

Poecilogony in the caenogastropod *Calyptraea lichen* (Mollusca: Gastropoda)

Kathryn A. McDonald,^{1,2,a} Rachel Collin,² and Maryna P. Lesoway^{2,3}

¹ Present affiliation: Department of Biological Sciences, Humboldt State University, Arcata, California 95521, USA

² Smithsonian Tropical Research Institute, Apartado Postal 0843-03092, Balboa, Ancon, Republic of Panama

³ Department of Biology, McGill University, Montreal, Québec, Canada H3A-1B1

Abstract. In marine invertebrates, polymorphism and polyphenism in mode of development are known as “poecilogony.” Understanding the environmental correlates of poecilogony and the developmental mechanisms that produce it could contribute to a better understanding of evolutionary transitions in mode of development. However, poecilogony is rare in marine invertebrates, with only ten recognized, well-documented cases. Five examples occur in sacoglossan gastropods, and five occur in spionid polychaetes. Here, we document the eleventh case, and the first in a caenogastropod mollusc. Females of *Calyptraea lichen* collected in the field or reared in the laboratory often produce broods of planktotrophic larvae. They can also be collected with mixed broods, in which each capsule contains planktotrophic larvae, nurse embryos, and adelphophagic embryos. Adelphophages eat the nurse embryos and hatch as short-lived lecithotrophic larvae, or even as juveniles. Mitochondrial COI and 16S DNA sequences for females with different types of broods differ by less than 0.5%, supporting conspecific status. Some females collected in the field with mixed broods subsequently produced planktotrophic broods, demonstrating that females can produce two different kinds of broods. *Calyptraea lichen* is therefore polyphenic in two ways: mode of development can vary among embryos within a capsule, and females can change the types of broods they produce.

Additional key words: adelphophagy, Calyptraeidae, polyphenism, mode of development, plasticity

Alternative phenotypes, the expression of more than one discrete phenotype either during the lifetime of an individual or among individuals of the same species, are thought to play a major role in generating biological diversity (West-Eberhard 2003; Schwander & Leimar 2011). Evolutionary transitions between different genetically fixed discrete monomorphic states may occur by passage through transitional stages that could include polyphenic intermediates (Schwander & Leimar 2011). The alternative phenotypes expressed by a polyphenic species can result from genetic polymorphism, a plastic response to different environmental cues, or a combination of the two. These underlying modes of control may be evolutionarily labile, changing repeatedly between environmental and genetic control through evolutionary time and across phylogeny (Schwander & Leimar 2011).

Most species of marine invertebrates are monomorphic with respect to mode of development (Thorson 1950; Hoagland & Robertson 1988; Allen & Pernet 2007). Some species produce offspring that develop as small swimming larvae that must feed to reach metamorphosis (planktotrophic development). Other species produce larger non-feeding lecithotrophic larvae, and yet others produce large embryos that bypass a free-living larval stage and hatch as crawling juveniles (direct development). Although many groups of marine invertebrates express interspecific variation in mode of development, very few intermediates or evolutionary transitional forms between these two common modes of development have been documented (Thorson 1950; Allen & Pernet 2007; Collin 2012). Understanding transitions among the different modes of development is a major goal of invertebrate biologists, and studies of potentially transitional forms are considered key in this endeavor (Strathmann 1978a,b, 1985; Allen & Pernet 2007; Collin 2012; Knott & McHugh 2012).

^a Author for correspondence.

E-mail: kamcdon@post.harvard.edu

The production of alternative phenotypes in mode of development is termed poecilogony (Hoagland & Robertson 1988; Bouchet 1989; Chia et al. 1996; Knott & McHugh 2012). As populations or females of poecilogonous species are capable of producing different offspring types, such species could represent a transitional stage between indirect and direct modes of development (Knott & McHugh 2012). An alternate view is that poecilogony is not a transitional but a stable strategy, adaptive in variable environments (Chia et al. 1996; Collin 2012). Both of these views could apply to poecilogonous species, as the term poecilogony has been used to describe polyphenism at different scales, from variation among populations to plasticity within a female (Collin 2012).

