

Ancestral form and function of larval feeding structures are retained during the development of non-planktotrophic gastropods

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ABSTRACT Mode of development (MOD) is a key feature that influences the rate and direction of evolution of marine invertebrates. Although many groups include species with different MODs, the evolutionary loss of feeding larvae is thought to be irreversible, as the complex structures used for larval feeding and swimming are lost, reduced, or modified in many species lacking feeding larvae. This view is largely based on observations of echinoderms. Phylogenetic analysis suggests that feeding larvae have been re-gained in at least one species of calyptraeid gastropod. Further, its sister species has retained the velum, the structure used for larval feeding and swimming. Here, we document velar morphology and function in calyptraeids with 4 different MODs. Embryos of *Crepidula navicella*, *Crepidula atrasolea*, *Bostrycapulus aculeatus*, *Bostrycapulus odites*, *Bostrycapulus urraca*, *Crepidatella dilatata*, *Crepidatella occulta*, *Crucibulum quiriquinae* and *Crepidula coquimbensis* all hatch as crawling juveniles, yet only *Crepidula coquimbensis* does not make a well-formed velum during intracapsular development. The velar dimensions of 6 species with non-planktotrophic development were similar to those of planktotrophic species, while the body sizes were significantly larger. All of the species studied were able to capture and ingest particles from suspension, but several non-planktotrophic species may ingest captured particles only occasionally. Video footage suggests that some species with adelphophagic direct development capture but frequently fail to ingest particles compared to species with the other MODs. Together these lines of evidence show that, among calyptraeids at least, species that lack planktotrophic larvae often retain the structures and functions necessary to successfully capture and ingest particles, reducing the barriers to the re-evolution of planktotrophy.


KEY WORDS: *adelphophagy*, *veliger*, *particle capture*, *Crepidula*

Introduction

Key evolutionary transitions in morphology, ecology, or development in a lineage of organisms can alter its evolutionary potential, changing the dynamics of subsequent evolution. For example, ecological specialization, such as the transition from generalist feeders to host plant specialists, is thought to increase the potential for host-race formation and therefore increase the rate of speciation of specialist lineages relative to generalists (e.g., Caillaud and Via 2000; Groman and Pellmyr 2000). Morphological modifications can also alter evolutionary potentials. For example,

the loss of flight in birds has resulted in higher recent extinction rates in flightless birds than in those that retain flight (Steadman 1995; Steadman and Martin 2003) and lineages of plants with bilaterally symmetric flowers have higher speciation rates than those with radially symmetric flowers (Sargent 2004). The direction of evolutionary change of such key features often appears to be biased. For example, it is commonly assumed that evolution goes from generalist to specialist, and there is evidence for such a bias in phytophagous insects (Crespi and Sandoval 2000; Nosil

Abbreviations used in this paper: MOD, mode of development.

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2002). Such biases are sometimes thought to reflect the presumed difficulty of regaining complex morphological features once they are lost (Collin and Miglietta 2008). However, the causes of these biases are not always clear, and investigation of the functional and morphological features underlying such biases is necessary to complement phylogenetic patterns that suggest bias (e.g., Igic et al., 2006; Collin and Miglietta 2008).

Mode of development (MOD) in marine invertebrates is one such key feature thought to influence both the rate and direction of evolution (Krug et al., 2015; Collin and Moran 2018). From the perspective of energetics and ecology, invertebrates can be roughly divided into those that have feeding, planktonic larvae (planktotrophic development) and those that do not (Fig. 1; Table 1) (see Strathmann 1978a, b; 1985 for reviews). Development and growth of feeding larvae occur in the water column where small ciliated larvae feed on phytoplankton and are subject to passive dispersal via ocean currents. Larvae of some invertebrates may remain in the plankton for as long as several years, but larval development usually ranges from several days to months (Strathmann 1985; Strathmann 1987). In contrast, direct development (used here to refer to species that lack a free-living larval stage; Table 1) can

include development within benthic egg capsules, vivipary, or internal or external maternal brooding, and results in juveniles that crawl away from the site of oviposition.

The location of development (i.e., pelagic versus benthic) has a significant impact on both the microevolution and macroevolution of marine invertebrates. Differences in MOD in marine invertebrates have been demonstrated to result in differences in dispersal, gene flow, and population structure: Species with planktotrophic larvae have greater gene flow, higher nucleotide diversity and fewer nonsynonymous substitutions than species with benthic development or abbreviated larval stages (Duffy 1993; Hunt 1993; Hellberg 1996; Hoskin 1997; Kyle and Boulding 2000; Collin 2001; Foltz 2003). MOD can also correlate with the size of a species' geographic range (Jablonski 1987; Emler 1995). Paleontological and phylogenetic studies have shown that extinction and speciation rates differ for species with different modes of development (Hansen 1978, 1980, 1982; Jablonski 1986a, b; Gili and Martinelli 1994; Krug et al., 2015).

Unlike many of the examples of key evolutionary traits listed above, which seldom vary among close relatives, MOD varies on almost all levels of the phylogenetic hierarchy. It appears that the

