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The Slipper Snail, *Crepidula*: An Emerging Lophotrochozoan Model System†

JONATHAN J. HENRY[1,*] RACHEL COLLIN[2], AND KIMBERLY J. PERRY[1]

[1]*University of Illinois, Department of Cell & Developmental Biology, 601 S. Goodwin Ave, Urbana, Illinois 61801; and* [2]*Smithsonian Tropical Research Institute, Box 0843-03092, Balboa, Republic of Panama*

Abstract. Recent developmental and genomic research focused on "slipper snails" in the genus *Crepidula* has positioned *Crepidula fornicata* as a *de facto* model system for lophotrochozoan development. Here we review recent developments, as well as earlier reports demonstrating the widespread use of this system in studies of development and life history. Recent studies have resulted in a well-resolved fate map of embryonic cell lineage, documented mechanisms for axis determination and D quadrant specification, preliminary gene expression patterns, and the successful application of loss- and gain-of-function assays. The recent development of expressed sequence tags and preliminary genomics work will promote the use of this system, particularly in the area of developmental biology. A wealth of comparative information on phylogenetic relationships, variation in mode of development within the family, and numerous studies on larval biology and metamorphosis, primarily in *Crepidula fornicata*, make these snails a powerful tool for studies of the evolution of the mechanisms of development in the Mollusca and Lophotrochozoa. By bringing a review of the current state of knowledge of *Crepidula* life histories and development together with some detailed experimental methods, we hope to encourage further use of this system in various fields of investigation.

Introduction

The Lophotrochozoa is one of the largest clades of metazoans and includes the greatest diversity of adult body plans in any of the three major metazoan superclades (Halanych *et al.*, 1995; Nielsen, 2001; Mallatt and Winchell, 2002; Passamaneck and Halanych, 2006; Dunn *et al.*, 2008; Paps *et al.*, 2009). The relationships among the lophotrochozoan taxa are still somewhat poorly resolved (Dunn *et al.*, 2008; Paps *et al.*, 2009), but the core group (lophotrochozoan *sensu stricto* of Paps *et al.*, 2009) includes molluscs, annelids, nemertines, the lophophorates, platyhelminths, and other smaller phyla). Molecular phylogenies sometimes recover other groups such as rotifers and gastrotrichs nested within the core lophotrochozoans (*e.g.,* Passamaneck and Halanych, 2006), although these groups sometimes fall outside, as sisters to the core group taxa (*e.g.,* Paps *et al.*, 2009). Although adult morphology varies vastly across the group, many core Lophotrochozoa (including the Mollusca, Annelida [including Vestimentifera, Pogonophora, and Echiura, and possibly *Myzostoma*, and Sipunculida], Gnathostomulida, Nemertea, dicyemid Mesozoa, Entoprocta, and some Platyhelminthes [including polyclad Turbellaria, Catenulida, and Macrostomida]) exhibit spiral cleavage, a highly conserved pattern of early development. Representatives of some phyla also share a trochophore larval stage (*e.g.,* some polychaetes, sipunculids, vestimentiferans, echiurans, entoprocts, and molluscs).

Understanding the hypothesized repeated losses of both spiral cleavage and trochophore larvae in the Lophotrochozoa (Dunn *et al.*, 2008; Paps *et al.*, 2009) requires not only a phylogenetic context but also a good grasp of the mechanisms of lophotrochozoan development. Unfortunately, relatively little is understood of the basic cell and molecular mechanisms underlying these processes because, unlike the deuterostomes and ecdysozoans, lophotrochozoans have not been widely used in modern cell and molecular biology studies, partly due to the lack of well-developed model systems.

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*To whom correspondence should be addressed. E-mail: j-henry4@illinois.edu

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Molluscs are one of the most diverse lophotrochozoan phyla, with over 93,000 described species (Brusca and Brusca, 2002). For more than 100 years, molluscs have been used in developmental investigations. Species studied include the patellogastropod limpet *Patella vulgata*; the caenogastropods *Ilyanassa obsoleta* and *Crepidula* spp.; the pulmonate gastropods *Lymnea* spp. and *Bythinia tetaculata*; the scaphopod *Dentalium dentale;* and the bivalve *Spisula solidisima*. Despite this long history of study, no single species has been favored as a model representative. A recent surge in developmental and genomic research focused on "slipper snails" in the genus *Crepidula* is resulting in the development of *Crepidula fornicata* (Linnaeus, 1758), as a *de facto* model system for molluscan development (see Fig. 1A). The utility of *C. fornicata* as a model system is augmented by a wealth of comparative information on the systematic relationships, variation in embryonic development, and life histories (reviewed below).

Recent key results include a well-resolved fate map of embryonic cell lineage (Conklin, 1897; Hejnol *et al.*, 2007; Henry *et al.*, 2007), elucidated mechanisms for axis determination and D quadrant specification (Henry *et al.*, 2006; Henry and Perry, 2008), the ability to characterize gene expression patterns and carry out loss- and gain-of-function assays (Dean *et al.*, 2009; Taris *et al.,* 2009; Henry *et al.,* 2010b; for on-line supplemental methods, see Henry *et al.,* 2010a), as well as the development of large numbers of expressed sequence tags (ESTs) and preliminary genomics work (Henry *et al.,* 2010b; F. Viard, A. Tanguy, and N. Taris, Station Biologique de Roscoff, unpubl. data). The variety of development exhibited by species in the genus and the availability of a fairly well-resolved phylogenetic tree (Collin, 2001, 2003a, b, 2004) also make this group ideal for studies of comparative genomics, evolution, and development. The successful application of a wide toolbox of methods, combined with the animals' abundance, wide geographic range, and the ease with which adults and embryos can be obtained, makes *C. fornicata* an ideal platform for a variety of studies in different fields.

A wide array of recent research is coming together to make *C. fornicata* the lophotrochozoan equivalent of other invertebrate models for development, including the deuterostome *Strongylocentrotus purpuratus* (a sea urchin) and the cnidarian *Nematostella vectensis* (a sea anemone). Although other lophotrochozoans are the focus of some developmental research (*e.g.,* the polychaetes *Platynereis dummerilii and Capitella* sp. I, and the gastropods *Lottia gigantea*, *Biomphalaria glabrata*, and *Ilyanassa obsoleta*), the rapid rate at which information is accumulating for *Crepidula* and the diversity of laboratories working on *C. fornicata* in particular make it timely to assess the state of knowledge for this organism. By bringing a review of the current state of *Crepidula* biology and development together with some detailed experimental methods (see Henry *et al.,* 2010a), we hope to encourage further use of this system for investigations of invertebrate development.

Calyptraeids and the Genus *Crepidula*

The caenogastropod family Calyptraeidae is made up of five familiar, relatively well-studied genera: *Crepidula* (often referred to as "slipper snails," Fig. 1A–B), *Crucibulum* (cup and saucer snails," Fig. 1C), *Calyptraea* ("hat snails," Fig. 1D), *Bostrycapulus* ("spiny slipper snails," Fig. 1E), and *Crepipatella* ("cupped slipper snails"), in addition to several other smaller, less well-known genera. There are currently at least 60 recognized species in the genus *Crepidula* (Collin, 2003a, b), although several species that were previously attributed to *Crepidula* have been recently reassigned to *Bostrycapulus* and *Crepipatella* (Collin, 2005; Collin *et al.*, 2007).

Like most calyptraeids, *Crepidula* species have a limpet-shaped shell and an expanded foot. The shells are generally flattened, with a posterior apex (as opposed to the central apex of *Crucibulum* and *Calyptraea*) and a slight lateral curve, but no distinct coiling (Collin and Cipriani, 2003). The shelf, or internal septum, of the shell separates the foot from the viscera and is flat in *Crepidula* and *Bostrycapulus* and cup-shaped in *Crucibulum*. Furthermore, all calyptraeids lack an operculum in the adult stage.

Calyptraeids range worldwide except for the Arctic and Antarctic, and generally occur from the intertidal to depths of 100 m. *Crepidula* species are most diverse in the Americas and Africa and unknown or hard to find in most of the Indo-Pacific. Several species, most notably *C. fornicata* and *Crepidula onyx* Sowerby, 1824, are highly invasive, and several other species are known to have established populations after human-mediated introductions (Deslou-Paoli and Heral, 1986; Huang *et al.*, 1983; Blanchard, 1997; Collin *et al.*, 2006, 2009; McGlashan *et al.*, 2008). Most species are found attached to rock rubble or other hard substrates, including mussels, other gastropods, and gastropod shells inhabited by hermit crabs (Fig. 1F). Species that occur on other animals may have quite restricted host preferences. This preference for attaching to shellfish is thought to have resulted in several of the human-mediated introductions, as shellfish were transported for aquaculture (Carlton, 1992).