Poecilogony is extremely rare. There are five documented cases in sacoglossan gastropods (Vendetti et al. 2012) and five cases in spionid polychaetes (Collin 2012). In six of these species, development varies either among populations (*Costasiella ocellifera* (SIMROTH 1895); *Elysia chlorotica* GOULD 1870; *Pygospio elegans* CLAPARÈDE 1863) or among females (*Elysia zuleicae* ORTEA & ESPINOSA 2002; *Elysia pusilla* (BERGH 1871); *Streblospio benedicti* WEBSTER 1879). Also, only four of these ten poecilogonous species produce both planktotrophic larvae and non-feeding offspring (reviewed in Collin 2012). In the spionid polychaete *Polydora cornuta* BOSC 1802 and the sacoglossan gastropod *Alderia willowi* KRUG, ELLINGSON, BURTON & VALDÉS 2007, females can change the kind of offspring produced between consecutive broods. Sometimes during these transitions they produce both kinds of offspring in the same clutch, but this is not typical (Krug 1998; MacKay & Gibson 1999; Krug et al. 2012). The spionids *Boccardia polybranchia* (HASWELL 1885) and *Boccardia proboscidea* HARTMAN 1940 normally produce both kinds of offspring from the same brood (Duchêne 1984; Gibson 1997); some of the embryos in each capsule of these mixed broods develop into planktotrophic larvae, while others within the same capsule consume nurse eggs, grow larger, and hatch as lecithotrophic larvae (Gibson 1997; Gibson et al. 1999; Oyarzun & Strathmann 2011; Oyarzun et al. 2011). Here, we report the discovery of an eleventh case of poecilogony, in the calyptraeid gastropod *Calyptrea lichen* BRODERIP 1834. This species shares features with *P. cornuta* and *A. willowi* (changes in brood composition between broods of the same female), and also with *B. proboscidea* and *B. polybranchia* (mixed broods). This report aims to describe the phenomenon and provide a basis for future research.

Methods

Individuals of *Calyptrea lichen* live on large cobbles and rock rubble in the low intertidal of the Bay of Panama. They are patchily distributed; of the calyptraeid species collected in the same location, *Crucibulum spinosum* (G.B. SOWERBY 1824), *Crucibulum radiata* (BRODERIP 1834), and *Crepidula navicella* (LESSON 1831) are frequently found together with *C. lichen*, but *C. lichen* seems to be less abundant than members of the other species. Like all calyptraeids, they are protandrous suspension-feeders that brood thin-walled transparent egg capsules between the substrate and neck lappets (Fig. 1A). Calyptraeids can produce broods of small eggs that hatch as planktotrophic larvae (~50% of species), broods of large eggs that hatch as crawling juveniles or very short-lived lecithotrophic larvae (~30% of species), or broods of adelphophagic embryos that consume nurse eggs/embryos and hatch as crawling juveniles (Collin 2003a). *Calyptrea lichen* has previously been reported to produce planktotrophic larvae (Collin 2003a).

Adults of *C. lichen* were collected from Playa Venado near Veracruz on the Pacific coast of Panama (8°52.9567'N, 79°35.8778'W) in 2010 and 2011. Any broods present were collected with their mother. Adults were kept in the laboratory individually or in pairs, if they were collected as pairs, in 350-mL plastic cups, and fed *Isochrysis galbana* PARKE 1949 daily (following Collin & Salazar 2010). Broods removed from their mothers were raised in UV-sterilized seawater passed through a 0.22- μ m membrane filter, with added antibiotics (6.3 $\times 10^{-5}$ mol L $^{-1}$ streptomycin sulfate salt, 1.4 $\times 10^{-4}$ mol L $^{-1}$ penicillin G potassium salt). Broods deposited in the laboratory were kept with the mother and allowed to hatch naturally to determine the hatching stage.

Three females with planktotrophic development and two females with mixed broods were preserved in 95% ethanol, and DNA was extracted using a phenol-chloroform extraction protocol. Fragments of two mitochondrial genes were sequenced; 650 base pairs of mitochondrial Cytochrome Oxidase subunit 1 (COI), and 500 base pairs of 16S rDNA. These were amplified and sequenced following the protocol and using the universal primers reported in Collin (2001). A fragment of the nuclear gene histone H3 was also amplified and sequenced following methods in Colgan et al. (2000). The COI and 16S sequences were aligned with previously published sequences of species in the Panamanian *Calyptrea* clade (planktotrophic *C. lichen*: AF546067, AF546007; *Calyptrea conica* BRODERIP 1834:

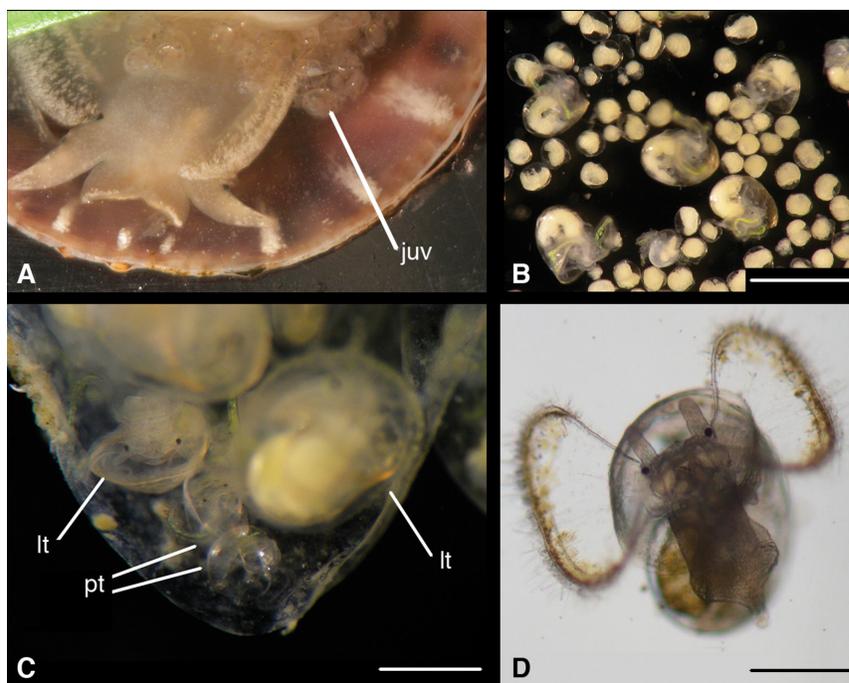


Fig. 1. Embryos and larvae of *Calyptraea lichen*. **A.** Ventral view of an adult snail, illustrating the brood area. This brood was mixed, and capsules were not hatched simultaneously; these particular capsules were retained intact until all larvae had metamorphosed. Several juveniles (juv) are visible, with approximate shell lengths of 1–1.2 mm. **B.** The contents of a capsule from a brood with mixed development illustrate size and developmental differences between nurse embryos and adelphophagic (nurse-embryo consuming) embryos. Scale bar=1.0 mm. **C.** Capsules in mixed broods contain planktotrophic-type embryos (pt) and adelphophagic embryos (lt), as well as the nurse embryos consumed by adelphophages. Scale bar=0.60 mm. **D.** Some adelphophagic embryos are hatched as swimming larvae. Scale bar=0.32 mm.

AF546063, AF546003; *Calyptraea mamillaris* BRODERIP 1834: AF546066, AF546006; *Calyptraea aspersa* C. B. ADAMS 1852: AF546060, AF546000) (Collin 2003b,c). The previously published *C. lichen* DNA sequences were from a single individual with planktotrophic development. Because the new sequences were either identical to or differed by only a single base pair from the previously published sequences for *C. lichen*, a new phylogenetic analysis was not necessary to place them in context.

Results

All lines of evidence detailed below support the conclusion that *Calyptraea lichen* is poecilogonous. Females of *C. lichen* produce two distinct kinds of broods: (1) those that consist entirely of capsules containing planktotrophic larvae, and (2) those that consist of capsules each containing a mix of nurse embryos, planktotrophic larvae which appear unable to consume nurse embryos, and larger lecithotrophic larvae which consume numerous nurse embryos. Hereafter, these will be referred to as “planktotrophic broods” and “mixed broods,” respectively.

These two types of broods cannot be distinguished early in development. Average uncleaved embryo size measured from two field-collected broods was ~200 μm (brood 1: mean embryo diameter = 202 μm , S.D. = 9.40, $N = 67$; brood 2: mean embryo diameter = 195 μm , S.D. = 19.8, $N = 73$). Calyptraeid embryos fail to develop further when they are excapsulated early in development, so we could not confirm brood type (planktotrophic or mixed) by continued observation of the measured embryos. Embryos from both field-collected and laboratory-reared broods appear similar in size and morphology during early development. All embryos appear to form blastulae and gastrulae typical for calyptraeids. After the gastrula stage, morphological differences between the nurse embryos and normal embryos become apparent (Fig. 1B–D), and the two brood types can be distinguished.