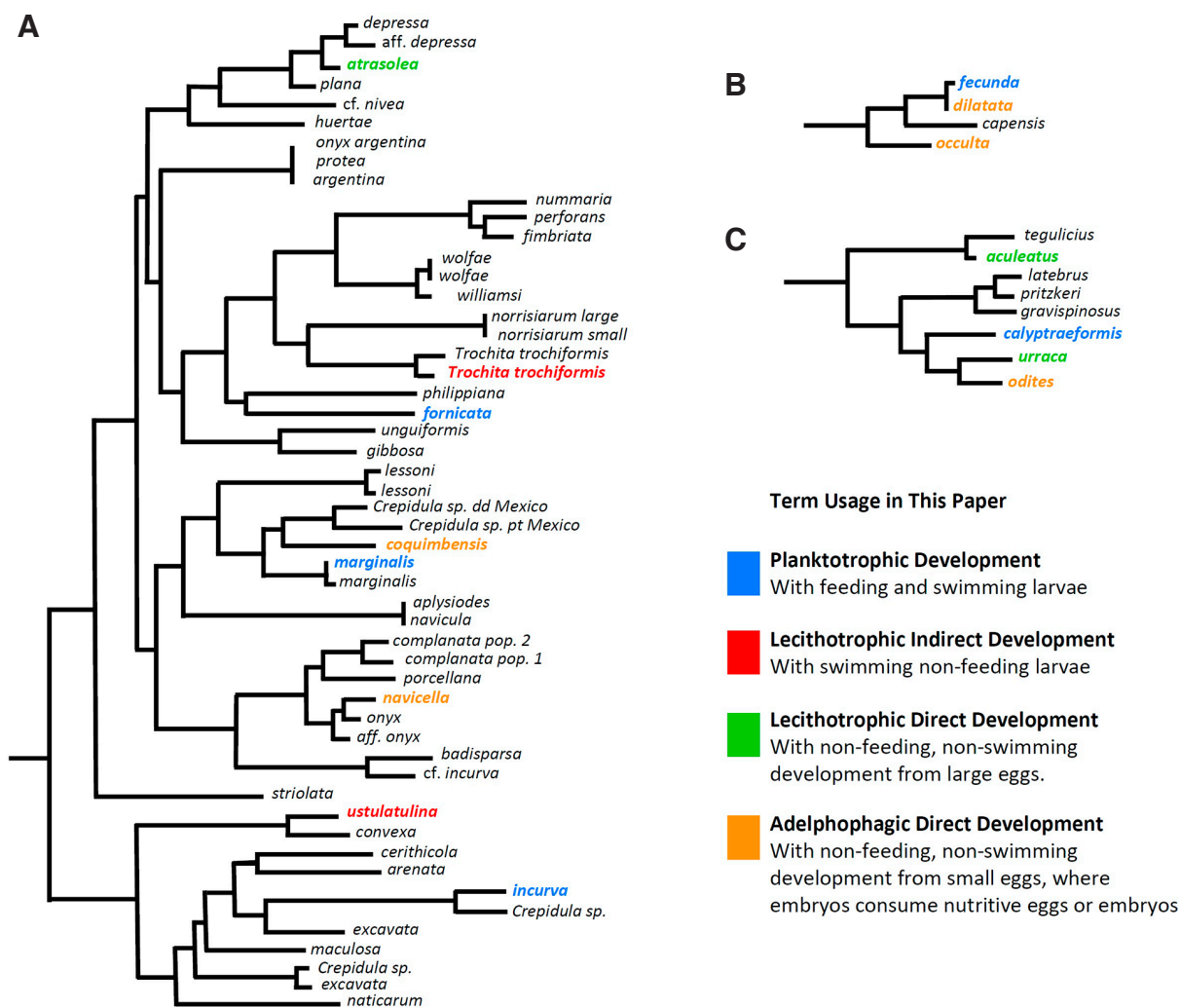


Fig. 1. Relationships of the taxa studied here, with the mode of development of the study taxa color-coded by development type. (A) Crepidula (after Collin 2004) **(B)** Crepipatella based on (Collin et al., 2007) and **(C)** Bostrycapulus based on (Collin 2005).

TABLE 1

**SUMMARY OF TERMINOLOGY AS USED IN THIS PAPER
WHICH FOLLOWS AN ECOLOGICAL DEFINITION
OF MODES OF DEVELOPMENT**

Term	Definition
Adelphophagic development	Development that relies on extraembryonic yolk provided as sibling eggs or embryos
Direct development	Development that results in a juvenile stage at eclosion
Embryo	Any developmental stage after fertilization and prior to eclosion
Larva	The free-living stage between eclosion and settlement
Lecithotrophic development	Development that relies on endogenous yolk supplies
Lecithotrophic larva	A free-living stage that does not feed
Non-planktotrophic development	Development that does not include a stage that feeds on plankton
Planktotrophic development	Development that includes a stage that feeds on plankton
Planktotrophic larva	A free-living stage that feeds on plankton

most diverse phyla of marine invertebrates each include species with planktotrophic larvae, non-feeding larvae, vivipary and brooded or encapsulated direct development, while the less diverse groups are often notable in being composed entirely of species with one MOD (see Strathmann 1978b). Species with different MODs occur in many speciose genera or families in the major phyla (e.g., Mollusca: *Conus*, *Cypraea*, *Sacoglossa*; Annelida: *Serpulidae*, *Spionidae*). When examined in detail, it is often apparent that development differs between sister species or other close relatives within a genus. For example, within the gastropod genus *Conus* development includes planktotrophic larvae, lecithotrophic larvae and lecithotrophic direct development but not adelphophagic direct development (Kohn and Perron 1994); asterinid starfish include species with planktotrophic larvae, lecithotrophic larvae, externally brooded or intragonadal lecithotrophic direct development (reviewed by Byrne 2006); the brittle star genus *Macrophiothrix* includes planktotrophic larvae, facultative feeding larvae, and lecithotrophic larvae (Allen and Podolsky 2007). This variation can even occur within a species, with either populations or females differing in the MOD (Pernet and McArthur 2006; see Collin 2012), or sometimes with MOD varying over the lifetime of an individual female (e.g., Gibson 1997; Krug 1998; Krug 2007; McDonald *et al.*, 2014).

Bias in evolutionary transitions of MOD

As currently understood, the predominant direction of evolutionary change in MOD is from species with planktotrophic larvae to those with direct development; and it is thought to be uncommon or virtually impossible for planktotrophic larvae to evolve from species with direct development without being obviously distinct from the ancestral planktotrophic form (Strathmann 1978a, b). There are two main lines of evidence that support this idea: (1) The complex structures used for larval feeding and swimming are generally lost, reduced or modified in species with direct development, and (2) phylogenetic reconstructions of changes in MOD tend to suggest that the presence of a pelagic larva is ancestral, and that direct development evolves repeatedly towards the tips of the trees (Krug *et al.*, 2015; Collin and Moran 2018). This provides evidence for multiple origins of direct development in each group (e.g., Duda and Palumbi 1999; Hart and Podolsky 2005; Byrne 2006; Krug *et al.*, 2015; but see Jeffery *et al.*, 2003). However, there are very few cases of the recent re-acquisition of larvae: McEdward (1992) gave

a single example of the re-evolution of a planktonic non-feeding larva in a starfish, and Collin *et al.*, (2007) gave an example of the recent re-evolution of feeding larvae in a gastropod.

Both the phylogenetic pattern of direct developing or lecithotrophic species occurring as twigs on phylogenies, and evidence of the common loss of structures in species without planktotrophic development consistent with this hypothesis are particularly well-developed for echinoid echinoderms (e.g., Emler 1991; Hart 1996; Wray 1996). This has been generalized to other marine invertebrates (see critique in Rouse 2000a), which often lack such complete data and are in need of additional study (e.g., Pernet 2003, 2020). Other groups, most notably gastropods and annelids, provide at most equivocal support for these two patterns. It is common for embryos of gastropod species lacking pelagic larvae and some lecithotrophic (i.e., non-feeding) annelid larvae to retain larval features such as the velum (in gastropods) and the opposed band ciliary mechanism used for feeding and swimming (Fioroni 1967; Moran 1999; Pernet 2003; Hofstee and Pernet 2011; Pernet 2020). In addition, the high proportion of species with non-planktotrophic development makes phylogenetic reconstructions of the direction of evolutionary transitions in development uncertain (Collin 2004; Collin and Moran 2018) and highly dependent on the assumptions about the transition probabilities and outgroup coding (Rouse 2000a,b; Collin 2004; Li and Foighil 2015). The interpretation of these comparative patterns is further complicated by the possibility of differential speciation and extinction of species with different modes of development (Krug *et al.*, 2015). Regardless of these caveats it seems clear that MOD changes frequently in these trochozoans.

MOD in calyptraeid gastropods

Calyptraeid gastropods, the focus of this study, are sedentary filter-feeding caenogastropods with a world-wide temperate and tropical distribution in the intertidal and shallow subtidal. Calyptraeid systematics has received recent detailed attention (Collin 2000; 2001; Collin *et al.*, 2007; Véliz *et al.*, 2012; Collin 2019) and the phylogeny of the group is well-resolved (Collin 2003a, b). A variety of species are commonly used in developmental biology (e.g., Conklin 1897; Henry *et al.*, 2006; Hejnal *et al.*, 2007; Henry and Perry 2008; Henry *et al.*, 2010; Lesoway *et al.*, 2014, 2016; Lesoway *et al.*, 2017), and *Crepidula atrasolea* is on its way to becoming a model system for Evo-Devo research (Henry *et al.*, 2017; Lesoway and Henry 2019).

