting aspect of Spiralian development is that they have two different sources of mesoderm (Lyons & Henry, 2014). Some mesoderm is derived from an endodermal precursor as "endomesoderm." Endomesoderm is believed to represent the ancestral source of mesoderm within the Metazoa. In many spiralians, such as *C. fornicata* (but not all cases), endomesoderm arises from the fourth quartet daughter of the D quadrant, 4d (the "mesentoblast" cell, Lyons et al., 2012). This source gives rise to some endodermal tissue, such as the intestine, as well as many larval and adult mesodermal tissues, including: various muscles (e.g., the retractor muscle, foot muscles, etc.), the kidney complex, the heart, and primordial germ cells. A second source arises from ectodermal precursors as "ectomesoderm." The combination of ectomesodermal cells varies between different spiralian species, but typically they arise from ventral daughters of the 2nd and/or 3rd quartet micromeres (Fig. 2). In *Crepidula*, these cells arise specifically from the 3a2 and 3b2 cells. Ectomesodermal progeny undergo EMT as they leave the edge of the blastopore (Lyons et al., 2015). These cells contribute to the formation of various tissues, including the larval retractor muscle, the radial muscles in the larval velum, as well as scattered mesenchyme, and also pigment cells in the velum, in addition to a few unidentified progeny associated with the apical organ (J. Henry and K. Perry, Unpublished data). Little is known about the evolutionary origins of this alternate source of mesoderm and there are many unanswered questions. Do endo- and ectomesoderm share a common embryonic origin? Did ectomesoderm arise by co-opting the same ancestral endomesodermal gene regulatory network (GRN) (Perry et al., 2015)? Alternatively, these two sources of mesoderm may be regulated by completely different GRNs. Furthermore, one can ask what genes regulate the process of EMT in the ectomesodermal progeny, and is this basic behavior regulated via a conserved EMT GRN (Osborne, Perry, Shankland, & Henry, 2018)? While there are some other examples of so-called ectomesenchyme that also undergo EMT, such as the neural crest in vertebrates, ectomesoderm appears to represent a spiralian novelty.

10. Shell development and biomineralization

Molluscs are well known for diversification of their shell structures (Kocot, Aguilera, McDougall, Jackson, & Degnan, 2016). The diversity is the result of organic extracellular matrix proteins (shell matrix proteins) directing the growth of mineral structures during larval and adult stages (Clark, 2020; Kocot et al., 2016). Shell matrix proteins (SMPs) have been identified by proteomics in many molluscs, but few have been studied in detail (Jackson & Degnan, 2016). *C. fornicata* is a good model for studying biomineralization at a mechanistic level. Intracellular fate-mapping showed that all 8second-quartet daughter cells (2a1/2a2, 2b1/2b2, 2c1/2c2, 2d1/2d2; Fig. 2) contribute to the shell gland (Hejnol et al., 2007; Lyons et al., 2015). Screens for transcription factors and signaling pathway components led to the identification of at least 24 genes that are expressed in the *C. fornicata* shell gland, which makes the larval shell (Figs. 3 and 4; Perry et al., 2015; Osborne et al., 2018; Lyons et al., 2020; Truchado-Garcia, Caccavale, Grande, & D'Aniello, 2021).

Using proteomics, we identified 185 SMPs occluded within the adult shell of *C. fornicata* (G. Batzel et al., in review). Differential gene expression analysis identified 20 SMP genes that are differentially expressed in the shell-producing mantle tissue (Figs. 3A and C, and 4A–C). Through in-situ hybridization we identified two genes, CfSMP1 and CfSMP2, that are expressed exclusively in the shell gland (Fig. 4D–F). These data reveal that the two most differentially expressed SMPs in the adult are also expressed in the shell gland. With this new knowledge about the terminal differentiation "nodes" (=SMPs), we can now carry out perturbation experiments of upstream "inputs" (=previously identified transcription factors) to build an experimentally-verified biomineralization GRN in a mollusc.

11. Sex determination

All calyptraeid gastropods are sequential hermaphrodites, meaning they transition from one sex to the other. As male-first (protandric) hermaphrodites, calyptraeids mature first to have an external penis and internal testis with sperm, before transitioning to having female external genitalia (female genital papilla) and producing oocytes. Sex determination in calyptraeids is environmentally determined, and the timing of sexual transitions is controlled by several factors including age, size, nutritional state, and social environment (reviewed in Lesoway & Henry, 2019a). Typically, males will delay their transition, when in the presence of females. In the absence of females, males change sex earlier and at smaller sizes (Lesoway & Henry, 2019a). Some species are less influenced by their social environment, which appears to correlate with the level of gregariousness of a particular species (Hoagland, 1978).