Planktotrophic broods

In broods with exclusively planktotrophic development, embryos develop following the normal pattern reported for planktotrophic calyptraeids

(Werner 1955; Collin 2000; Chaparro et al. 2005). In two planktotrophic broods that were deposited and hatched naturally (by the female snail) in the laboratory, veliger larvae hatched at 300–400 μm shell length (brood 1: mean shell length=366 μm , S.D.=27.8 μm , $N=6$; brood 2, mean shell length=407 μm , S.D.=25.1 μm , $N=6$). Measurements of near-hatching larvae from one field-collected planktotrophic brood showed a similar (but somewhat greater) mean shell length, and a unimodal size distribution (Fig. 2). The larval shell is unsculptured, and the velum has a fine, black line and a scattering of opaque, yellow-green pigment spots edging the food groove. Hatched veligers swam and fed actively in culture, but no attempt was made to raise these larvae to metamorphosis.

Mixed broods

In mixed broods, each capsule contained a combination of nurse embryos, planktotrophic larvae, and adelphophagic larvae that consumed the nurse embryos (Fig. 1B,C). The majority of embryos in each capsule developed into nurse embryos that appeared to arrest development at or near the gastrula stage (Fig. 3). One brood collected before the normal embryos began to consume the nurse embryos had a ratio of approximately one normal embryo per 10 nurse embryos (14:130, 17:191, and 15:152 normal embryos to nurse embryos in three capsules). The nurse embryos were morphologically similar to nurse embryos in a number of other calyptraeids (Véliz et al. 2001, 2003; Collin et al. 2007; Lesoway et al. unpubl. data). They developed a thin layer of clear, partially ciliated ectoderm that enclosed the yolk (Fig. 3). Unlike the normal embryos, they did not show directional swimming. At later stages, nurse embryos retained only a thin tube of yolk running through the center of a hollow ball of ectoderm. Occasional free yolk blobs were also present in the capsules.

The planktotrophic larvae produced in these mixed broods followed the same developmental trajectory as the planktotrophs from purely planktotrophic broods, and did not appear to consume nurse embryos. In capsules removed from the mother and maintained in culture, planktotrophic larvae eventually starved if capsules were not hatched artificially, even when there were still nurse embryos present. No attempt was made to raise the planktotrophic larvae from mixed broods in culture.

Adelphophages in mixed broods developed the early veliger morphology typical of calyptraeid embryos before beginning to consume the nurse