Calyptraeids are diverse in their modes of development (Collin 2003c). Development not only includes planktotrophic larvae (50% of species) and lecithotrophic larvae (5% of species) but also two kinds of direct development: Direct development where large juveniles develop from large eggs (lecithotrophic direct development; 30% of species) and adelphophagic development where large juveniles develop from small eggs that grow into large juveniles by eating other eggs or embryos within the same egg capsule (adelphophagic direct development; 15% of species) (Collin 2003c). In a number of cases, extant sister species have different modes of development (Collin 2004). Phylogenetic analyses of calyptraeid gastropods suggest that the transition from planktotrophic development to lecithotrophic direct development is irreversible, while transitions from planktotrophic development to adelphophagic direct development are reversible (Collin 2004). If it is true that feeding larvae cannot be regained due to the loss

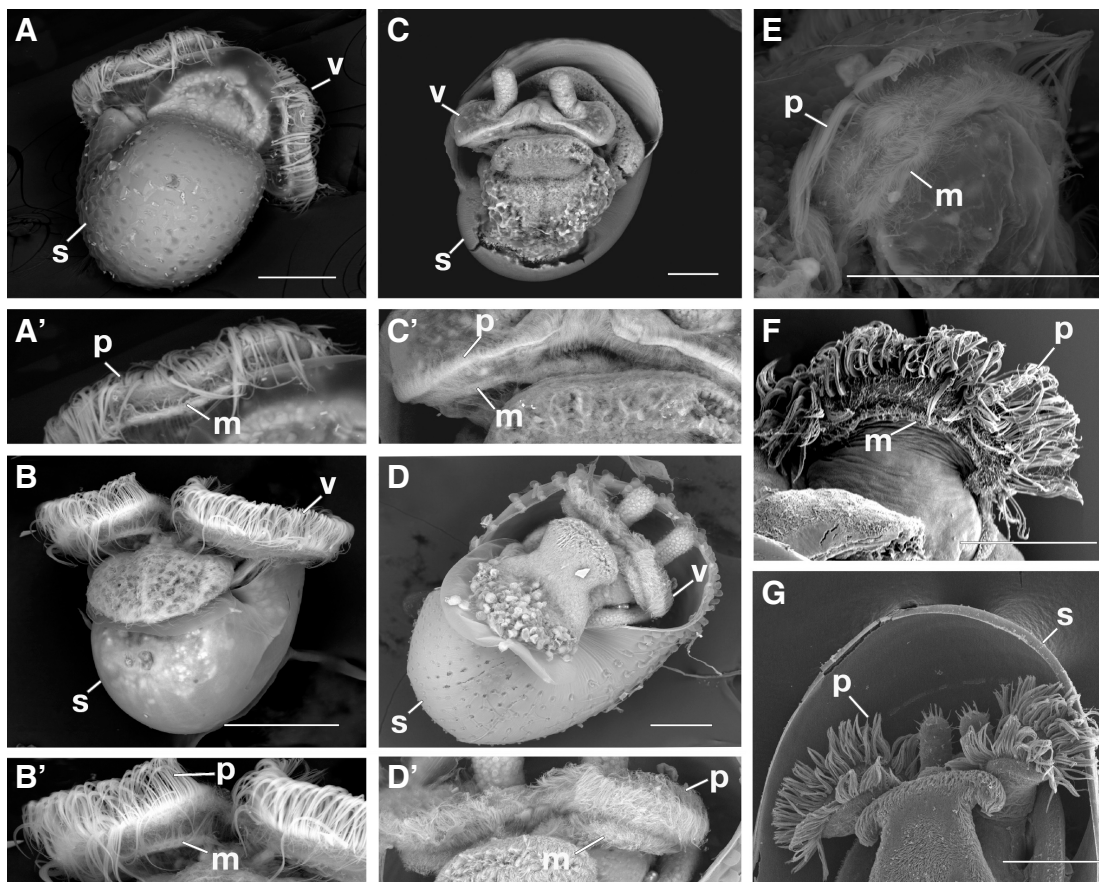


Fig. 2. Scanning electron micrographs of the velum and associated ciliary bands of calyptroid embryos with different modes of development. **(A)** Late stage embryo of the planktotroph *Bostrycapulus calyptraeformis*, ventral view. **(B)** Late stage embryo of the planktotroph *Crepidula incurva*, posterior view. **(C)** Mid-stage embryo of the lecithotrophic direct developer *Crepidula atrasolea*, ventral view. **(D)** Mid-stage of the lecithotrophic direct developer *Bostrycapulus urraca*, ventral view. **(E)** Velum of mid stage adelphophagic direct developer *Crepidula navicella*, posterior view. **(F)** Velum of mid-stage lecithotrophic indirect developer *Crepidula usulatulina*, posterior view (by B. Pernet). **(G)** Velum of late stage lecithotrophic indirect developer *Crepidula usulatulina* which appears to lack the metatrochal and food groove cilia, ventral view. **(A', B', C', D')** The ciliary bands present on the velum. Scale bars, 100 μm . m, metatroch; p, protroch; s, shell; v, velum. Images by Jeanette Hofstee unless otherwise noted.

of complex structures used for feeding, we might expect to find that, in this group, species with adelphophagic direct development retain these structures and their function, while lecithotrophic direct developers do not. To test this hypothesis, we documented the structure and compared the function of the velum (the structure used for feeding and swimming) in embryos of calyptroid species with 4 different MODs.

Results

We collected particle ingestion data for 16 calyptroid species: 5 with planktotrophic larvae, 2 with lecithotrophic larvae, 3 lecithotrophic direct developers and 6 adelphophagic direct developers (Table 2). Eight species occur in the monophyletic clade that is largely comprised of *Crepidula* species and include all 4 modes of development (Fig. 1A). The three *Crepipatella* species include the putatively re-evolved planktotroph (*C. fecunda*) as well as its adelphophagic direct developing sister (*C. dilatata*) and another closely related adelphophagic direct developer (*C. occulta*) (Fig. 1B). The four species of *Bostrycapulus* include another putatively re-evolved planktotroph, two lecithotrophic direct developers and one adelphophagic direct developer (Fig. 1C).

Our results clearly show that embryos with all 4 MODs could capture and ingest particles. The results for the cocktail (shown) and the individual solutions (not shown) were similar. Within a brood, there was a lot of variation between embryos in the number and size of beads ingested, we therefore show the results as the average number of spheres per embryo for each trial (i.e., brood; Fig. 3). Regardless of this variation, some embryos from most broods contained plastic microspheres after an hour of incubation, and some embryos from every species had ingested some spheres (Fig. 3, Table 2). All species except for the adelphophagic direct developers *Bostrycapulus odites* and *Crepidula coquimbensis* contained an average of more than 1 microsphere per embryo in samples from at least one of the developmental stages (Table 2). In the non-planktotrophs the counts were often very un-even among embryos, with some having consumed numerous spheres while their siblings in the same vial contained no spheres or only spheres of a different size. This wide variation was particularly evident in the adelphophages which can also vary significantly in size and somewhat in developmental stage from the same brood. Overall the counts of ingested 2 μm and 10 μm spheres were surprisingly low for the adelphophages.