Adult *Crepidula* and other calyptraeids are sedentary suspension feeders that collect phytoplankton on a mucous net associated with the gills (Jørgensen *et al.*, 1984; Chaparro *et al.*, 2002a, 2004; Barillé *et al.*, 2006). This mucus is transported to the mouth in a string and prior to ingestion is occasionally concentrated in the food pouch located in the mantle margin in front of the head (Chaparro *et al.*, 2002a). Under high food concentrations the food is concentrated in the food pouch and subsequently rejected. For example, in *Crepipatella fecunda* (Gallardo, 1979) (previously *Crep-*

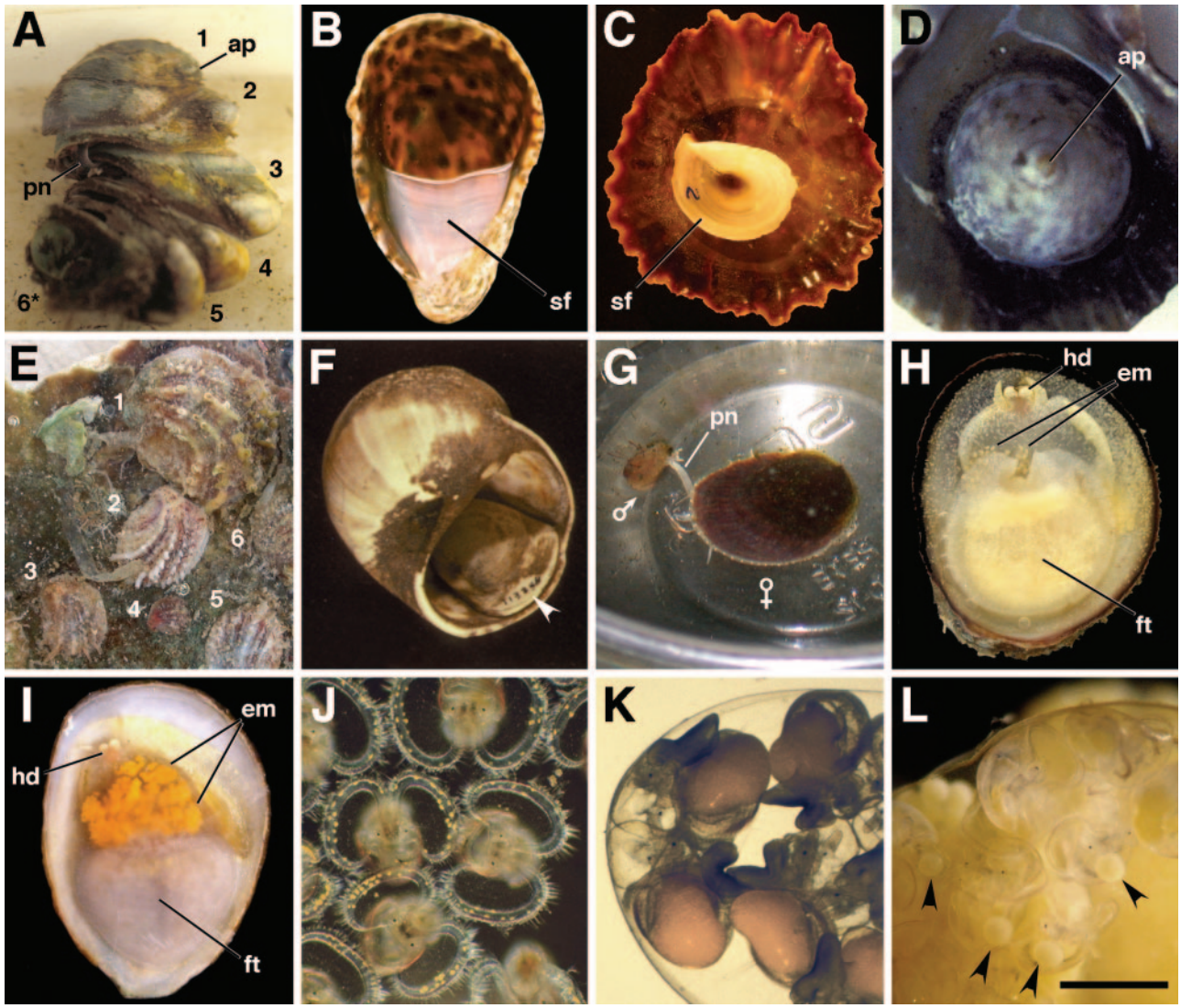

Figure 1. Various species of calyptraeids and some modes of their development. (A) Stack of *Crepidula fornicata*. There are five live adults as numbered (1–5), with the fifth being attached to an empty shell (6*). Notice also that a male (2) is mating with the female below him (3). The apex is located at the posterior end of the shell. (B) Inferior view of the shell of *Crepidula badisparsa.* Note the characteristic "slipper-shaped" morphology and presence of a flattened shelf. (C) Inferior view of the shell of *Crucibulum scutellatum* reveals that the "shelf" is not flat but funnel-shaped like a small cup placed on a saucer (*i.e.,* the main shell). (D) Symmetrical cone-shaped appearance of *Calyptraea conica*. (E) Examples of six spiny *Bostrycapulus aculeatus* attached to a rock, as numbered. A small juvenile snail is seen amongst this group (4). (F) Flattened *Crepidula plana* (white arrowhead) attached inside the opening of a moon shell (*Lunatia heros*) that was once occupied by a hermit crab. (G) Mating pair of *Crepidula onyx* being reared in a plastic cup. (H) Inferior view of *Bostrycapulus urraca* with a small brood of embryos located in the region of the propodium. (I) Inferior view of *Crepidula atrasolea* with large brood of embryos. (J) Planktotrophic veliger larvae of *C. fornicata*. (K) Direct-developing embryos within an egg capsule of *Crepidula adunca*. (L) Egg capsule containing veligers of *Crepipatella dilatata* feeding on nurse eggs (black arrowheads). ap, apex; em, egg/embryo mass; ft, foot; hd, head; pn, penis; sf, shelf. Scale bar in L equals 2.5 cm for A, F, and G; 5 mm for B; 2 cm for C; 1 cm for D and H; 1.5 cm for E and I; 1 mm for J; 800 μm for K; and 400 μm for L.

idula fecunda), food concentrations above 15×10^4 cells/ml lead to decreased feeding rates due to rejection (Chaparro *et al.*, 2004). Although many species are tolerant of large changes in salinity, feeding does not occur below salinities of 20 psu (Chaparro *et al.*, 2008a). Hatchlings and small juveniles can also feed by grazing (Montiel *et al.*, 2005).

Like all calyptraeids, *Crepidula* species are protandric hermaphrodites, which become males after an initial juve-

nile phase and subsequently change to females. Size at sex change is influenced by associations with conspecifics: males change sex at a larger size in the presence of females (Coe, 1938; Collin *et al.*, 2005). Adult *C. fornicata* are typically arranged in stacks containing 2 to 20 animals (Fig. 1A). The largest and oldest animals (females) are located at the base of the stack, often attached to a single empty shell or other hard substrate. Smaller, younger individuals (males) are located on top (Collin, 1995). Animals often grow to precisely fit the substrate to which they are attached and are therefore not mobile. However, the smallest individuals (juveniles or males) are more active and may crawl over the surface of the stack. Stacking behavior is linked to variation in sex ratio (Collin *et al.*, 2006), and in *C. fornicata*, offspring from a female are generally fathered by males from the same stack (Dupont *et al.*, 2007; Proestou *et al.*, 2008; Le Cam *et al.*, 2009). Some other species of *Crepidula* are more often found in pairs or as solitary individuals (Collin *et al.*, 2006; Fig. 1E–F).

Individuals of *Crepidula fornicata* are thought to live for up to 11 years (Botton and Ropes, 1988; Blanchard, 1997). Modal decomposition analyses shows that field populations are usually composed of 4 or 5 age groups, with the latest groups composed of a mix of age cohorts (Deslou-Paoli and Heral, 1986; Grady *et al.*, 2001; Dupont *et al.*, 2007). Individuals may presumably live more than 6 years in the field. Both growth rate and size at maturity depend heavily on temperature, food availability, and associations with conspecifics. Male sexual maturity, however, can be attained as early as 2 months after settling, once they reach a length of 4 mm (Werner, 1955), and the average time to first egg production after settling in the laboratory can be as little as 300 days (Nelson *et al.,* 1983). However, female reproduction in the field is unlikely before the 3rd year (Deslou-Paoli and Heral, 1986). In the laboratory, several other species of *Crepidula* (*C. atrasolea,* Collin, 2000; *C. ustulatulina,* Collin, 2002; *C.* cf. *marginalis* and *C. incurva,* Broderip, 1893) can reach a reproductive female stage in 6 months or less (R. Collin, pers. obs.), and *C. onyx*, a species similar to *C. fornicata,* is reported to reach maturity in 8 weeks (Zhao *et al.,* 2003).

Obtaining and Rearing Adult *Crepidula fornicata*

Adult *C. fornicata* are abundant in shallow water along the east coast of North America (especially in New England) and along the northern coast of Europe, where they can reach densities of up to 2000/m^2 (Dupont *et al.,* 2007). Within the United States, adult *C. fornicata* are easily collected from the intertidal or shallow subtidal, and both *C. fornicata* and *C. plana* Say, 1822, can also be shipped by the Marine Resources Department of the Marine Biological Laboratory, Woods Hole, Massachusetts (see section A1 in Henry *et al.,* 2010a).

Adult *C. fornicata* and most other *Crepidula* species are extremely hardy. The animals can be easily maintained in sea tables supplied with fresh running seawater (*e.g.,* Gould, 1950; Nelson *et al.,* 1983). They can also be raised in individual cups of seawater if changed every 2 or 3 days (*e.g.,* Collin *et al.,* 2005; Fig. 1G). A mixed diet is probably preferable, but several species of *Crepidula* can be raised for two complete generations on only *Isochrysis* (Collin and Salazar, 2010). The animals can also be fed a diet of commercial live marine phytoplankton (DT'S Live Marine Phytoplankton, Sycamore, IL.). *Crepidula fornicata* can also be maintained in recirculating inland glass aquaria containing artificial seawater (see section B1 in Henry *et al.*, 2010a).

Reproductive Period, Fertilization, and Egg Laying

In the wild, female *C. fornicata* lay their eggs in the spring and summer months, as soon as the water temperature begins to rise. Costello and Henley (1971) reported that animals from Woods Hole, Massachusetts, breed from mid-June through mid-August. However, it is likely that animals begin to spawn in May, as we have found females brooding egg capsules that contained advanced veliger larvae in early June, and a few animals continue to lay eggs in August. In northern France the reproductive season is somewhat longer, and females can be found brooding early embryos from mid-February until September (Richard *et al.,* 2006).