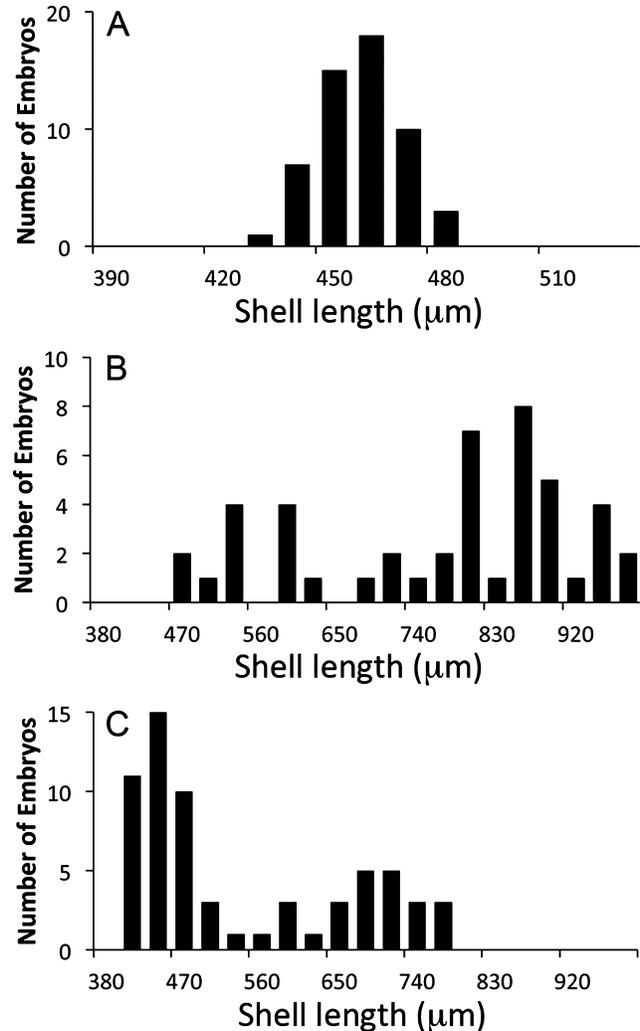


Fig. 2. Distribution of shell lengths of late-stage planktotrophic (A) and mixed broods (B, C). A. The veligers measured were a haphazard subsample from a field-collected planktotrophic brood. They appeared ready to hatch, and the shell lengths are similar to those measured for broods that hatched naturally in the laboratory. B, C. All viable embryos in the brood were measured, planktotrophs as well as adelphophages. Embryos in the brood shown in (C) retained more yolk and appeared to be at a somewhat earlier stage of development compared to the brood shown in (B), which accounts for their smaller size.

embryos: this included an early shell, velar lobes, a ciliated head vesicle, and a small foot. Adelphophages grew larger than the planktotrophs in the same capsule, and eventually developed the cap-shaped shell characteristic of calyptraeid larvae that are competent to metamorphose. Some adelphophages metamorphosed prior to hatching, but some retained the velum after hatching: we observed naturally hatched adelphophages swim in culture for at least 2 d (Fig. 1D).

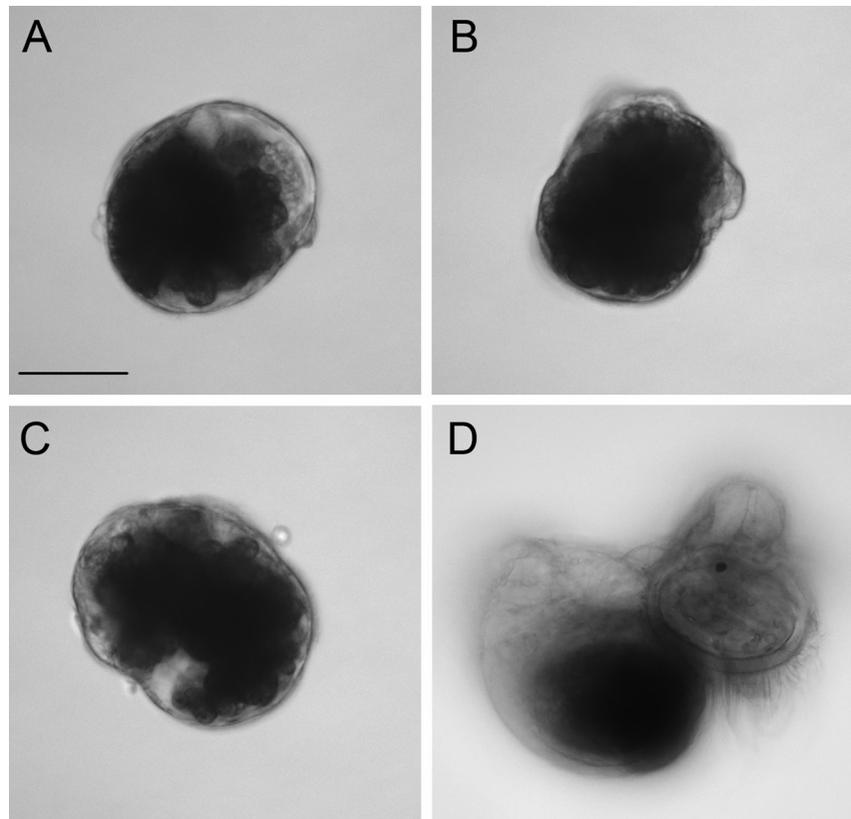


Fig. 3. Nurse embryos and one viable embryo taken from the same capsule of a mixed brood. **A–C.** Nurse embryos (nonviable embryos). **D.** A viable veliger excapsulated at the same time as the nurse embryos shown in (A–C). Scale bar=100 μm ; scale is the same for all panels.

Adelphophagic embryos that hatched as juveniles were ~ 1 mm in shell length (brood 1: mean shell length=1.00 mm, S.D.=0.024, $N=4$; brood 2: mean shell length=1.16 mm, S.D.=0.025, $N=5$). Adelphophagic embryos that hatched as swimming larvae from the same capsules were quite variable and generally slightly smaller than their siblings that metamorphosed prior to hatching. The shell lengths of embryos from two field-collected mixed broods, removed from their mothers late in the developmental period, demonstrated the range in size of viable embryos in mixed broods. Offspring size in those broods showed a bimodal distribution (Fig. 2).