Overall, 2 μm and 10 μm spheres were consumed in appre-

ciable numbers and 45µm and 90µm spheres were consumed at similar low frequencies across all 4 modes of development (Fig. 3). Planktotrophic embryos consumed more 10µm spheres as they approached hatching (Fig. 3; top right) and 2 species, *B. calyptraeformis* and *C. marginalis* also consumed 25µm spheres

later in development. Only one non-planktotroph species, *C. atrasolea*, consumed appreciable numbers of 25µm spheres. The two species with lecithotrophic larvae consumed fewer spheres than the planktotrophs, but showed different patterns from each other. *Crepidula ustulatulina* consumed no large spheres and

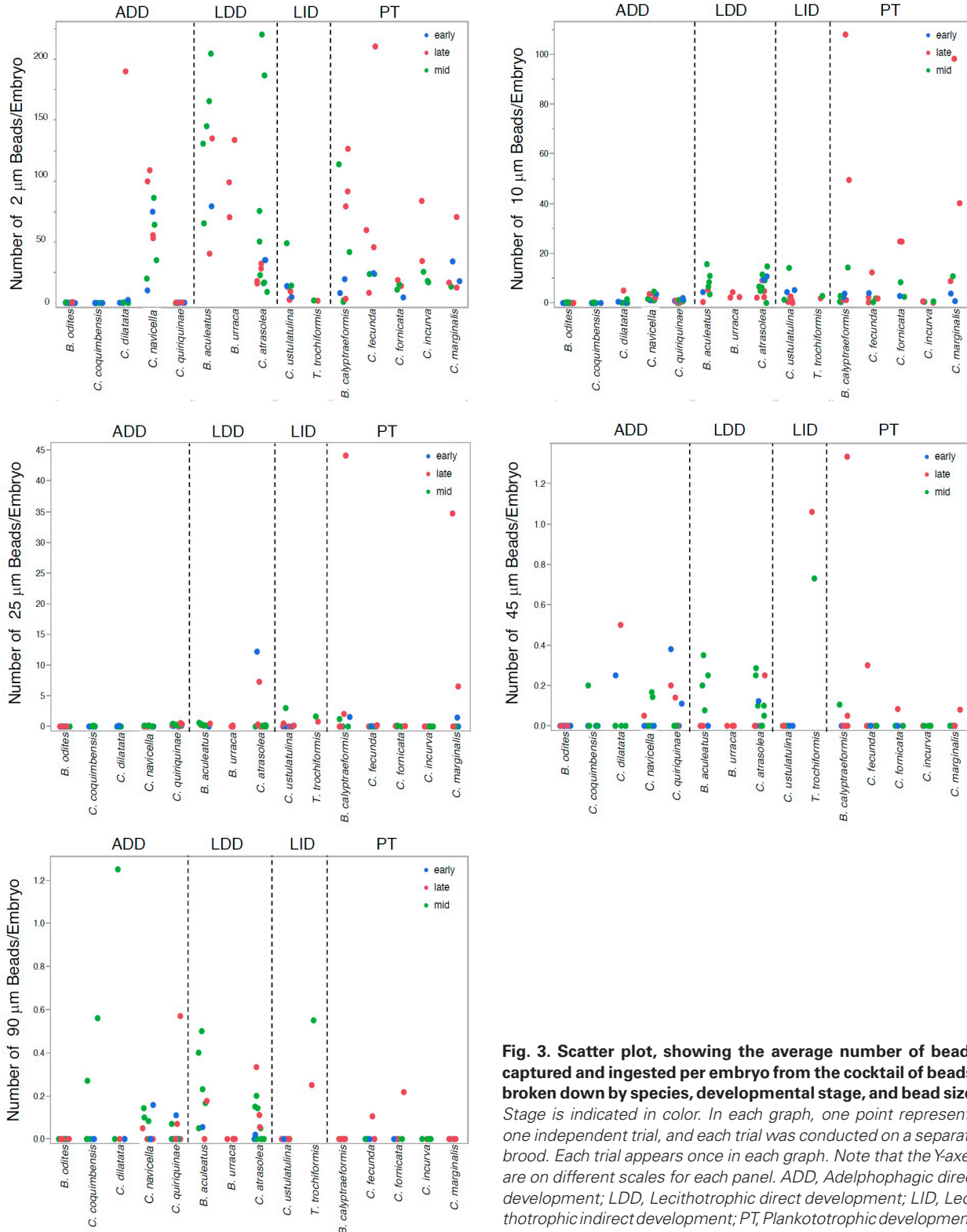


Fig. 3. Scatter plot, showing the average number of beads captured and ingested per embryo from the cocktail of beads, broken down by species, developmental stage, and bead size. Stage is indicated in color. In each graph, one point represents one independent trial, and each trial was conducted on a separate brood. Each trial appears once in each graph. Note that the Yaxes are on different scales for each panel. ADD, Adelphophagic direct development; LDD, Lecithotrophic direct development; LID, Lecithotrophic indirect development; PT, Planktotrophic development.

TABLE 2

SUMMARY OF SPECIES STUDIED AND OBSERVATIONS OF VELAR FUNCTION AND BEAD CAPTURE

Species	Location	Velum	Broods measured	Swimming Ability	Food Groove Present	Capture Observed/Recorded	Bead size with >1/embryo (mm)
Planktotrophic Development							
<i>Bostrycapulus calyptraeformis</i>	Panama City, Panama	large	10	yes	yes	yes	2, 10, 25
<i>Crepidatella fecunda</i>	Coquimbo, Chile	large	7	yes	yes	-	2, 10
<i>Crepidula fornicata</i>	Fort Pierce, Florida	large	5	yes	yes	-	2, 10
<i>Crepidula incurva</i>	Panama City, Panama	large	6	yes	yes	-	2, 10
<i>Crepidula marginalis</i>	Panama City, Panama	large	6	yes	yes	yes	2, 10, 25
Lecithotrophic Larval Development							
<i>Crepidula ustulatulina</i>	Fort Pierce, Florida	large	8	yes	yes	rare	2, 10
<i>Trochita trochiformis</i>	Coquimbo, Chile	large	-	yes	-	yes	2, 10, 45
Adelphophagic Direct Development							
<i>Bostrycapulus odites</i>	San Antonio Oeste, Argentina	distinct	-	no	yes	very rare	none
<i>Crepidula coquimbensis</i>	Coquimbo, Chile	Highly reduced	-	no	no	one	none
<i>Crepidatella dilatata</i>	Coquimbo, Chile	distinct	1	no	yes	yes	2, 10
<i>Crepidatella occulta</i>	Coquimbo, Chile	distinct	2	no	yes	-	2, 10, 25
<i>Crepidula navicella</i>	Panama City, Panama	distinct	8	no	yes	-	2, 10
<i>Crucibulum quiriquinae</i>	Coquimbo, Chile	distinct	-	no	yes	yes	10
Lecithotrophic Direct Developers							
<i>Bostrycapulus aculeatus</i>	Fort Pierce, Florida	distinct	8	no	yes	yes	2, 10
<i>Crepidula atrasolea</i>	Fort Pierce, Florida	distinct	12	no	yes	yes	2, 10, 25
<i>Bostrycapulus urraca</i>	Panama City, Panama	distinct	3	no	yes	-	2, 10

- means no observation.

fewer spheres late in development than during mid development, despite having a large velum at hatching. In contrast, mid and late stages of *Trochita trochiformis* consumed low, but similar numbers of spheres of all sizes.