Egg production in *C. fornicata* can be inhibited by a reduction in temperature (Gould, 1950; and pers. obs.), and individuals collected from November through April will readily spawn within a week or two of being placed in warm (25 °C) water (see section B1 in Henry *et al.,* 2010a). Likewise, egg laying may be reliably induced through July by holding the animals in chilled seawater at 14–16 °C and subsequently warming them when needed. In the wild, *C. fornicata* lays several broods each season (Conklin, 1897; Richard *et al.,* 2006), and many *Crepidula* species produce a brood within days of natural hatching of the previous brood (R. Collin, pers. obs.).

Eggs can be laid at any time but are commonly laid during the first half of the day, and the entire brood is usually laid within 4–12 h. Eggs are fertilized after they leave the ovary, as they pass the seminal receptacles (which can store sperm for months or possibly years), and are subsequently surrounded by albumin and encased in the capsule before being deposited through the female genital papilla. Each triangular capsule is shaped individually by the foot and attached to the substrate with the other capsules, at the base of a flattened stalk (Hoagland, 1986). The numerous embryos contained within each egg capsule develop synchronously but differ somewhat in age from their siblings in other capsules. Females brood their embryos between the neck and the propodium until they hatch (Fig.

1H–I). The shell can be used to seal the brood chamber from unfavorable external conditions like reduced salinity, from which the thin egg capsules afford little protection (Chaparro *et al.,* 2008b).

Freshly laid eggs are somewhat flattened, and all of the eggs in a capsule are tightly pressed together, giving the impression of a smear of yolk that cannot be separated into individual eggs. However, the eggs rapidly round up when left in the capsule for 30 min to an hour. Eggs and young embryos are pale or bright yellow, while older embryos and larvae appear dark brown due to the dark larval pigments (Fig. 1H–I). The eggs contain a lot of yolk and a small area of clear cytoplasm, which is located at the animal pole and contains the pro-nuclei. Apart from the egg capsule, the eggs and embryos are devoid of any external investments, such as a vitelline envelope.

The number of eggs produced by a female *Crepidula* depends on her size and the mode of development of the species. In general, both the number of capsules and the number of eggs per capsule increase with female size. In *C. fornicata,* virtually all of the 1,000–50,000 embryos develop to hatch as swimming veligers (Richard *et al.*, 2006; Brante *et al*., 2009), although an occasional empty shell may be found within the capsules. In direct-developing species it is more common for broods to be small (*e.g.,* average brood of 64 ± 29 [1SD] eggs for *Crepidula adunca* Sowerby, 1825; Collin, 2000b), and smaller species with planktotrophic development likewise produce fewer embryos than *C. fornicata* (*e.g.,* average brood of 4300 ± 2000 eggs for *C. lingulata*; Gould, 1846; see Collin, 2000b).

Embryogenesis: Obtaining and Culturing Eggs and Embryos

If females are cultured directly attached to aquarium walls, plastic cups, or other transparent substrates, the appearance of eggs can be easily observed below the female (Fig. 1H–I). If they are cultured in stacks, eggs can be located and cultured following sections C1 and D1 in Henry *et al.* (2010a). Earlier it was reported that embryos would not survive long once they were removed from the protective care of their mothers (Conklin, 1897; Gould, 1917). The use of modern antibiotics has, however, eliminated these problems, and embryos can be cultured outside their egg capsules in either filtered or artificial seawater containing penicillin G and streptomycin sulfate (Henry *et al.*, 2006; see section D1 in Henry *et al.,* 2010a). One may also use UV-sterilized and filtered seawater with streptomycin (Brante *et al*., 2009).

Early Development

The fertilized egg of *C. fornicata* measures about 180 μm in diameter (Fig. 2A). First cleavage occurs about 7.3 h after the egg is laid (Fig. 2B, K–M, Hejnol *et al.*, 2007; Henry and Perry, 2008) at 21–22 °C. The two meiotic reduction divisions occur shortly after the eggs are laid, resulting in the production of two polar bodies, which remain at the animal pole during early cleavage. The second polar body forms about 2 h after the first polar body. It has been proposed that the fertilized egg and early embryos exhibit the phenomenon of "gonomery," in which the products of the two pronuclei apparently do not fuse but remain distinct until the 69-cell stage (Conklin, 1901, 1902); however, because the nuclei in some other gastropods are bilobed, this claim needs to be verified.

Cleavage Pattern and Embryonic Fate Map

The cleavage pattern of *C. fornicata* is well-characterized (Conklin, 1897; Hejnol *et al.*, 2007; Henry and Perry, 2008). The early holoblastic cleavages are diagrammed in Fig. 2A–J, L–R, along with a timeline of early development at 21–22 °C (Fig. 2K). First cleavage is essentially equal; however, this cleavage is accompanied by the production of a small polar lobe, which fuses with one of the two resulting blastomeres. Second cleavage also appears to be equal, though a second, small polar lobe is formed by the first cleavage blastomere that inherited this structure during the preceding division. The second polar lobe ultimately fuses with the presumptive D cell. Polar lobe formation in *C. fornicata* was nearly missed by Conklin (1897), and the potential significance of these structures appeared only as a footnote in his first published description of development (see also Henry *et al.*, 2006). Polar lobes have also been observed in a number of other calyptraeids with both small and large eggs. In all cases the polar lobes are small and easy to miss (R. Collin, pers. obs.).

Third cleavage leads to the formation of the first quartet of micromeres located at the animal pole (1a–1d). The first quartet is formed in a dexiotropic fashion, as shown in Figure 2D. In preparation for third cleavage, the presumptive D blastomere becomes somewhat elongated along the animal-vegetal axis, though the formation of a discrete polar lobe at its vegetal pole is not obvious.

Three additional quartets of micromeres are formed (2a-2d, 3a-3d, and 4a-4d; Fig. 2E–J, N–Q). Unlike the micromeres of the first three quartets, the fourth quartet derivative of the D quadrant is formed precociously to generate the 25-cell stage (*i.e.,* 4d; Fig. 2J, Q). The other fourth quartet micromeres (4a, 4b, 4c) form synchronously after several hours have elapsed. Unlike the other micromeres, the fourth quartet micromeres are larger and contain a substantial quantity of yolk. The 4d micromere plays a significant role, giving rise to the paired mesentoblast cells MEL and MER. These two daughters generate endoderm (the hindgut or intestine), as well as the mesodermal bandlets (see Figs. 3, 4, and below). Conklin (1897) also described the formation of a "fifth quartet" of micromeres. These large and yolky