We observed maternal behavior at the time of hatching for a single mixed brood. The mother opened two or three capsules in which the adelphophagic embryos still had a large velum. She then ejected the swimming larvae (Fig. 1D) from her shell, using her tentacles and propodium to maneuver them into the excurrent flow from the mantle cavity. The three or four remaining capsules were still intact 3 d after the initial hatching event. In the meantime, the adelphophagic embryos within these

capsules completed development to the juvenile stage (Fig. 1A). Several days later, these metamorphs had hatched, and many remained beneath the maternal shell while others crawled actively around the cup.

Changes in brood type

Females of *C. lichen* can change the type of broods they produce. Animals collected from the field with mixed broods frequently produced planktotrophic broods in the laboratory: of the five females that were collected with mixed broods in January 2010, or produced mixed broods immediately after collection, two snails produced planktotrophic broods subsequently in the lab and the other three failed to reproduce again. Six of seven snails collected with mixed broods in 2011 went on to produce only planktotrophic broods in the laboratory. The remaining snail from that collection produced three subsequent broods in the laboratory, two of which were planktotrophic and one of which was mixed. We did not observe the production of

mixed broods by animals collected in the field with planktotrophic broods.

We have not monitored individuals in the field to observe whether switches in brood type occur under natural conditions. Seventeen of 29 broods collected in the field in 2010 (January, March, November) and 2011 (January, August, September) were planktotrophic, while 10 of 14 broods collected in the same location in December 2013 were mixed. Overall, most broods deposited in the laboratory were planktotrophic.

DNA sequences

Sequences from all three gene fragments support the conclusion that the animals observed here belong to a single species. Of the five individuals sequenced, all three COI sequences from females with planktotrophic broods were identical to the GenBank sequence for a planktotrophic *C. lichen* (AF546067). The two females with mixed broods shared a sequence that differed from the others by 1 silent substitution (GenBank KF656790). The 16S sequences also show little variation. Three of the animals, two with planktotrophic broods and one with a mixed brood, had haplotypes identical to AF546007, a sequence previously obtained from a female with a planktotrophic brood. A new 16S haplotype with a single base difference was obtained from one planktotrophic female and one mixed brood female (GenBank KF656789). The nuclear H3 sequences showed no variation among the five samples (GenBank KF656788). Phylogenetic reconstruction of the 16S and COI sequences showed that *Calyptreaea aspersa* is sister to *C. lichen* (Collin 2003b), and the new sequences did not alter this conclusion. Taxon sampling in this genus is sparse compared with other calyptreaeids, and increased taxon sampling may uncover a closer sister. Insufficient calyptreaeid reference sequences for histone H3 are available to make comparisons with this nuclear gene.

Discussion

Poecilogony is so unusual that we have little understanding of how and why it arises, and whether it is an evolutionary stable developmental strategy. Our understanding of the genetic, developmental, and ecological factors associated with the evolution of poecilogony is limited by its phylogenetic distribution as well as the scarcity of documented cases. Independent phylogenetic comparisons are unlikely to be informative at the family level, when only two distantly related taxa include poecilogonous

species (Collin 2012; Knott & McHugh 2012). The discovery of poecilogony in *Calyptreaea lichen*, a caenogastropod, provides the first instance of this phenomenon outside the spionid polychaetes and sacoglossan gastropods. With this discovery, we now know of a gastropod that, like the spionid polychaetes, utilizes varying levels of adelphophagy to produce alternative phenotypes: in four of the five species of poecilogonous spionids, poecilogony results from differences in nurse-egg consumption by adelphophagic embryos (Knott & McHugh 2012), whereas all previously documented poecilogonous gastropods derive differences in offspring phenotype from differences in egg size (Vendetti et al. 2012).