Morphological analysis was completed for a subset of species. Amongst the 5 planktotrophic species velum perimeter increased linearly with shell length. ANCOVA analysis conducted for each MOD separately (different MODs could not be combined for the analysis as there was insufficient overlap in the shell lengths) showed no statistically significant difference between planktotrophic species in the relative velum size compared to shell length (Table 3; Fig. 4a). *Crepidula ustulatulina*, the sole species with lecithotrophic larvae measured, showed no significant increase ($p > 0.1$) in velum perimeter with shell length across 8 broods ranging from 350-700 microns in shell length. Velum perimeter ranged from 1100 to 1700 microns, roughly the same size as the velum of the much smaller planktotrophs across the same pre-hatching developmental stages (Fig. 4). In contrast the velum size decreased significantly with shell

length in the species with lecithotrophic direct development (Table 3; Fig. 4), as expected as they approached hatching. *Bostrycapulus aculeatus* had a statistically significant larger velum than the other lecithotrophic direct developer, *Crepidula atrasolea* (Table 3). At their largest, the velum perimeter of *Bostrycapulus aculeatus* was similar to those of late stage planktotrophic embryos. Insufficient replicates were available to repeat this analysis for the adelphophagic direct developers. Cilia length and food groove widths showed a similar pattern (Fig. 4), with statistically significant factors of shell length for planktotrophs and species for lecithotrophic direct developers (Table 3). Both were roughly similar sizes across the modes of development (Fig. 4).

Video footage of planktotrophic embryos showed 10 μm particles approaching the velum, getting entrained in the food groove, and moving along it to the mouth. This happened in a manner typically reported for planktotrophic gastropod veliger larvae, demonstrating that this mechanism functions similarly in pre-hatching stages. Videos demonstrating the same style of particle capture and move-

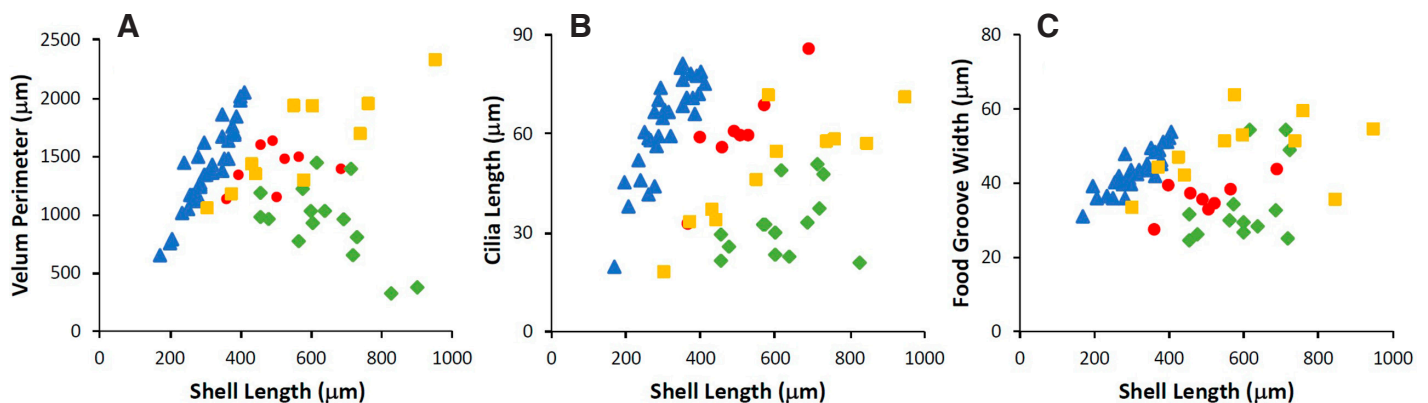


Fig. 4. Scatter plots showing how velar morphology relates to shell length for the 4 modes of development. Planktotrophs, blue triangles; lecithotrophic direct development, green diamonds; adelphophagic direct developers, yellow squares; lecithotrophic larvae, red circles.

ment through the food groove was also obtained for all species with non-planktotrophic development in at least one stage of development. Although our observations did not allow us to quantify these differences, non-planktotrophic species differed in their propensity to retain and ingest the beads under our experimental conditions. We observed that some commonly rejected particles after they had been transported to the mouth by the food groove, while others swallowed the particles. The lecithotrophic direct developers *C. atrasolea* (Fig. 5) and *B. aculeatus* (Fig. 6), the lecithotrophic indirect developer *T. trochiformis* and the adelphophagic direct developer *C. dilatata* all showed frequent successful captures with beads moving around the food groove to the mouth. The adelphophagic direct developer *C. quiriquinae* (Fig. 7) frequently captured particles but often subsequently rejected or lost them at the mouth. For *Bostrycapulus odites*, the adelphophagic direct developer that consumed virtually no small spheres in the feeding trial, video footage showed that many spheres approaching the velum were pushed away (Fig. 8). The occasional captures that were observed showed that the particle moved very slowly along the food groove and was usually rejected or fell off near the mouth (Fig. 8). Likewise, older embryos of *C. ustulatulina*, a species with lecithotrophic larval development, were seldom observed to capture particles. In this species, despite having a large and active velum, late stage embryos seemed to avoid approaching particles, although younger embryos of this species were filmed making successful captures. Finally, films of the adelphophagic direct developer *C. coquimbensis*, which has such highly modified embryos that the velum is reduced to nothing more than a ciliated ridge below the tentacle, generally showed eclectic movement of beads around the embryos. However, when exposed to extremely high concentrations, one bead was observed becoming entrained on this small ridge and being transported to the mouth. It was not possible to determine if this ridge retained the 3 classic trochal bands of cilia,

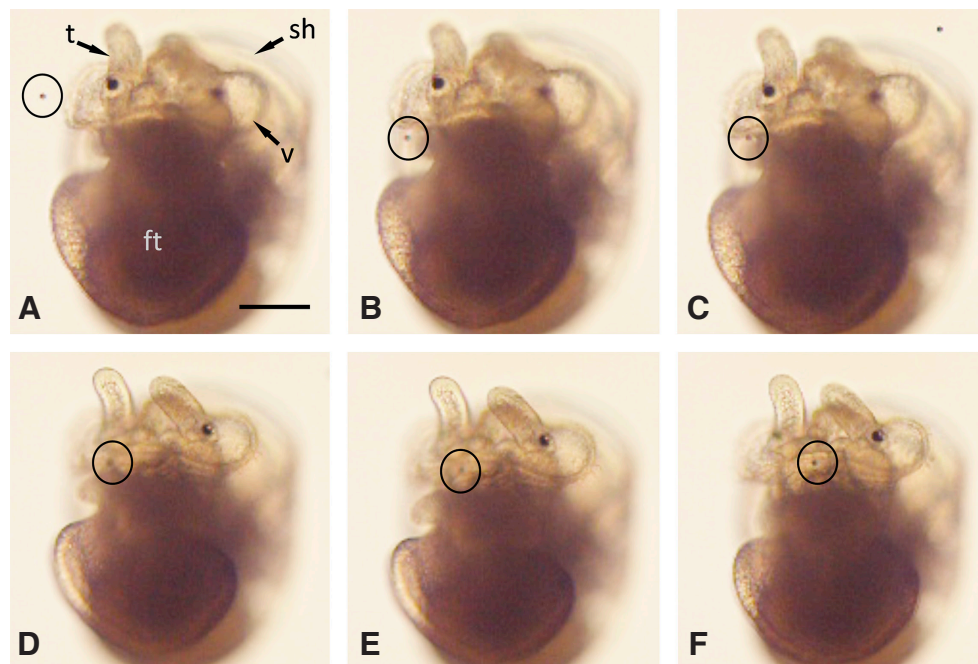


Fig. 5. Video frame sequence of a lecithotrophic direct developing embryo of *Crepidula atrasolea* capturing a 10 µm sphere. ft, foot; sh, shell; t, tentacle; v, velum. Scale bar ~130 µm.