While the ecological and evolutionary consequences of sex determination in calyptraeids is of long-standing research interest (e.g., Collin, 1995, 2007), knowledge of the molecular underpinnings has lagged. Previous experiments implicated the left pedal ganglion in inducing male development (Le Gall, 1981; Le Gall & Streiff, 1975), including a partial characterization of a potential neuroendocrine peptide that triggers cell division in the gonad Le Gall, Feral, Lengronne, and Porchet (1987). Recent work has shown that targeting specific components of the retinoic acid pathway including retinoic acid receptors (RAR) and retinoid X receptors (RXR) induces penis development in juveniles, and blocking RXR reduces penis length (Lesoway & Henry, 2021). That study showed that both agonists and an antagonist of the RAR receptor increase penis length, suggesting that RAR may function in inhibitor release. Furthermore, exposure to tributyltin (TBT) resulted in high rates of penis induction in females of four different calyptraeid species. Interestingly, females of the small-bodied, solitary species *C. convexa* showed reduced rates of penis induction, correlating with reduced social control over sex determination. Interactions between levels of social control and specific pathways, including initiation of sexual transitions, remain areas for future exploration. More broadly, hermaphroditism, both sequential and simultaneous, has evolved repeatedly in gastropods and other molluscs (Lesoway & Henry, 2019a). Detailed knowledge of shared and divergent mechanisms will play an important role in elucidating evolutionary patterns of sexual development and sex determination in these and other animals.

12. *Crepidula atrasolea*: A complementary model system

While *C. fornicata* is a useful model system for study, another related *Crepidula* species has proved to be even more convenient. Working with *C. fornicata* at the Marine Biological Laboratory and at the University of Illinois, Jon and Kim Perry showed that they could tag endogenous Beta-catenin protein by CRISPR-mediated homology-directed repair (Perry & Henry, 2015). That study demonstrated for the first time the effectiveness of CRISPR-Cas9 mediated gene editing in a representative of the spiralian Lophotrochozoa, setting the stage for the development of transgenic lines carrying fluorescent reporters to study cell and developmental processes.

The F0s generated in the Perry and Henry (2015) study were not raised through metamorphosis to establish a stable line; the life cycle of *C. fornicata* is rather long (at least 1 year), and even if they wanted to raise animals in the lab, they lacked the necessary culturing infrastructure. Therefore, the present authors decided that it was necessary to develop an additional *Crepidula* species that could be easily reared in inland-aquaria throughout its entire life cycle, and one that would have a shorter generation time. Earlier, Rachel Collin had suggested that Jon and Mark use *C. atrasolea* (Figs. 1A–C and 3D–F), which she had recently described (Collin, 2000). This species is native to the warm waters of the southern Atlantic, and could be readily collected near the Smithsonian Marine Station (SMS) at Fort Pierce, Florida. It was first detected as a cryptic species that had previously been lumped with *Crepidula plana* by Elaine Hoagland, who examined the two with allozymes and described developmental differences (Hoagland, 1984, 1986), and who referred to it as *Crepidula* cf. *plana* without formally naming the species. Unlike *C. fornicata*, the larger embryos (280μm) of *C. atrasolea* are direct-developing and hatch out as “crawl-away” juveniles (Fig. 3D–F). The adults do not form stacks, and grow to an adult size of about 1–2cm (Fig. 1A–C). Importantly, their generation time is much shorter than that of *C. fornicata* at 4–6months in captivity (Henry, Lesoway, & Perry, 2020; Henry, Lesoway, et al., 2017), comparable to the generation times of *Danio rerio* and *Xenopus tropicalis* (Showell & Conlon, 2009).

In the winter of 2015 Dede first collected *C. atrasolea* specimens from the waters around the Fort Pierce Marine station, and sent them to Jon’s lab to rear. Jon made a trip to Fort Pierce in 2016 and supplemented the colony with additional specimens. All of the animals now in culture are derived from the animals collected during those two trips. Back in Illinois, Jon, Kim, and Maryna Lesoway, who was then a postdoc in Jon’s lab, developed methods for raising the collected animals through their entire life cycle, described their development, made developmental transcriptomes, and ultimately developed methods for raising them in an automated, recirculating rack system (Henry et al., 2020; Henry, Lesoway, et al., 2017). With the implementation of automated aquatic systems (see below), it has become much easier to raise lots of animals through successive generations. We are now rearing animals in the 7th generation.