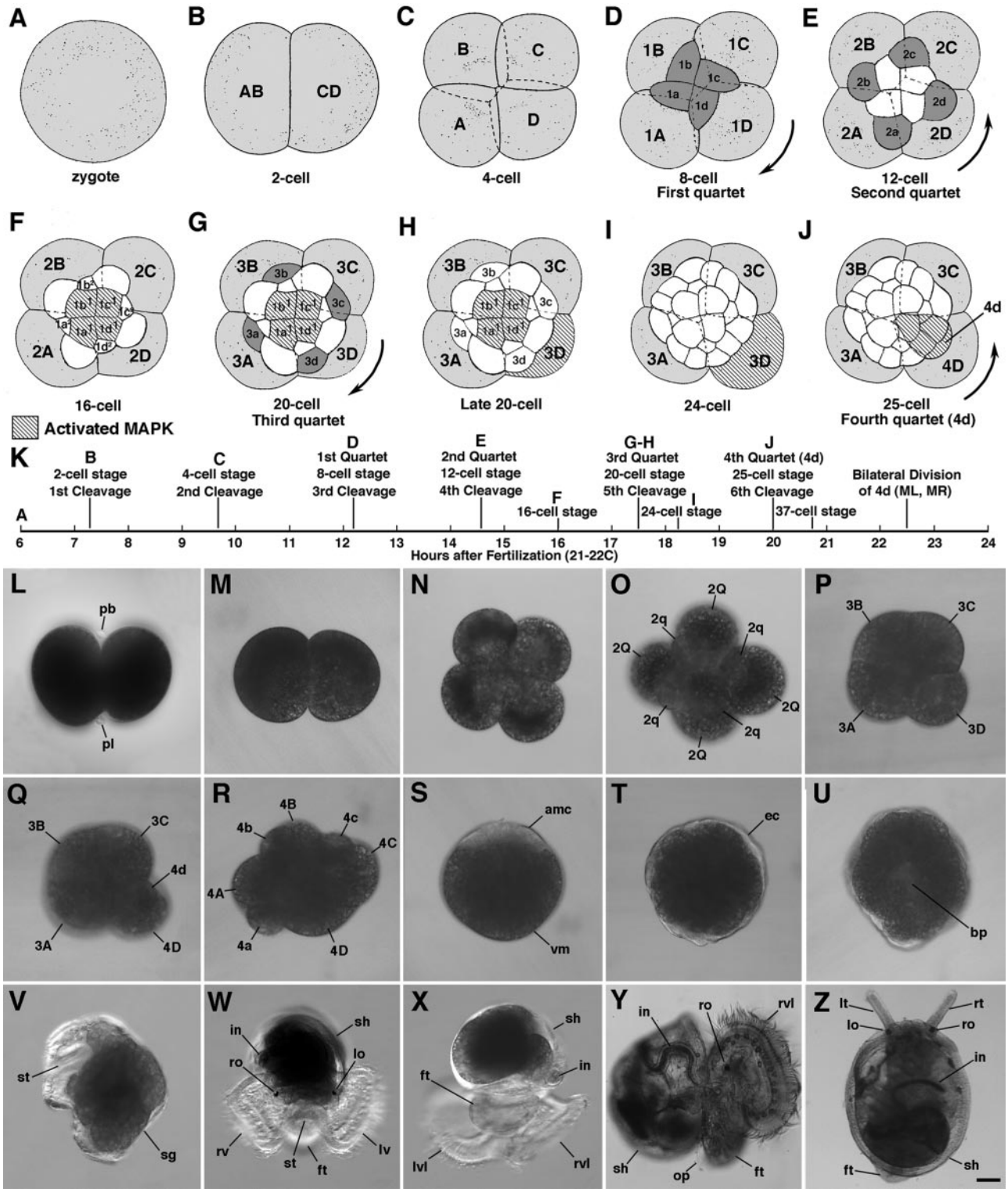

Figure 2. A series of developmental stages in the snail *Crepidula fornicata*. (A–J) Diagrams of early cleavage showing the spiral cleavage pattern and the synchronous formation of the first three quartets of micromeres. This is followed by the precocious formation of the fourth quartet micromere (the mesentoblast, 4d) in the D quadrant to produce the 25-cell stage. Stages are as depicted. The birth of successive quartets of micromeres is shown with dark-gray shading at the 8- (D), 12- (E), and 20-cell stages (G), and the mesentoblast (4d) is shaded with diagonal hatch marks at the 25-cell stage (J). Vegetal macromeres are shown with light-gray shading. Diagonal hatch marks highlight the general pattern of activation of MAPK in specific cells. Nomenclature follows that of *(continued)*

cells contribute to the formation of endodermal tissues, and it is not clear if they actually constitute a fifth quartet of micromeres.

In *C. fornicata*, individual blastomeres may be pressure-injected with fluorescent lineage tracers, including fluorescent dextrans, at any stage of development (see Hejnol *et al.*, 2007; and section E1 in Henry *et al.,* 2010a). Using this approach, a high-resolution cell lineage fate map has recently been compiled using confocal microscopy for each blastomere through the formation of the fourth quartet of micromeres (Fig. 3; Hejnol *et al.*, 2007; Henry *et al.*, 2007).

In general, the fate map is consistent with those of other spiralians, revealing a high degree of conservation (Hejnol *et al*., 2007). The first three quartets give rise to ectodermal tissues, including the two ocelli (derived from 1a and 1c micromeres), as well as ectomesoderm (from 2c, 3a, and 3b micromeres). The fourth quartet cells contribute to the formation of the internal kidney (4a, 4b, 4d) as well as to the stomach and style sac endoderm (4c). The mesentoblast, 4d, contributes to the formation of endodermal structures (the intestine or hindgut) as well as the left and right mesentoblast cells. In addition, the external "embryonic kidneys," described by Rivest (1992), are derived from 4d (see below). The macromeres 4A, 4B, and 4C contribute to the stomach, the left and right midgut lobes, the style sac, and possibly the salivary glands. 4D appears to be nutritive, ending up within the developing stomach. A similar nutritive fate of one or more fourth quartet macromeres is found in other spiralians (*e.g.,* Clement, 1962; Henry, 1989).

This analysis revealed a number of additional findings. For instance, the accessory trochoblast cells that contribute to the formation of the prototroch in many spiralians (2q derivatives) have been co-opted to form the secondary opposed feeding band ("metatroch") in *C. fornicata* (Henry *et al.*, 2007). These findings suggest that the secondary ciliated band in the veliger is not homologous to the metatroch found in the trochophore larva of other spiralians, such as the annelid *Polygordius lacteus* (reportedly derived from 3c and 3d; Woltereck, 1904; Jägersten, 1972; Nielsen, 2001, 2004). This lends support to the hypothesis that postoral ciliary bands have arisen multiple times in the Trochozoa (Rouse, 1999, 2000a, b).

A detailed fate map of the 4d daughter cells has not been published for any spiralian, though the pattern of early divisions has been recently described for *Ilyanassa* (Swartz *et al.*, 2008; Rabinowitz *et al.*, 2008; using a nomenclature based on that of Goulding, 2001). We studied the pattern of early divisions in the 4d sublineage in *C. fornicata*, using fluorescent lineage tracers to the larval stage (Figs. 3B, 4; Henry and Martindale, unpubl. data), specifically following cells ML, MR, 1mL, 1mR, 1ML, 1MR, 2mL, 2mR, 3mL, and 3mR). The progeny of 4d are named here using a new nomenclature modified after that of Conklin (1897), where the teloblasts are designated with a capital letter, M, and the daughters with a lower case letter, m. L and R refer to left and right, respectively). The initial daughters of 4d (ML and MR) serve as teloblasts that form the paired mesentoblast bands. Within each of these sublineages, successive daughters (divisions) are numbered sequentially (1ML, 2ML, 1mL, 2mL, *etc.*). In *C. fornicata,* the intestine is derived from the 1mL, 1mR, 3mL, and 3mR cells. The two external left and right embryonic kidneys are derived from 2mL and 2mR, located at the tips of the mesodermal bands. The true internal kidney, mesenchyme, and various muscles, including the retractor muscles, are derived from other progeny of the 3ML and 3MR teloblast cells.

(Fig. 2 continued) Conklin (1897). Refer to Fig. 3A for the ultimate fates of these cells. Arrows depict the handedness of each set of micromere divisions that occur in alternating clockwise and counterclockwise directions. A developmental timeline is depicted in (K), which shows the initiation of each stage depicted in A–J. Note also that the exact time of fertilization is uncertain as this occurs internally. (L–Z) Photomicrographs depicting different stages of development. (L–M) Two-cell stage showing transient formation of the small vegetal polar lobe (pl, in L). The animal polar bodies (pb) are located at the opposite animal pole. (N) Essentially equal four-cell stage. (O) 12-cell stage, showing the formation of the second tier of animal micromeres (2q) that lie on top of the vegetal macromeres (2Q). The first quartet micromeres are difficult to see in this image. (P) 24-cell stage showing protrusion of 3D cell in preparation for the formation of the mesentoblast. (Q) 25-cell stage showing the mesentoblast, 4d. (R) Later formation of the other 4th quartet micromeres 4a, 4b, and 4c. (S) Early stage of gastrulation that occurs by epiboly. The transparent animal micromere cap of cells (amc) is seen at the animal pole overlying the large vegetal macromeres (vm). (T) Later stage of development, following gastrulation, showing the outer layer of animal ectodermal cells (ac) has now completely surrounded the internal large yolk-filled macromeres. The embryo is beginning to flatten along the animal-vegetal axis. (U) A later stage showing the prominent remnant of the blastopore (bp) on one side of the embryo. The posterior dorsal side of the embryo is located at the bottom, where the protruding mass of tissue is seen. (V) Preveliger stage showing the development of the stomodeum (st) within the head and the dorsal-posterior shell gland (sg). (W–X.)Young veliger larvae showing the shell (sh), left and right velar lobes (lvl, rvl), stomodeum, right and left ocelli (ro, lo), and intestine (in). (Y) Older veliger following feeding. Waste products can be seen in the lumen of the intestine (in). The operculum (op) is also seen attached to the foot. (Z) Juvenile snail following metamorphosis. The left and right tentacles (lt, rt) and ocelli (lo, ro) can be seen. The diagrams depicted in A–J and the timeline shown in K are from Henry and Perry (2008). Scale bar in Z equals 40 μm and applies to L–Z.