TABLE 3

ANALYSIS OF COVARIANCE - THE EFFECTS OF SPECIES IDENTITY AND SHELL LENGTH ON VELUM PERIMETER, CILIA LENGTH AND FOOD GROOVE WIDTH IN CALYPTRAEID EMBRYOS

Factor	df	F ratio	p	Factor	df	F ratio	p
VELUM PERIMETER							
Planktotrophs				Direct developers			
Species	4	2.26	0.09	Species	2	8.19	0.003
Length	1	19.50	<0.0001	Length	1	6.42	0.02
R ² =0.88, N = 32				R ² =0.46; N=23			
CILIA LENGTH							
Planktotrophs				Direct developers			
Species	4	1.68	0.19	Species	2	4.97	0.02
Length	1	54.35	<0.0001	Length	1	2.34	0.14
R ² =0.77, N=32				R ² =0.59, N=22			
FOOD GROOVE WIDTH							
Planktotrophs				Direct developers			
Species	4	0.74	0.57	Species	2	25.89	<0.0001
Length	1	69.23	<0.0001	Length	1	1.03	0.32
R ² =0.80, N=32				R ² =0.78, N=20			

No interactions between species and length were significant and were therefore removed from the analysis. Bold highlights statistically significant effects.

as they were not visible at the resolution of the video. Typically, much of the area around the mouth is ciliated in calyptraeid embryos, including the head vesicle and the foot (see Collin 2000). The ability of these cilia to change direction and to move particles toward the mouth has not been investigated.

Discussion

The phylogenetic hypothesis for 94 species of calyptraeids (Collin 2003a, b, 2004) has allowed the evolution of MOD to be examined in more detail for this group than for any group of marine mollusks other than sacoglossan sea slugs (see Krug *et al.*, 2015; reviewed in Collin and Moran 2018). Phylogenetic reconstructions show that transitions between modes of development happen frequently and rapidly, and that the evolution of direct development is more common than the re-evolution of feeding larvae. Parsimony reconstruction of MOD showed that direct development has arisen 19 times, and has been lost (i.e., feeding larvae have re-evolved) three times. Maximum likelihood reconstructions show that losses and gains of larval feeding are equally likely and the differences in number of transitions in the 2 directions are a result of the abundance and distribution of character states on the phylogeny (Collin 2004). In the three cases where the phylogeny indicates the re-evolution of feeding larvae, planktotrophy appears to have arisen from an ancestor with adelphophagic

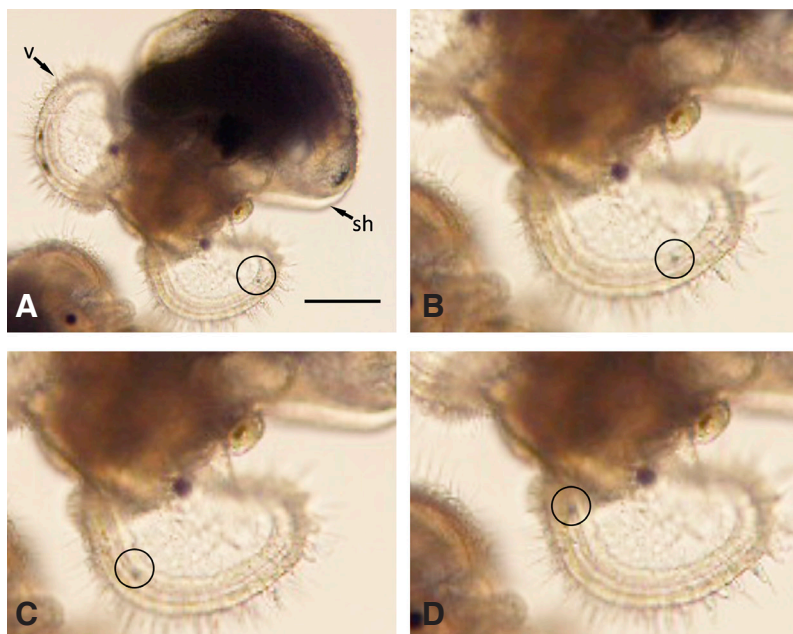


Fig. 6. Video frame sequence of a lecithotrophic direct developing embryo of *Bostrycapulus aculeatus* capturing a 10 μm sphere. *sh*, shell; *v*, velum. Scale bar $\sim 200 \mu\text{m}$.

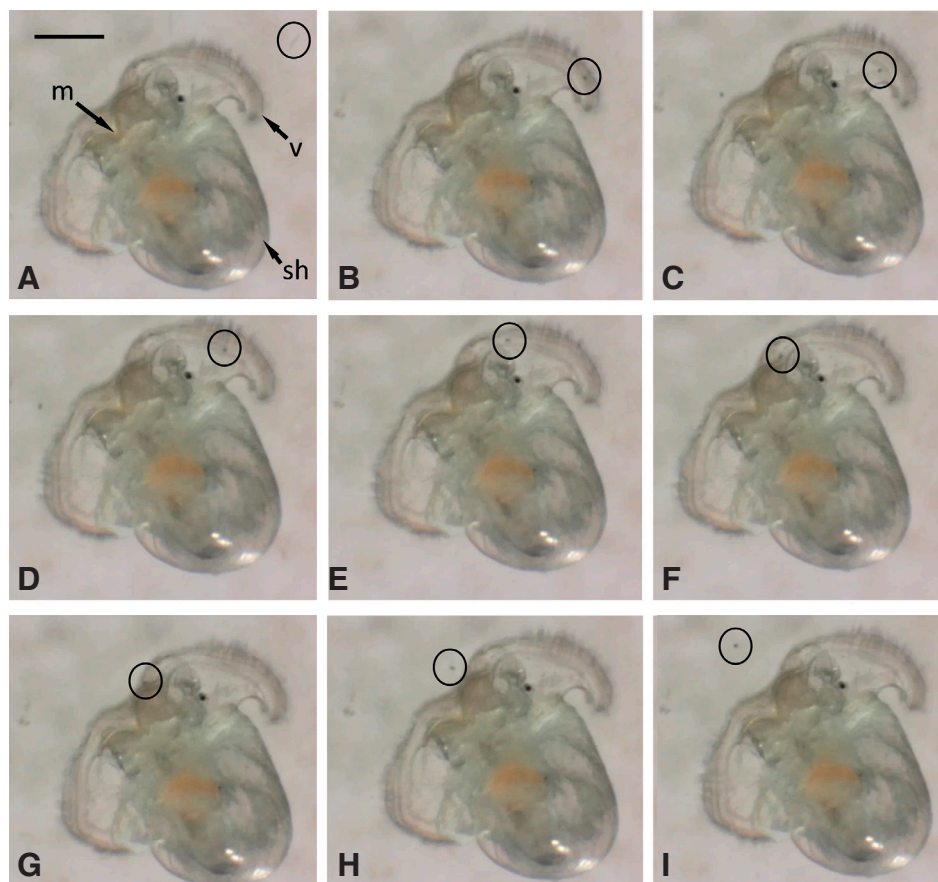


Fig. 7. Video frame sequence of an adelphophagic direct developing embryo of *Crucibulum quiriquinae* capturing and rejecting a 10 μm sphere. *m*, mouth; *sh*, shell; *v*, velum. Scale bar $\sim 200 \mu\text{m}$.

direct development. This phylogenetic pattern suggests that species with adelphophagy retain the potential to revert to development with feeding larvae, while species with lecithotrophic direct development from large eggs do not (Collin 2004). Based on this, we would expect that adelphophages would retain the velar structures required for feeding, while lecithotrophs would not. Our observations of particle capture and measurements of embryonic allometries show that this is not the case. The structure and function of the velum are commonly retained amongst species with both lecithotrophic direct development and adelphophagic direct development.