13. Development of an automated rack system to culture *Crepidula*

A major challenge is the amount of effort needed to rear marine animals in the laboratory, especially when one is rearing animals through successive generations and plans to maintain multiple inbred and transgenic lines. Recently, we succeeded in developing a fully automated marine aquatic rack system to rear *Crepidula atrasolea* through successive generations (Henry et al., 2020). This multi-tank system takes care of monitoring and adjusting all critical water quality parameters and also accomplishes automated feeding of commercially available liquid phytoplankton food (Fig. 5). Furthermore, the filtration system is designed to process waste and greatly minimize the need to carry out water changes. This aquatic system has decreased the overall amount of effort required to care for animals on a day to day basis and has allowed us to dramatically scale up production. It was instrumental in allowing us to maintain these animals throughout the Covid 19 pandemic with minimal hands-on effort. This system, and the protocols we have developed, will be of broad use to those working with different marine organisms. Jon Henry has recently moved to the Marine Biological Laboratory in Woods Hole, MA to develop additional systems and related tools to culture other aquatic species.

14. What's on the horizon for *Crepidula* research?

One of our highest priorities is sequencing and assembling the genomes for both species. While the *C. fornicata* genome is slated to be sequenced by the Darwin Tree of Life project (https://www.darwintreeoflife.org/), we are leading the effort to sequence the genome of *C. atrasolea*. Not only will these genome sequences form critical backbones for supporting other genomics studies like ATAC-seq, single cell RNA-seq, and spatial transcriptomics, they will provide a valuable opportunity for comparative genomics. For example, we are eager to identify evolutionarily conserved regions within putative promoter sequences, and use them for making knock-in lines of *C. atrasolea*. With stable lines of transgenic animals expressing cell type-specific fluorescent proteins, or photoconvertible proteins like kaede, we could extend our lineage tracing studies to later stages including in the adult. To our knowledge, as of our writing, no lineage tracing studies linking embryonic cell lineages to adult organs have been published in molluscs. With the ability to undertake long-term lineage tracing, many interesting questions about adult molluscan biology could be addressed, including but not limited to: (1) how the shell gland tissue differentiates into the adult mantle tissue organ, (2) how the adult brain forms and grows, (3) how the immune system matures and responds to environmental changes, (4) how the primordial germ cells and gametes develop, and (5) how regeneration of adult tissues is regulated. The cellular and molecular underpinnings of these processes will be studied with gene perturbation studies, especially CRISPR/Cas9 genome editing, ideally with Cas9 being driven by inducible or tissue-specific promoters to perturb gene function in time and space. Access to a fast-growing, laboratory model like *C. atrasolea* enhances our ability to explore these late-stage developmental questions, which we hope will shed light on molluscan biology in general.

15. Join #TeamCrepidula

We encourage more people to use *Crepidula* species for their research and/or teaching. *C. fornicata* can be ordered from the Marine Biological Laboratory's Marine Resource Center. *Crepidula atrasolea* can be requested by emailing Dede Lyons. Our papers describe the methods for working with the adult and embryonic material, as well as accession numbers for transcriptomic data for *C. fornicata* and *C. atrasolea* (Henry, Collin, et al., 2010; Henry & Lyons, 2016; Henry, Lesoway, et al., 2017; Henry, Perry, Fukui, & Alvi, 2010; Truchado-Garcia, Harland, & Abrams, 2018). Additional information about rearing animals in closed aquaria, and collecting, handling, and raising embryos can be found in the supplement to the Henry, Collin, et al. (2010) paper and on Jon Henry's website (https://www.life.illinois.edu/henry/tools.html, see also https://github.com/HenryLabUIUC). Rachel Collin's lab has produced an excellent series of videos describing how to collect and work with marine invertebrates, including *Crepidula* spp. (https://www.youtube.com/user/CollinLabPanama/videos). We welcome anyone interested in joining the larger community working on molluscs and spiralians to follow the growing "SpiraliaBase" community (https://www.spiraliabase.org/; @SpiraliaBase), where many labs share their latest results and "hacks" for working with spiralian animals and their genomes.