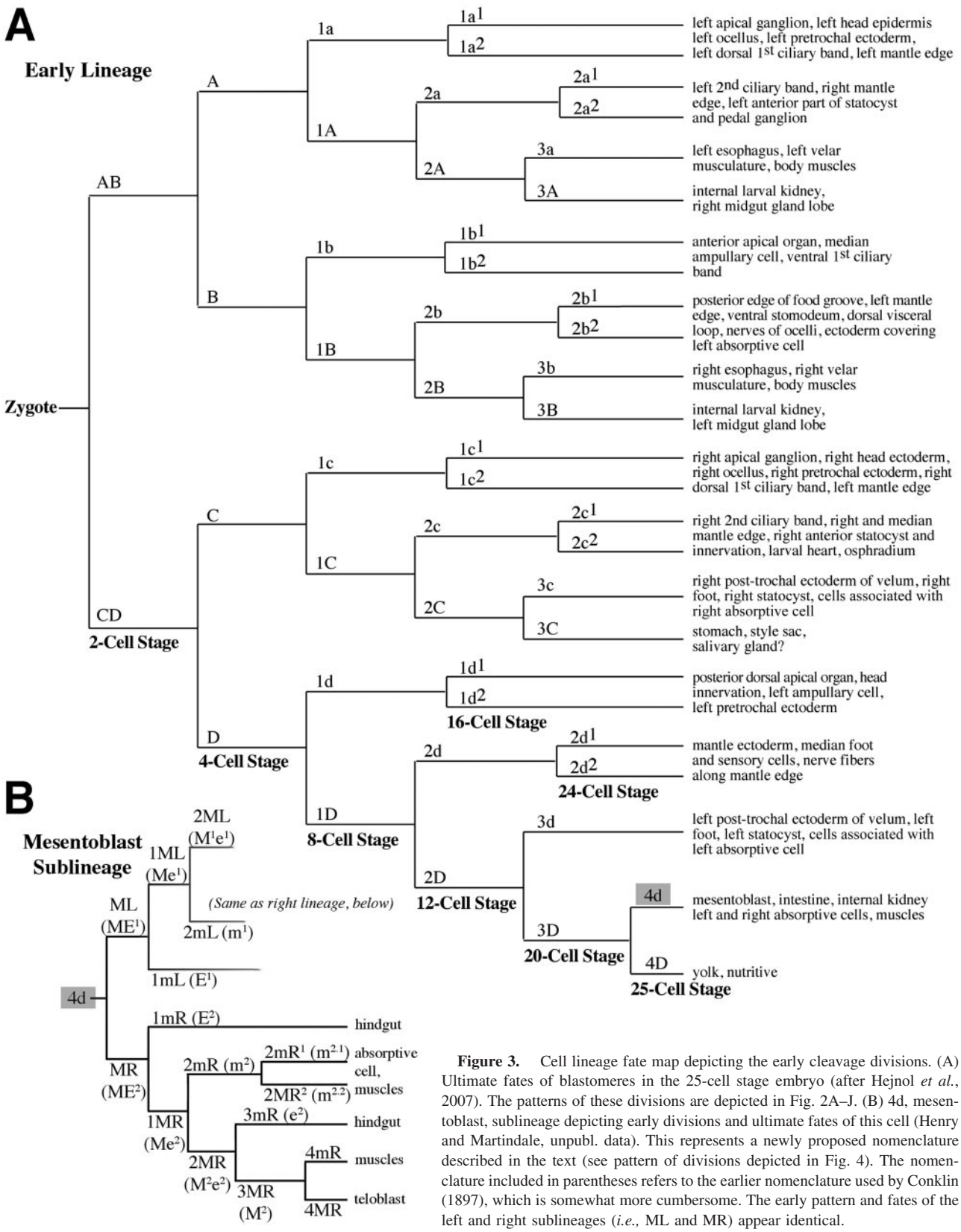

Figure 3. Cell lineage fate map depicting the early cleavage divisions. (A) Ultimate fates of blastomeres in the 25-cell stage embryo (after Hejnol *et al.*, 2007). The patterns of these divisions are depicted in Fig. 2A–J. (B) 4d, mesentoblast, sublineage depicting early divisions and ultimate fates of this cell (Henry and Martindale, unpubl. data). This represents a newly proposed nomenclature described in the text (see pattern of divisions depicted in Fig. 4). The nomenclature included in parentheses refers to the earlier nomenclature used by Conklin (1897), which is somewhat more cumbersome. The early pattern and fates of the left and right sublineages (*i.e.,* ML and MR) appear identical.

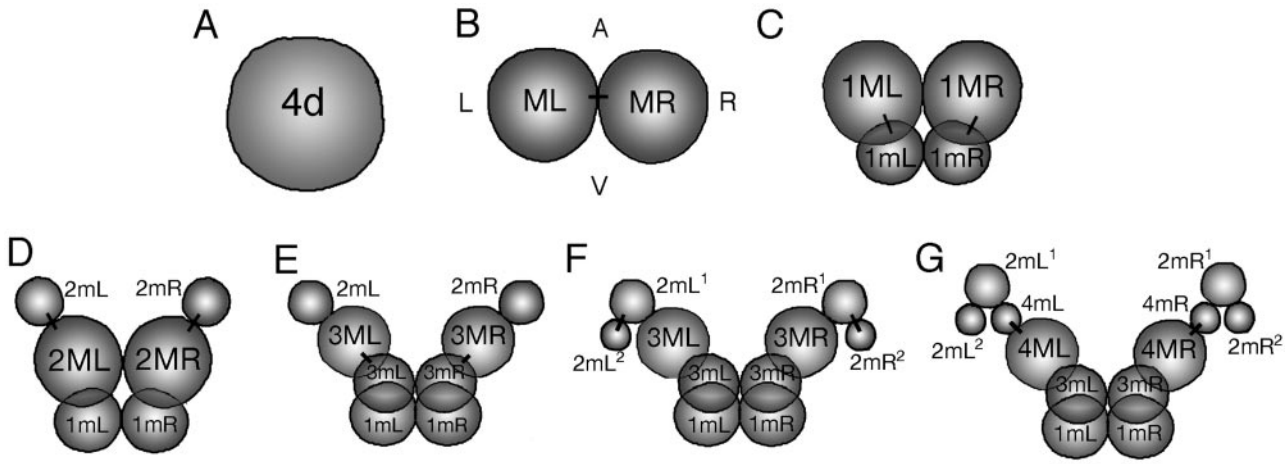

Figure 4. The symmetrical early cleavage pattern of the 4d sublineage. The diagram depicts dorsal views with the animal pole located toward the top of the figure. The separation of the endodermal precursors (1mR, 1mL, 3mR, 3mL) from the other cells constituting the mesodermal bands is clearly seen. The #ML and #MR cells represent the successively older teloblasts that contribute to the growing mesodermal bands (and the endoderm). The alternating, "spiral" nature of their mirror symmetrical divisions can be appreciated. Refer to Fig. 3B and the text for the ultimate fates and nomenclature for these cells.

Comparative Mechanisms of Cell Fate and Axis Determination: D Quadrant Specification and Organizer Activity in *Crepidula*

Two mechanisms have been identified that lead to the specification of the four cell quadrants and the dorsoventral "DV" axis in spiralians (Verdonk and Cather, 1973, 1983; van den Biggelaar and Guerrier, 1983; Verdonk and van den Biggelaar, 1983). In unequal-cleaving molluscs with polar lobes (*e.g., Ilyanassa obsoleta*) and polychaete embryos (*e.g., Chaetopterus variopedatus* and *Sabellaria cementarium*) the first two cell divisions differentially partition vegetally localized factors responsible for establishing the identity of the larger D blastomere (*e.g.,* autonomous specification, see Verdonk and Cather, 1973, 1983; Guerrier *et al.*, 1978; van den Biggelaar and Guerrier, 1983; Render, 1983, 1989; Henry, 1986, 1989; Henry and Martindale, 1987; Dorresteijn *et al.*, 1987; Wall, 1990). If these localized factors are removed (*e.g.,* by removal of the vegetal region, including the polar lobe), D quadrant identity and DV polarity are abolished. Likewise, if these factors are distributed to both blastomeres during first cleavage by equalizing this division, twinning occurs *via* the production of two D quadrants (Tyler, 1930; Henry and Martindale, 1987; Dorresteijn *et al.*, 1987; Render, 1989).

In equal-cleaving embryos without polar lobes the dorsal (D) cell quadrant is determined later during development by virtue of cell-cell inductive interactions between derivatives of the first quartet micromeres ($1a^1$–$1d^1$) and one of the vegetal macromeres (presumptive 3D macromere) after the birth of the third quartet (van den Biggelaar and Guerrier, 1979; Arnolds *et al.*, 1983; Martindale *et al.*, 1985; Kuhtreiber *et al.*, 1988; Boring, 1989; van den Biggelaar, 1996; Henry, 2002).

In both equal- and unequal-cleaving embryos the D quadrant subsequently acts as a key organizer to polarize the dorsoventral axis and regulates development of adjacent quadrants. Experiments in the unequal-cleaving and equal-cleaving molluscs indicate that the organizing influence of the D quadrant is manifested following formation of the third quartet micromeres, during the interval between the fifth and sixth cleavages (Clement, 1956, 1962; Verdonk and Cather, 1983; van den Biggelaar and Guerrier, 1983; Verdonk and van den Biggelaar, 1983; Martindale, 1986; Labordus and van der Wal, 1986; Sweet, 1998). The 3D macromere serves as the organizer, and its removal at successively later stages during this time interval leads to fewer developmental defects.

In *C. fornicata* the early cleavage divisions appear equal, but the embryos form small polar lobes during the first two cell divisions. This seemed to suggest that the D quadrant is specified *via* an autonomous mechanism typical of other species with small polar lobes (*e.g., Bythinia*; Cather and Verdonk, 1974; Cather *et al.*, 1976). For most species examined, polar lobes contain critical vegetal factors required to establish the dorsal D quadrant and its organizing function to polarize the dorsal-ventral axis and regulate the development of adjacent cell quadrants. In a few species, however, polar lobes have been shown to play only minor roles in development (*e.g.,* first polar lobe of *Chaetopterus variopedatus*, Henry, 1986, 1989; second polar lobe of *Bithynia tentaculata*, Dohmen and Verdonk, 1974; Cather and Verdonk, 1974; van Dam *et al.*, 1982). In *C. fornicata*,

the polar lobe and individual blastomeres may be isolated to study subsequent development and to assay for developmental potential and the presence of specific cell-cell inductive interactions (see section F1 in Henry *et al.,* 2010a).

In *C. fornicata* the small polar lobe formed at the first cleavage division may be removed, and subsequent development leads to the formation of a normal-looking veliger larva (Henry *et al.*, 2006). In fact, several lines of evidence support the idea that in *C. fornicata,* D quadrant selection occurs through a conditional mechanism like that deployed in equal-cleaving spiralians. Unlike the situation found in unequal-cleaving spiralians, the first and second cleavage planes in *C. fornicata* exhibit a variable relationship relative to the arrangement of the A, B, C, and D quadrants and the dorsoventral axis (Henry *et al.*, 2006). In most cases the first cleavage plane separates the AB and CD blastomeres; however, in some cases it separates the equivalent of a DA blastomeres from a BC blastomere.