With the exception of a few species with highly modified development, the velum is not lost in most non-planktotrophs examined here. Our results show that, although the body size is significantly larger in the embryos of non-planktotrophs, the absolute velum size during the intracapsular period is similar across all modes of development. This departure from the tight allometry exhibited by the planktotrophs results in embryos that cannot swim because they are so large, but which retain a velar perimeter presumably adequate to capture almost as many particles as planktotrophs at similar stages.

This pattern was previously demonstrated in the detailed comparison between *Crepidatella fecunda* and *C. dilatata* (see Chaparro et al., 2002). In addition, in most lecithotrophic direct developers and adelphophagic direct developers, the velum retains similar long prototrocal cilia which beat in organized metachronal waves, and a food groove which can transport particles to the mouth. This contrasts with the non-feeding development of *Littorina obtusata*, *L. saxatilis*, *L. sitkana*, and *L. subrotundata*, all of which retain the velum in encapsulated embryos, but have lost the ciliary mechanisms to capture particles (Hofstee and Pernet 2011). All of the species we examined that retained the velum also retain the opposed band ciliary arrangement.

Some direct developing calyptraeids do lose the velum completely. For example, among the species studied here, *C. coquimbensis* retains only a tiny ridge as a residual velum (Fig. 9). A similarly reduced velum has also been documented in *Crepidula norrisiarum* development (as *C. adunca* in Collin 2000) and can be seen on *Crepidula williamsi* (Fig. 10). Across the entire family, there is limited evidence that the frequency of velum loss differs between lecithotrophic direct developers and adelphophagic direct developers. Of the 39 non-planktotrophs for which some observations of development are available, in only 12 species has a velum not been observed (31% overall and 39% of the 31 for which the character state has been reported; reviewed in Collin

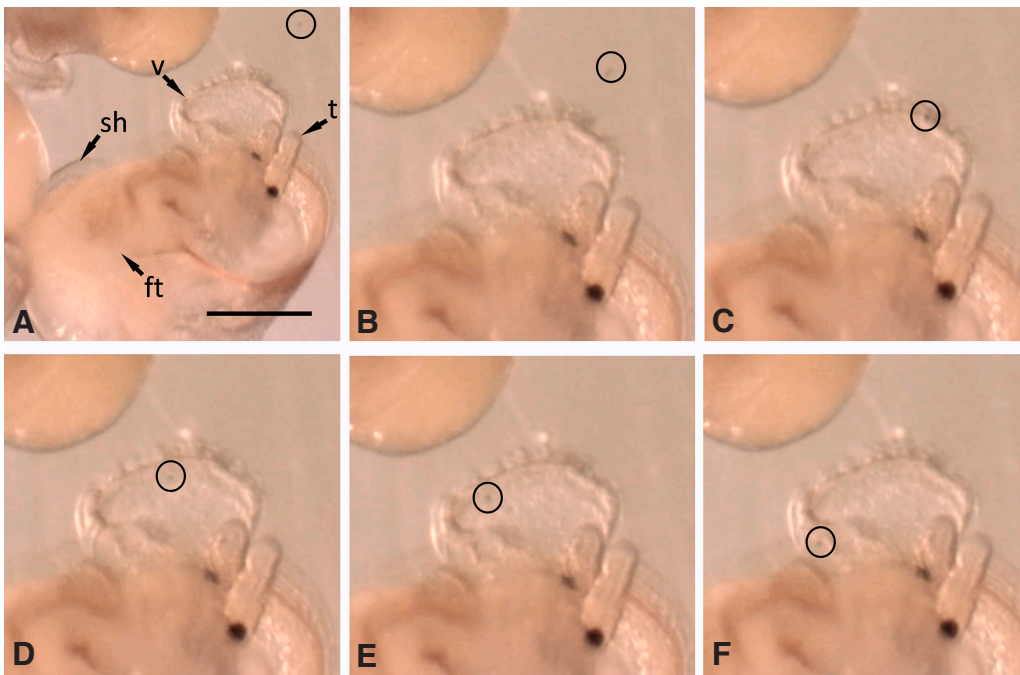


Fig. 8. Video frame sequence of an adelphophagic direct developing embryo of *Bostrycapulus odites* capturing a 10 µm sphere. ft, foot; sh, shell; t, tentacle; v, velum. Scale bar ~200 µm.

2004). In some species, the velum is quite transitory, occurring for only a narrow window during development, and the appropriate stage may not have been observed, so this should be taken as an upper-bound estimate of the proportion of species that lack a velum. Of these 12 species, 42% (8 of 19) of lecithotrophic direct developers lack a velum, while 33% (4 of 12) of adelphophagic direct developers lack a velum. Although this trend suggests that reductions are more frequent in lecithotrophic direct developers, this difference is not significant with a Chi-square test ($p > 0.2$). Nevertheless, these numbers indicate that ~60% of non-planktotrophs retain a velum, which, extrapolating from our results, are also likely to retain the ability to capture particles.

Despite the retention of the velum and the ability to capture particles in the typical way for planktotrophic larvae, the embryos of adelphophagic direct developing species, especially *C. quiriquinae* and *B. odites* were observed to lose or reject most 10 µm particles with only the very occasional capture resulting in the ingestion of a particle. On the other hand, the lecithotrophic direct developers *B. aculeatus* and *C. atrasolea* could be seen to capture and ingest numerous particles in rapid succession. These differences among species could be due to slight differences in the developmental stage of the larvae, or species-specific negative responses to the flavor or nutritional content of the particle, both of which have been shown to reduce capture or ingestion rates (Gallager 1988; Baldwin 1995; Pedrotti 1995; Bricelj and MacQuarrie 2007). Most of our observations of lecithotrophic direct developers were made in Florida, while most observations of adelphophages were made in a subsequent year in Chile. Environmental conditions including the quality of the seawater at each site, as well as possible changes in the surface properties of the spheres as they aged may also impact feeding rates (Rosa *et al.*, 2017). Satiated veligers reduce their feeding rates (Mapstone 1970; Sprung 1984) and it is difficult to assess and control for how satiated adelphophagic embryos may

be immediately after excapsulation. Despite all of these reasons why embryos might not ingest particles, it is also possible that these differences are due to selection on the function of the mouth and velum in the intracapsular environment. In adelphophages, the velum and mouth must function in the “capture” and ingestion of much larger nutritive eggs and embryos. This remains a mysterious and little-studied process. Where this has been observed, for example in *C. dilatata*, the embryo appears to use the velum to hold the nutritive egg in front of the mouth, and either deform and consume the egg whole. In some cases, a stream of yolk can be seen moving into the embryo as the egg rotates on the velum (R.C. pers. obs.; Chaparro *et al.*, 2002). Embryos of *C. navicella* are able either to ingest large nutritive embryos (~160 µm) whole, or to