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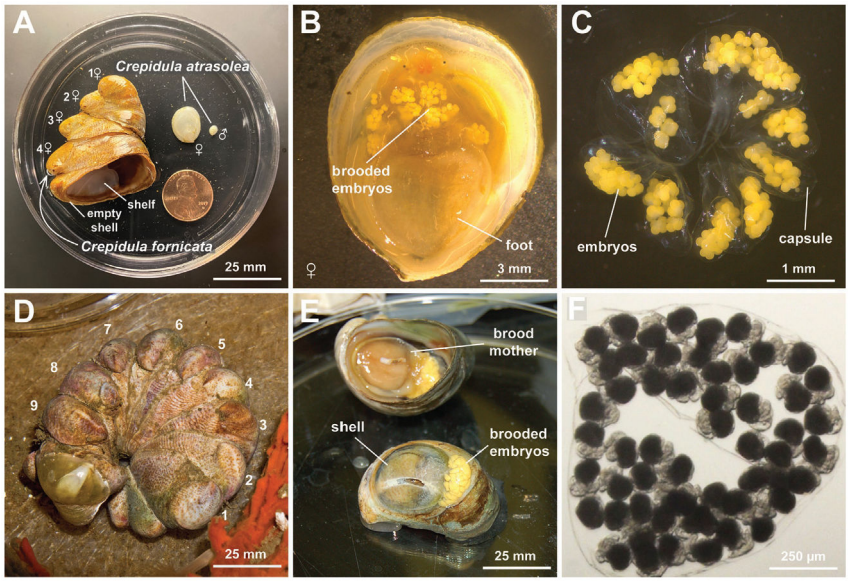

Fig. 1.

Two species of *Crepidula* and their embryos. (A) Adults of *Crepidula fornicata* and *Crepidula atrasolea* reach different sizes at adulthood. For *C. fornicata*, groups of individuals stack on top of each other, whereas adults of *C. atrasolea* remain unstacked, but firmly attached to a substrate. Both species have males that will eventually become female over their lifetime. (B) Ventral view of a brooding adult female of *C. atrasolea*. (C) Clutch of brooded embryos of *C. atrasolea*. (D) Stack of *C. fornicata* adults numbered 1–9. (E) Brooded embryos of *C. fornicata* attached to the dorsal shell of another individual. Note: the mother who laid the brood is seen ventral side up in the background (at top of image). (F) Individual capsule containing embryos of *C. fornicata*.

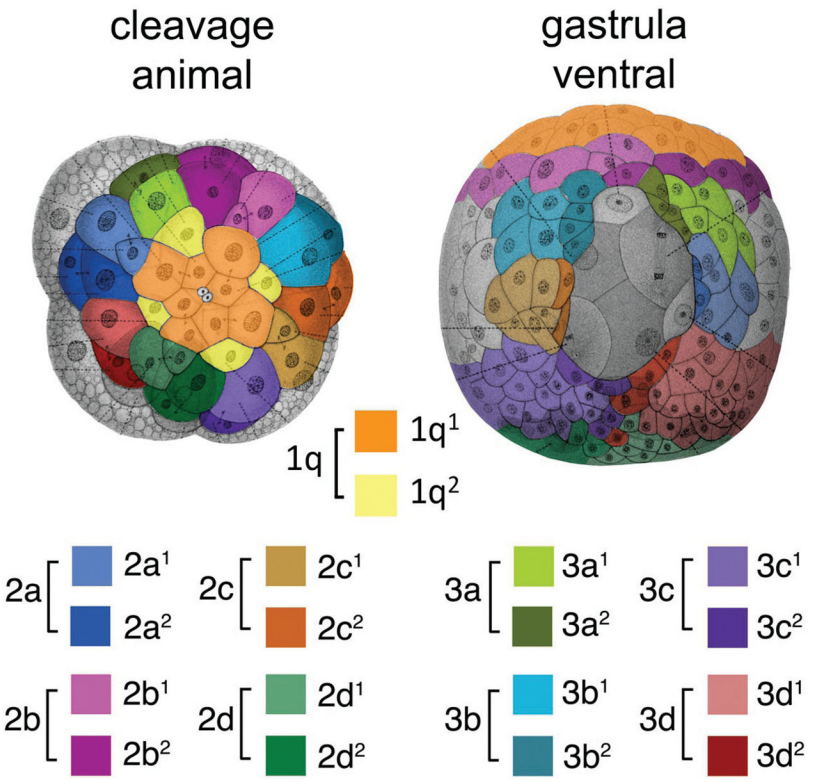

Fig. 2.