In equal-cleaving spiralians, any quadrant may become the D quadrant (van den Biggelaar and Guerrier, 1979; Arnolds, *et al.*, 1983; Martindale *et al.*, 1985; Kuhtreiber *et al.*, 1988; Boring, 1989; van den Biggelaar, 1996; Henry, 2002; Henry *et al*., 2006). While there is a bias favoring the two vegetal cross-furrow macromeres due to their central location beneath the first quartet derivatives, only a single quadrant ultimately becomes the D quadrant (Verdonk and van den Biggelaar, 1983; Henry, 2002). Normally the small polar lobe in *C. fornicata* ends up fusing with the prospective D blastomere (Henry *et al.*, 2006). However, it seems unlikely that the tiny polar lobe in *C. fornicata* could increase the size of the D quadrant enough to favor its spatial relationship relative to the other quadrants. While development appears to be normal following removal of the polar lobe at first cleavage, we have observed multiple quadrant activation (phosphorylation) of ERK1/2, MAPK in two or three macromeres at the 24-cell stage after polar lobe removal (Henry and Perry, 2008; see further discussion below). Removal of the polar lobe does not appear to result in the loss of or formation of multiple, functional D quadrants, as abnormal development was rarely observed. Likewise, embryos containing supernumerary dorsoventral axes (*e.g.,* twins or triplets) were never observed after removal of the polar lobe, or after compression to distribute the contents of the polar lobe to both blastomeres. However, we have observed spontaneous cases of *C. fornicata* with two protruding 3D macromeres that each form mesentoblast cells. These twin embryos exhibit grossly abnormal forms of development, but do differentiate ectopic ocelli (three to four) and four external, embryonic kidneys (Henry and Martindale, unpubl. obs.).

As is the case in equal-cleaving spiralians, cell ablation experiments indicate that D quadrant selection involves inductive interactions from the derivatives of the first quartet micromeres in *C. fornicata* (Henry *et al.*, 2006). Removal of the first quartet micromeres leads to the absence of a D quadrant and to subsequent radial development. Furthermore, embryos from which the D quadrant blastomeres have been removed (*e.g.,* D at the four-cell stage, or even 3D early during the 24-cell stage, as determined *via* the earlier pattern of polar lobe fusion at first and second cleavage) can regulate to form a normal-looking veliger larva.

We have carried out ablations of the mesentoblast (4d) and its daughters (Henry and Martindale, unpubl. data). While removal of 4d just after its formation leads to radialized development, removal of both ML and MR does not. The latter results in the absence of the internal and both external kidneys and intestine (specific fates normally formed by this cell), but development is otherwise normal with respect to the head, velum, foot, and shell. These experiments indicate that, unlike the case seen in other spiralians where 3D serves as the organizer, the key organizer is 4d in *C. fornicata* (Henry *et al.*, 2006). Furthermore, the ablation experiments in *C. fornicata* demonstrate that the 4d organizer signal is sent prior to its division to form the left and right daughters (ML and MR). At the time of its formation, the 4d cell in *C. fornicata* is elongated and extends to the center of the embryo. This finger-like extension may serve to induce the development of cells in the other quadrants. Although this condition appears to represent an evolutionary heterotopic and heterochronic shift compared to the situation encountered in other spiralians, it has recently been suggested that 4d might serve as the organizer in annelids and that the organizer activity of 3D might be continued by its daughter (4d) in *Ilyanassa obsoleta* (Lambert and Nagy, 2003; Lambert, 2008).

Molecular Basis of D Quadrant Selection and Organizer Activity

Mitogen-activated protein kinases (MAPK, or extracellular signal-related kinases, ERK) are conserved serine/threonine kinases activated by different extracellular stimuli (Ferrell, 1996; Garrington and Johnson, 1999; Cobb and Goldsmith, 2000). ERK1/2, MAPK is a target of receptor tyrosine kinases, such as the FGF and EGF receptors (Gabay *et al.*, 1997; Christen and Slack, 1999; Cobb and Goldsmith, 2000), as well as that of other pathways. The ERK 1/2, MAPK cascade plays roles in regulating a variety of developmental events in metazoans (Henry and Perry, 2008). Lambert and Nagy (2001) demonstrated that activated ERK1/2, MAPK is required in the D quadrant (*i.e.,* 3D) and subsequently in specific micromere progeny of the A and C quadrants for proper cell specification and dorsoventral (DV) axis formation in the gastropod *Ilyanassa*. These findings suggest that an unknown, short-range diffusible signal(s) is involved in this process. When the activation of ERK1/2, MAPK is inhibited in 3D and subsequently

in the micromeres using the MEK inhibitor U0126, development resembles that of embryos from which the polar lobe or the organizer (3D) has been removed (Lambert and Nagy, 2001). The timing of these events, and possibly D quadrant induction, may differ between species in some equal-cleaving spiralians (Lartillot *et al.*, 2002; Lambert and Nagy, 2003; Koop *et al.*, 2007). Unlike the situation in *I. obsoleta*, ERK1/2, MAPK is only activated in 3D in the molluscs *Patella vulgata*, *Lymnaea palustris*, *Tectura scutum*, *Cheatopleura apiculata*, and *Haliotis asinina,* and in 4d in the annelid *Hydroides hexagonus*.

The pattern of ERK1/2, MAPK activation in *C. fornicata* embryos is illustrated in Figure 2A–J (diagonal hatch marks) and is somewhat similar to that exhibited by *I. obsoleta*. Interestingly, ERK1/2, MAPK is initially activated in the progeny of the first quartet micromeres (the four $1q^1$ cells) at the 16-cell stage, and subsequently in 3D at the 20–24-cell stages, followed by 4d at the 25-cell stage, and weakly in a small subset of micromeres at later stages (Henry and Perry, 2008). Sequential treatments with the MEK inhibitor U0126 revealed that activated ERK1/2, MAPK is required for normal development in the progeny of the first quartet during the 16- to early 20-cell stage, and early during the 20-cell stage in 3D. These early treatments lead to radialized development. Therefore, as 4d serves as the organizer in *C. fornicata* (Henry *et al.*, 2006), ERK1/2, MAPK is not required for 4d organizer activity or in other animal micromeres at subsequent stages of development in *C. fornicata*. Significantly, the early activation of ERK1/2, MAPK in the animal 1q daughters in *C. fornicata* embryos, which themselves specify the 3D macromere, suggests that these micromeres may be responding to an even earlier, unidentified signal.

Genomics Data

At this time the genome of *C. fornicata* has not yet been sequenced. However, the Henry laboratory (Henry *et al*., 2010b) has undertaken a large-scale EST sequencing project using GS FLX Titanium 454 pyrosequencing of a normalized cDNA library, representing messages derived from a mixed population of embryonic stages in *C. fornicata* (covering the first 5 days of development). The total output from a single bulk plate and three initial titration runs was almost 1.3 million reads with an average of 379 bases (approx. 491 megabases of sequence). All sequences have now been assembled and annotated (39,897 contigs, each composed of 2 to 6965 overlapping sequences). There are an additional 149,292 unassembled sequences ranging from 7 to 553 bases in length. This information is available *via* the National Center for Biotechnology Information and is accessible through NCBI's SRA and TSA databases (SRA Accession # SRA012219, see http://www.ncbi.nlm.nih.gov/Traces/sra/; TSA Accession numbers EZ540111-EZ579863, see http://www.ncbi.nlm.nih.gov/Traces/assembly; see also Henry *et al.,* 2010b). The Henry laboratory has also constructed a *Crepidula* embryonic cDNA library, and along with RT-PCR using degenerate primers and 3′ and 5′ RACE, we have amplified, cloned, and sequenced more than 220 unique developmental genes (Henry *et al.*, 2010b). Along with the 454 sequences, above, these data include a large array of candidate genes involved in early development and will serve as a tremendous resource for continuing studies of *Crepidula* development.

In addition, F. Viard's team from the Station Biologique de Roscoff (France) has developed a small-scale EST library from which a microarray of unigenes has been produced (F. Viard, pers. comm.), as part of the "Marine Genomics Europe" Network of Excellence, funded by the European Commission. This work is focused on gene expression in response to various metamorphic inducers, and several genes have already been shown to have differential expression during larval development and metamorphosis (Taris *et al.,* 2009; F. Viard, A. Tanguy, and N. Taris, unpubl. data, see below). *C. fornicata* is also one of the targeted models of the European project ASSEMBLE (ASSEMBLE, 2010)

Experimental Molecular Approaches in *Crepidula*

Gain- and loss-of-function assays to study the roles of specific genes work well in *Crepidula fornicata* because at any stage of development its eggs and embryos are easy to microinject with synthetic RNAs and morpholinos. RNAs are readily expressed. For instance, both endogenous and *Nematostella vectensis* beta-catenin tagged with green fluorescent protein can be expressed in the early embryo (Henry *et al.*, 2010c; see Fig. 5A). Likewise, photo-activatible Kaede protein can be expressed from synthetic RNA, which stably alters its emission spectra from green to red wavelengths after UV irradiation (Tomura *et al.*, 2008; see Fig. 5B–E). The latter is useful for labeling specific blastomere sublineages later during development. Morpholinos can also be injected to knockdown translation of particular messages (see section I1 in Henry *et al.,* 2010a). For instance, injection of morpholinos directed against endogenous beta-catenin transcripts leads to characteristic defects and a significant decrease of expression, as verified by Western blots (Henry *et al*., 2010c).