The calyptreaeid gastropods are known for rapid evolution of mode of development. *Calyptreaea lichen* demonstrates a novel combination of mechanisms for producing variation in offspring type: mixed development in which planktotrophs, adelphophages, and nurse embryos are produced together within a single brood is combined with maternal “switches” between mixed development and planktotrophy. While mixed-brood development could represent a fixed trait, the alternation between planktotrophy and mixed development suggests a plastic response to external conditions. Maternal choice in hatching time (and therefore size and stage) of offspring in different capsules of a mixed brood may represent yet a third mechanism for controlling offspring phenotype in this species. Such maternal control over ratio of offspring phenotypes is known in the poecilogonous spionid *Boccardia proboscidea* (Oyarzun & Strathmann 2011). Capsules of *B. proboscidea* embryos contain a mixture of obligate planktotrophs, adelphophages, and nurse eggs; long brooding periods produce offspring that are close to metamorphosis at the time of hatching by the mother, whereas early hatching produces mainly planktotrophic larvae (Oyarzun & Strathmann 2011). For *B. proboscidea*, external conditions, and not the developmental stage of offspring, seem to regulate the time at which the mother opens her capsules (Oyarzun & Strathmann 2011). One or two levels of environmental control over offspring phenotype, or ratio of phenotypes (planktotrophic vs. adelphophagic larvae, short-lived veligers vs. crawl-away juveniles), could give *C. lichen* considerable flexibility to adjust reproductive investment to match or track environmental conditions. We observed one snail with a mixed brood hatch her capsules over a series of days: most capsules were opened after the adelphophages within had metamorphosed; therefore, the prior hatching of capsules

increased the representation of swimming adelphophages among offspring released. Swimming adelphophages from another mixed brood that hatched naturally in the laboratory were able to capture and ingest cells of *Isochrysis galbana*, which suggests that adelphophages hatched prior to their metamorphosis are viable larvae that may continue to feed and grow as they complete development. If a particular type of poecilogony improves offspring survivorship by improving the fit between hatchling size/stage and environment, then that strategy would be best viewed as an environmentally mediated adaptive maternal effect. Additional data on offspring fitness under a variety of environmental conditions are necessary to distinguish adaptive plasticity from bet-hedging.

Common features in the ecology of species that share poecilogony, or common patterns in the relationship between offspring phenotype and adult ecology, may help highlight the selective factors that give rise to this unusual kind of development. Uncovering new examples of poecilogony also increases our ability to evaluate apparent associations between poecilogony and specific adult ecologies. Poecilogony was first documented in species that inhabit estuarine mud flats, which suggested that features of this habitat, especially low salinities, might favor development with alternative phenotypes (Chia et al. 1996). Recent discoveries of poecilogonous species living on coral reefs, and in other high-salinity habitats, suggest that poecilogony is not peculiar to estuaries (Vendetti et al. 2012).

As more poecilogonous species are described, we will also be better able to determine how well micro-evolutionary models of alternative phenotypes predict the occurrence of different types of poecilogony. For example, Sasaki & Ellner (1995) used density-dependent models of selection to demonstrate that temporally unstable environments should favor the production of offspring of varying phenotype. They posited that “mixed” strategies (i.e., each female produces a variety of offspring phenotypes with a distribution determined by the maternal genotype) should be more common than “pure” strategies (i.e., genetic polymorphism in which each female can produce only one offspring phenotype), because a bet-hedger can always successfully invade a population that is genetically polymorphic, while the reverse is not true. Contrary to this prediction, our knowledge to date suggests that polymorphism more often underlies poecilogony than do mixed strategies: with the addition of *C. lichen*, we know of three species with mixed strategies and 6 species in which poecilogony results from polymorphism.

Although it is debatable if poecilogony plays a vital role in transitions between modes of development in marine invertebrates (Collin 2012; Knott & McHugh 2012; Vendetti et al. 2012), further development of a phylogenetic context, comparative developmental data, and genomic information could make poecilogonous species valuable model systems in which to investigate mode of development on a number of organizational levels (Knott & McHugh 2012). *Calyptraea lichen* may be a particularly attractive model, as calyptraeids exhibit several modes of development (Collin 2003a), and evolutionary transitions between them are rapid and frequent (Collin 2004; Collin et al. 2007). Mechanisms of early development have been described in detail for some calyptraeid species (Henry et al. 2010) and genomic information is already available for a planktotrophic and an adelphophagic species (Taris et al. 2009; Riquet et al. 2013; Lesoway, unpubl. data). *Calyptraea lichen* is thus a promising system in which to examine the genetic changes that underlie transitions in mode of development.

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