Crepidula fornicata embryonic fate map. Conklin's (1897) original drawings, over-laid with our updated lineage-tracing results for the four founding quadrants, and the first three quartets of micromeres. *Data compiled from Hejnol, A., Martindale, M.Q., Henry, J.Q., 2007. High-resolution fate map of the snail Crepidula fornicata: The origins of ciliary bands, nervous system, and muscular elements*. Developmental Biology 305, 63–76. *Henry, J.Q., Hejnol, A., Perry, K.J., Martindale, M.Q., 2007. Homology of ciliary bands in Spiralian Trochophores*. Integrative and Comparative Biology 47, 865–871. *Lyons, D.C., Perry, K.J., Lesoway, M.P., Henry, J.Q., 2012. Cleavage pattern and fate map of the mesentoblast, 4d, in the gastropod Crepidula: A hallmark of spiralian development*. EvoDevo 3, 21. *Lyons, D.C., Perry, K.J., Henry, J.Q., 2015. Spiralian gastrulation: Germ layer formation, morphogenesis, and fate of the blastopore in the slipper snail Crepidula fornicata*. EvoDevo 6, 24. *Lyons, D.C., Perry, K.J., Henry, J.Q., 2017. Morphogenesis along the animal-vegetal axis: Fates of primary quartet micromere daughters in the gastropod Crepidula fornicata*. BMC Evolutionary Biology 17, 217.

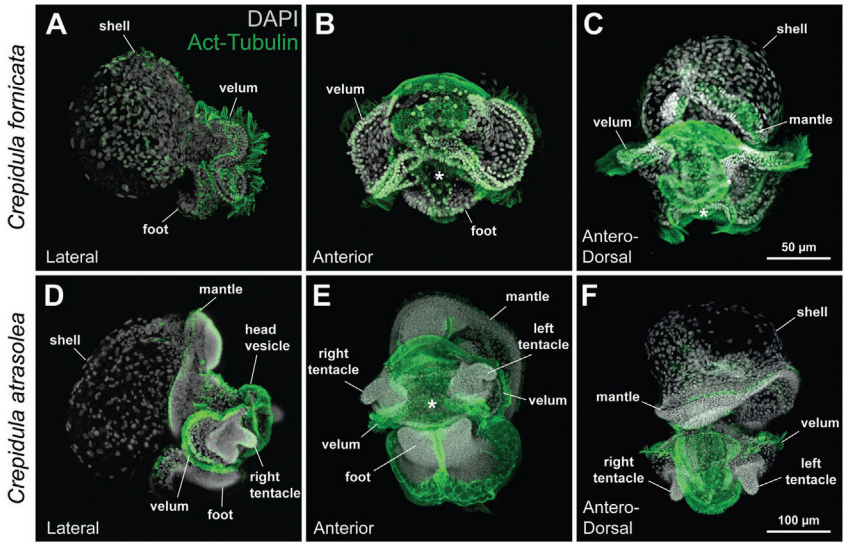

Fig. 3.

Acetylated tubulin (Act-Tubulin) immunoreactivity in veliger larvae of *Crepidula fornicata and Crepidula atrasolea*. Green–Cilia, Acetylated Tubulin; Gray–Nuclei, DAPI. (A–C) Different views of a veliger larva of *C. fornicata*, with Act-tubulin immunoreactivity primarily found in cilia of the velum and the mantle. (D–F) Acetylated tubulin immunoreactivity in a veliger larva of *C. atrasolea*. Acetylated tubulin immunoreactivity is present in cilia of the velum and mantle edge. Note the differences in larval features between the two species, especially in regards to a reduced larval velum, presence of left and right tentacles, pronounced mantle cavity, and difference in overall veliger size in *C. atrasolea* compared to *C. fornicata*.