Gene expression can be readily examined by in *situ* hybridization based on the protocol of Finnerty *et al.* (2003; examples are shown in Fig. 5F-I; see section H1 in Henry *et al.,* 2010a). Protein localization has been determined readily using various antibodies (*e.g.,* anti-dpERK1/2, MAPK, anti-seratonin, anti-FMRFamide, anti-hunchback-like; Henry *et al.*, 2006; Hejnol *et al.*, 2007; Henry and Perry, 2008; Dean *et al.,*

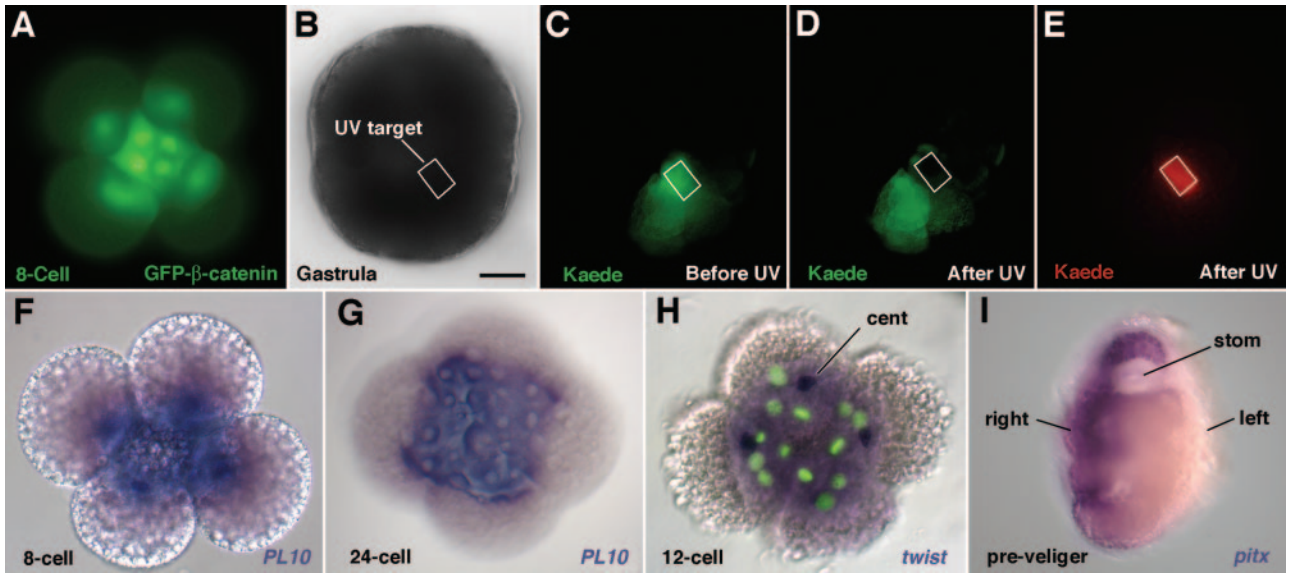

Figure 5. Expression of fluorescently tagged synthetic RNAs in *Crepidula fornicata*. (A) Expression of GFP-tagged beta-catenin RNA injected into the zygote. The *Nematostella vestensis* β–catenin-GFP fusion is as described in Wikramanayake *et al.* (2003). (B–E) Expression of Kaede synthetic RNA injected into one macromere at the eight-cell stage. Before exposure to UV irradiation the protein exhibits green fluorescence. After targeted UV irradiation, the protein can be converted to exhibit red fluorescence. Kaede encoding RNA is as described in Tomura *et al.* (2008). (F–I) mRNA localization by whole-mount *in situ* hybridization in *Crepidula fornicata*. (F–G) Animal pole views showing widespread localization of *PL10* mRNA (purple) in blastomeres of the 8- and 24-cell-stage embryos. (H) Animal pole view showing localization of *twist* mRNA in the 12-cell-stage embryo. Notice the intense accumulation of *twist* mRNA (purple) in the centrosomes (cent) associated with the nuclei (green) of the four macromeres. (I) Ventral view showing asymmetric localization of *pitx* mRNA in the preveliger-stage embryo. stom, stomodeum. Scale bar in B equals 40 μm for A–E, 30 μm for F–H, and 50 μm for I.

2009). Likewise, muscle cells containing filamentous actin have been labeled using phalloidin (Hejnol *et al.*, 2007).

Mode of Development in Calyptraeids

Calyptraeid gastropods are ideal for comparative studies, as five distinct modes of development are present within the family. Among 78 species reviewed, 50% develop from small eggs into planktotrophic veliger larvae, 30% develop directly from large eggs and produce crawl-away juveniles, 15% develop directly from small eggs by consuming other embryos (or nurse eggs) in the same capsule, and 5% produce large eggs that develop into short-lived nonfeeding pediveliger larvae (Collin, 2003c). In two species, embryos consume other embryos in the same capsule and hatch as short-lived nonfeeding pediveliger larvae. In many cases, sister species differ in mode of development (Collin, 2000a, 2003c, 2005; Collin *et al*., 2007). In general, the species with planktotrophic larvae have egg diameters of 120–256 μm and hatch at a shell length of 220–500 μm. Direct developers with large eggs have egg diameters of 240–540 μm and hatch at a shell length of 700–3000 μm (Collin, 2003c). Overall, the duration of development is temperature-dependent and for planktotrophs ranges from 6 days in the tropics (at 21–23 °C) to 33 days in Friday Harbor, Washington (at 10–12 °C), and 14–120 days in species with large eggs (reviewed in Collin, 2003c).

Embryonic development of calyptraeids is typical of caenogastropod development. Embryos do not pass through a distinct trochophore larval stage, and most develop directly into veliger larvae (Fig. 2W–Y). An intracapsular veliger stage is present in most species (figured in Collin, 2000b, 2005; Collin *et al.,* 2007); however, some of the direct-developing species have lost this stage, and development proceeds directly to the juvenile morphology (Gallardo, 1977; Collin, 2000b, 2004). Species with large yolky eggs are more likely than those with nurse eggs to have lost definitive larval features like the velum and operculum, and loss of such features is also more likely in species with large molecular divergences from their planktotrophic sister species (Collin, 2004). Detailed descriptions of organogenesis are available for the planktotrophs *C. fornicata* (Conklin, 1897) and *C. lingulata* (Fretter, 1972), as well as for the direct-developer *C. adunca* (Conklin, 1897; Moritz, 1939). The development of the early nervous system has also been described for *C. fornicata* (Dickinson *et al.,* 1999). The calyptraeid veliger larva is typical of most caenogastropod

larvae and has been described in detail for *C. fornicata* (Werner, 1955) and *C. lingulata* (Fretter, 1972; Collin, 2000b).

One unusual feature of calyptraeid development is the frequent presence of nurse eggs in the egg capsules. Nurse eggs are common in muricid gastropods but are unknown in other well-studied groups like *Conus* (Kohn and Perron, 1994) and littorinids. Development with nurse eggs has evolved multiple times within calyptraeids and appears to be most common in the southern hemisphere (Collin, 2003c, 2004). Nurse eggs can either be eggs that do not cleave (*e.g., Crepipatella dilatata* (Lamarck, 1822) Gallardo, 1977; Chaparro *et al.,* 2002b), or eggs that begin to develop but do not continue normally, forming yolky ciliated embryoids (*e.g., C. coquimbensis* Brown and Olivares, 1996; Collin, 2004), which are consumed by normal embryos during development. The mode of consumption of nurse eggs varies among species, with *C. dilatata* consuming the entire nurse egg whole, and *Crepipatella* sp. gradually sucking the yolk from the embryos (see Fig. 1L; see Collin *et al.*, 2007). In most cases, the normal embryos develop a veliger morphology before consuming the nurse eggs, but in some cases the embryos begin feeding on the nurse eggs without growing a distinct foot or velum (*Crepipatella capensis* (Quoy and Gaimard, 1832–1833) in Collin *et al.,* 2007). What distinguishes the developmental trajectory of normal embryos from those of nurse embryoids is an intriguing question in developmental biology.

The relatively rapid generation time makes classical genetic studies of developmental features in calyptraeids possible. A common "garden" experiment using *C. atrasolea* and *C. ustulatulina* has demonstrated that both genetic and environmental factors have significant effects on egg size and hatching size (Collin and Salazar, 2010). A large portion of this variation is due to genetic differences among females (Collin, 2010), and a quantitative genetic analysis is underway to determine the size of the additive genetic component of each feature. Wide sense heritibilities have also been calculated for larval growth and swimming speed in *C. fornicata*, and it is estimated that about 30% of the variation in each trait has a genetic basis (Hilbish *et al.,* 1999). Development of microsatellite markers for *C. fornicata* (Dupont and Viard, 2003; Proestou *et al.,* 2008), *C. convexa* (Daguin-Thiebaut *et al.,* 2009) and *C. coquimbensis* (Daguin *et al.,* 2007), and RAPD markers for *Crepipatella dilatata* and *Crepipatella fecunda* (previously *Crepidula*; Schmidt *et al.,* 2006) have further facilitated genetic studies of calyptraeid reproduction and population biology (Dupont *et al*., 2006; Proestou *et al*., 2008; Le Cam *et al*., 2009).

Larval Biology and Metamorphosis

The larvae of *C. fornicata* hatch at a shell length of 445–489 μm and settle at a length of 940–1000 μm (Pechenik *et al.,* 1996a, b), but most other planktotrophic species are somewhat smaller at hatching (Collin, 2003c). The shell is smooth and has a right-handed coil once it begins to grow. The velum is bilobed in all species and in *C. fornicata* has numerous yellow or greenish/brown spots, which also occur on the foot. Planktotrophic veligers of other calyptraeid species are very similar in appearance to those of *C. fornicata*, although the patterns of pigmentation on the velum and shell sculpture vary among species (Collin, 2000b, 2005; Collin *et al.,* 2007). Larvae can be reared easily in the laboratory on the same diet as the adults (Pechenik and Lima, 1984; Pechenik *et al.*, 1996a, b; Collin, 2000b; Klinzing and Pechenik, 2000), but are very sensitive to food concentrations (Pechenik *et al.,* 1996b, for *C. fornicata*; Zhao *et al.,* 2003, for *C. onyx*). Techniques for anesthetizing, observing, and fixing the larvae are included in section G1 in Henry *et al.* (2010a).