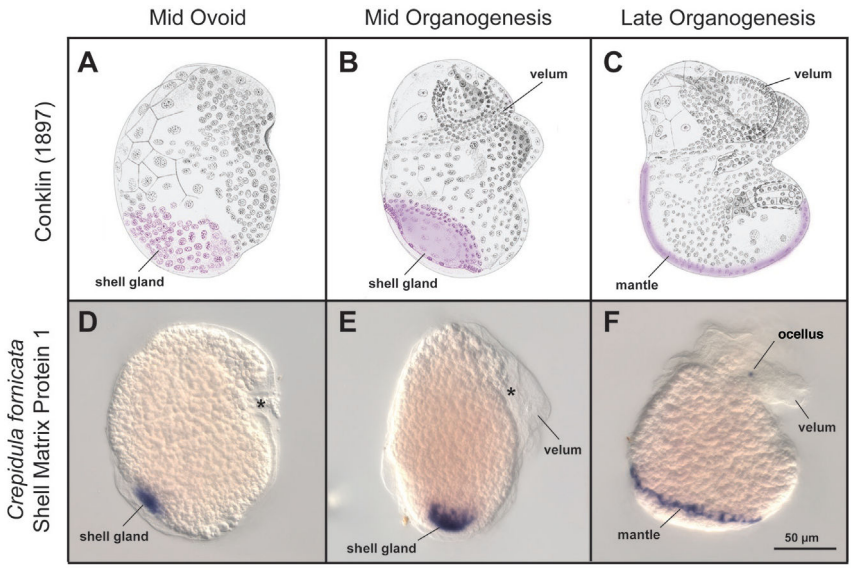

Fig. 4.

Expression of *Crepidula fornicata* Shell Matrix Protein 1 (CfSMP1) during larval shell gland development. (A–C) Drawings of embryos (160–228 hpf) undergoing larval shell gland development. Purple shading indicates the cells that Conklin originally labeled as "shell gland" cells or "shell gland edge" cells in his original publication. (D–F) CfSMP1 expression in embryos of a similar stage as panels (A–C). CfSMP1 is found exclusively expressed in the shell gland (panels D–E) during shell gland development, and later (panel C) in the mantle edge in early veliger larvae. *Panels (A–C) modified from Conklin, E.G., 1897. The embryology of Crepidula: A contribution to the cell lineage and early development of some marine Gasteropods. Ginn.*

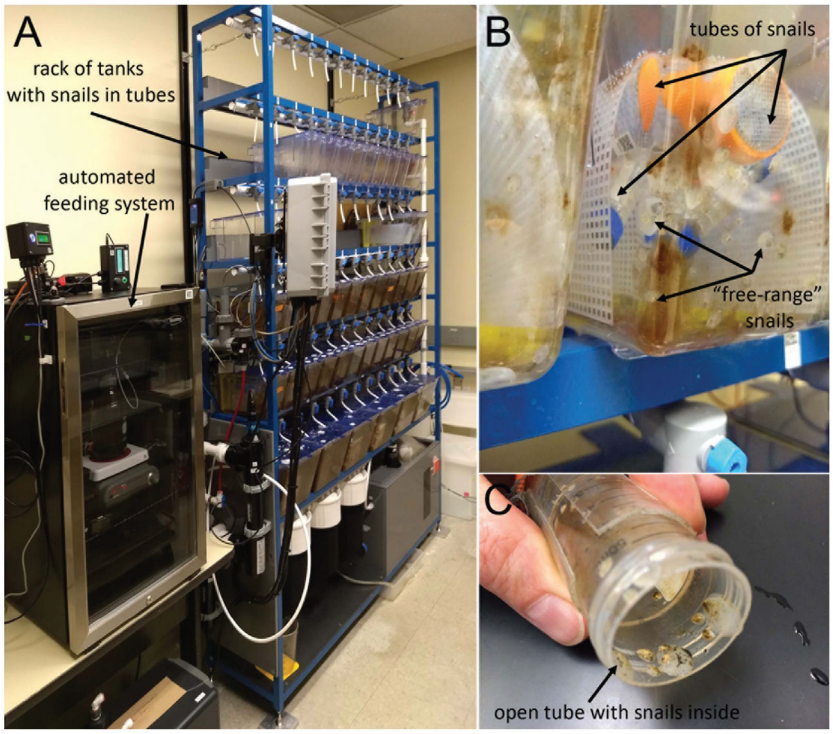

Fig. 5.

Automated rack system to culture *Crepidula atrasolea*. (A) Recirculating rack with tanks, Iwaki controller, and automatic feeding apparatus. (B) Close up of individual tank, holding tubes of embryos, as well as individual snails. (C) Individual tube opened up to reveal a mating colony of snails. Details of the system can be found in Henry, Lesoway, et al. (2017).