The larvae of *C. fornicata* have been the subject of numerous studies of the factors affecting larval growth rate and how this contributes to the timing of metamorphosis and settlement (Pechenik, 1980; Pechenik and Lima, 1984; Pechenik *et al.*, 1996a, b). The larvae of *C. plana* and *C. onyx* have been studied to a somewhat lesser extent (Lima and Pechenik, 1985; Zimmerman and Pechenik, 1991; Zhao *et al.,* 2003). Paternity analyses with microsatellite markers have shown that the more different individual males contribute paternity to a brood, the greater the growth rate of the resulting larvae (Le Cam *et al.,* 2009). Larval morphology also responds to food concentration: the size of the velum relative to the shell length increases with reduced food (Klinzing and Pechenick, 2000). However, under the same conditions, inherently fast-growing larvae have relatively smaller velar lobes than inherently slow-growing larvae (Klinzing and Pechenick, 2000). In addition, nutritional stress in larval stages carries over into the juvenile stage. Larvae that are food-limited metamorphose at smaller sizes and grow more slowly as juveniles than animals that are well fed as larvae (Pechenik *et al.,* 1996a, b).

Metamorphosis of competent larvae can be induced by exposure to adult-conditioned seawater, substrates with well-developed microfilms, or artificial cues (see below; Pechenik and Gee, 1993). In the absence of appropriate cues for settlement, *C. fornicata* larvae will continue to grow and differentiate even after reaching a stage or size at which they are competent to settle (Pechenik, 2006). The precompetent stage can last for up to 11 days, and this can be followed by a delay of metamorphosis of up to 16 days if the appropriate cues are not available to trigger metamorphosis (Pechenik, 2006). During this time the larvae may reduce growth, but they continue to differentiate the shell and gills (Pechenik and Lima, 1984, for *C. fornicata*; Lima and Pechenik, 1985, for *C. plana*). When metamorphic cues are not available, larvae will eventually metamorphose spontaneously. Continued differentiation followed by spon-

taneous metamorphosis suggests that metamorphosis is triggered when a fixed developmental trajectory reaches a more or less set endpoint (Pechenik, 1980; Pechenik and Lima, 1984). Delayed metamorphosis *per se* does not affect the juveniles' growth or survival (Pechenik and Eyster, 1989).

Metamorphosis, a key switch point in development, is easy to observe in calyptraeids with planktotrophic larvae. Metamorphosis can be induced in larvae that are competent to metamorphose by a variety of mechanisms, including excess K^+ (usually in the form of KCl) and dibromomethane (Pechenik and Gee, 1993; Taris *et al.*, 2009). Metamorphosis can be inhibited by depleting endogenous L-DOPA (Pires *et al.,* 2000), and the dopamine antagonists chlorpromazine, R(+)-Sch-23309, and spiperone can all act to block metamorphosis in response to excess K^+ (Pechenik *et al.,* 2002). These agents have also been shown to stimulate metamorphosis in the absence of excess K^+, or with long-term exposure, making the interpretation of the underlying mechanisms unclear (Pechenik *et al.,* 2002). Recent work has shown that nitric oxide (NO) is an endogenous inhibitor of metamorphosis (Pechenik *et al.,* 2007), and work on the nitric oxide synthase (NOS) pathway and regulation of the expression of neuronal NOS (nNOS) has shown a pattern of expression consistent with this function (Taris *et al.*, 2009).

Sex Change

An unusual developmental feature of calyptraeids is that after the juvenile stage all animals develop as males and subsequently undergo sex change to become females. All species that have been studied in the family show this pattern of protandrous sex change (*i.e.,* males changing to females). This has been the subject of a number of experimental, anatomical, genetic, and behavioral studies over the last century (Orton, 1909; Gould, 1917, 1919, 1952; Coe, 1936, 1938, 1948, 1953; Hoagland, 1978; Warner *et al.,* 1996; Collin, 1995, 2000b; Collin *et al.,* 2005, 2006; Dupont *et al.,* 2006; Proestou *et al.,* 2008). Sex change occurs when the penis is absorbed; the area associated with the vas deferens is elaborated into a capsule gland, albumin gland, and female genital papilla; and the gonad converts from a testis to an ovary. The occurrence of such morphogenic changes well into adult life is unusual among gastropods.

Sex change is influenced by conspecific associations: males associated with females delay sex change relative to solitary males or males associated with other males (Collin *et al.,* 2005). However, these associations do not explain the wide variation observed in natural sex ratio across the family (Collin *et al.*, 2006), and preliminary data suggest that some physical factors could also influence the timing of sex change (Mérot and Collin, unpubl. data). Recent application of microsatellites to assign paternity to broods in several species has shown that the varying social structure among species also affects patterns of paternity allocation (Dupont *et al.,* 2006, 2007; Proestou *et al.,* 2008; Le Cam *et al*., 2009). Once they are better understood, patterns of paternity will be included in models that predict when sex change should occur. Despite a fairly good understanding of the factors that influence sex change, little is known about the hormonal changes and mechanisms underlying the transformation of the gonad and secondary sexual features. Sex change is ripe for the application of genomics data to understand the differences in gene expression that underlie expression of the different sexual phases, the transition between them, as well as the cues that initiate this transition.

Advantages of *Crepidula* as a Model System

As is clear from the number and diversity of studies conducted on the slipper limpet *C. fornicata*, this species offers many advantages as an animal system. Here we have focused on its strengths as a model to study spiralian, lophotrochozoan development, especially in a comparative context, but the advantages of using *C. fornicata* are applicable to many research fields. The adults are abundant and easily collected in shallow waters, and both adults and developmental stages are easily maintained in the laboratory and are robust to experimental manipulation. Brooding females provide a large amount of embryonic material, and the reproductive period can be significantly extended in the laboratory. The embryos are easily collected and once released from their egg capsules are devoid of any external investments, such as a vitelline envelope. Development proceeds rapidly to the veliger larval stage at room temperature (22–23 °C), and the larvae are transparent, which permits visualization of internal structures.

Early development is well characterized for *C. fornicata*, and a detailed cell lineage fate map is available (Conklin, 1897; Hejnol *et al.*, 2007; Henry *et al.*, 2007). In fact, *Crepidula* represents one of only a small number of spiral-cleaving lophotrochozoans for which a fate map has been established for each blastomere through the birth of the four quartets of micromeres (Hejnol *et al.*, 2007; Henry *et al.*, 2007). Furthermore, this system offers significant advantages for experimental manipulation and modern cell and molecular studies, as the embryos may be readily injected with lineage tracers, synthetic RNAs, and morpholinos for either loss- or gain-of-function analyses (Henry *et al.*, 2006, 2007; and described above). Furthermore, *C. fornicata* exhibits the hypothesized ancestral pattern of spiralian development with equal cleavage and conditional specification of early cell fates (Freeman and Lundelius, 1992; see also van den Biggelaar and Haszprunar, 1996; Henry, 2002). Therefore, studies of *Crepidula* embryos complement those of the "unequal-cleaving" spiralian *Ilyanassa,* in which there is autonomous specification of the D quadrant (Lambert, 2009).

There are several advantages to evolutionary studies us-

ing calyptraeid gastropods and *Crepidula* in particular. The species-level phylogeny for the family is largely known (Collin, 2003a, b), and the taxonomic status and identification of the most commonly used species is well known and clear-cut. The placement of the family within the gastropods is clearly in the non-neogastropod caenogastropods, and the sister families are known, making evolutionary comparisons with other gastropod families informative and the use of phylogenetic comparative methods possible.

The time to sexual maturity is short, making genetic analyses possible in this organism. The genome of *C. fornicata* has not yet been sequenced, and only a few other lophotrochozoans (*e.g., Lottia gigantea*, *Biomphalaria glabrata*, *Capitella* sp. I, *Helobdella robusta*, *Aplysia california*) have been sequenced at this time. The genome size of *C. fornicata* is unknown, but that of *C. unguiformis* Lamarck, 1822, is 6.36 pg (Libertini *et al.*, 2009) compared to 3.2 pg for *I. obsoleta* (Gharbiah *et al.*, 2009), which is large compared to other metazoans and one of the largest known for gastropods. Although there is evidence of polyploidy in some calyptraeids (*C. fecunda* and C. *dilatata* relative to their undescribed Chilean sister species; F. Winker, Universidad Católica del Norte, Chile; pers. comm.), there is no evidence of polypoidy in *C. fornicata* (F. Viard, pers. comm.). Given the pace of technological advances and the number of research groups focused on *Crepidula,* it will soon be feasible to sequence the *C. fornicata* genome.

For experimental studies *Crepidula* offers many significant advantages over other "competing" gastropod molluscs. For instance, the limpet *L. gigantea* cannot be readily cultured and is highly seasonal; essentially no experimental work has been done with this system. Another gastropod, *B. glabrata,* is a pulmonate (as is *Lymnaea*), a group that displays highly derived development compared to other gastropods, and which has encapsulated embryos that are difficult to work with. The embryos are not amenable to manipulation and microinjection. Only recently has it been possible to culture the embryos of a pulmonate, *L. stagnalis,* outside of their egg capsules, and this must be done in small capillaries containing natural capsule fluid (Kuroda *et al*., 2009). In contrast to the situation found in most other gastropods, *B. glabrata* exhibits sinistral cleavage and shell coiling, which is important to understand from a comparative standpoint. Although *Crepidula* species develop without the formation of a trochophore larva, the absence of a trochophore is typical of development for most gastropods, being present only in patellogastropods (limpets) and vetigastropods (trochoideans, abalones, and other unusual basal taxa). In no gastropods is there a feeding trochophore. The presence of a feeding trochophore is considered ancestral for the trochozoans. Finally, *C. fornicata* (an invasive species) is now found in both North America and Europe, making it more widely available than some other species, such as *I. obsoleta*.

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