ingest smaller yolk droplets (14–46 µm), which are seen both in the mouth, and entrained on the velum (M.P.L. pers. obs.; Lesoway *et al.*, 2014). In other cases, whole nurse embryos can be seen intact inside the developing embryos (Fig. 9A,B and R.C. pers. obs. for *C. coquimbensis*, and *Crepipatella capensis*). Adelphophagic species in other families are also able to ingest nutritive embryos whole, for example *Searlesia dira*, and *Buccinum undatum* (Rivest 1982, 1983; Smith and Thatje 2013a, b). It seems likely that the mouth and surrounding cilia could be modified to support this novel function, especially in light of the importance of sibling competition for eggs (Rivest 1982, 1983; Smith and Thatje 2013b, a). Our observations offer some evidence of such modifications. For example, the mouth area in *C. quiriquinae* and early *C. coquimbensis* embryos appeared to be large open funnels, quite unlike the mouth in other calyptraeid embryos (Fig. 9). It is worth noting that embryos that ingest the nutritive eggs whole (e.g., *C. coquimbensis*, *C. capensis*) have often more or less lost the velum, while those that seem to suck or peel yolk from the nutritive eggs, or ingest yolk globules retain the distinct velum. This is not unprecedented, as the velum has also been modified in various direct developing *Littorina* species in association with the evolution of intra-capsular albumin uptake, a novel function of the velum (Hofstee and Pernet 2011).

In contrast, there is no clear functional reason why the arrangement of the ciliary currents and swallowing mechanism should have been modified for a different function in the direct developers. Natural selection for efficient function in adelphophagy may maintain some kind of function with respect to ingestion of small particles in adelphophagic direct developers, but similar function may be completely lost through genetic drift or pleiotropic interactions in lecithotrophic direct developers. Observations from other gastropod taxa are not available to support or contradict this scenario, however a study of one lecithotrophic direct developer and one adelphophagic direct developer in the genus *Nucella*, Hookham and

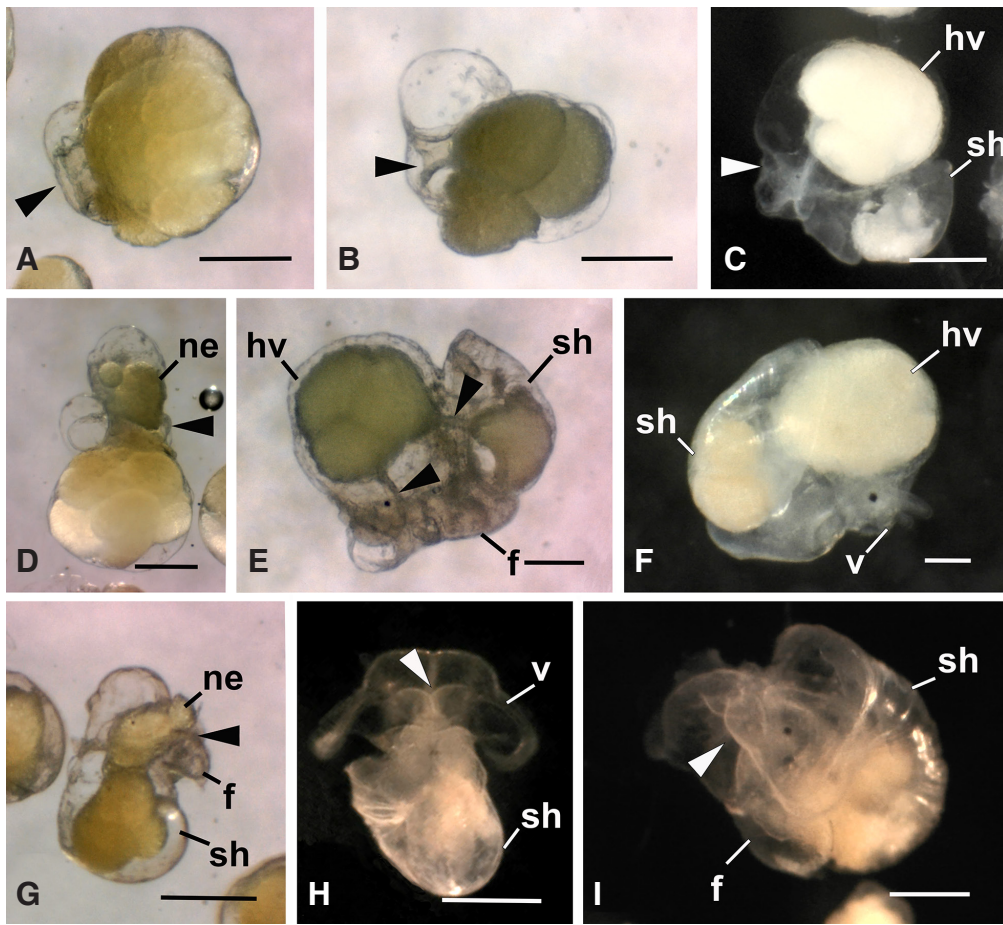


Fig. 9. Adelphophagic direct developing embryos of *Crepidula coquimbensis* (A-F) and *Crucibulum quiriquinae* (G-I). (A) An early stage embryo of *C. coquimbensis* which has already consumed a number of nutritive embryos. (B) A similar stage to A but this embryo has consumed fewer nutritive embryos which are distinctly visible inside the embryo. (C) A mid-stage embryo of *C. coquimbensis*, showing a clear shell covering the viscera, which contain some yolk as well as the large accumulation of yolk in the head vesicle. (D) An early stage *C. coquimbensis* embryo consuming a nutritive embryo. (E) A mid-stage embryo of *C. coquimbensis*, with a well-developed shell covering the viscera, a large accumulation of yolk in the head vesicle and arrows pointing to the tubes connecting what is presumably a pouch in the esophagus to the mouth anteriorly and to the gut posteriorly. (F) A somewhat later stage embryo of *C. coquimbensis*, with a small velar ridge below the tentacle. (G) Early embryo of *Crucibulum quiriquinae* with a partially consumed nutritive embryo. (H) A mid-stage embryo of *Crucibulum quiriquinae* which failed to consume many nutritive embryos and is therefore lacking yolk. (I)

A mid-stage embryo of *Crucibulum quiriquinae* showing the funnel shaped area around the mouth, created between the foot and the velar lobes. In all images the arrow indicates the mouth, unless otherwise noted. f, foot; hv, head vesicle; ne, nutritive embryo; sh, shell; v, velum. Scale bar, 200 microns.

Page (2016) found that the lecithotrophic direct developer retained a more distinct metatroch and food groove than the adelphophagic direct developer. In addition, they found that 2 species with encapsulated development did develop a transient larval esophagus, another feature necessary for the re-evolution of feeding larvae. The calyptraeid species we studied here all appeared to have an intact esophagus and the plastic spheres generally accumulated in the gut, although some did accumulate in the extremely modified esophageal pouch that stores nutritive embryos in the head vesicle of *Crepidula coquimbensis* embryos (Fig. 9E).

One key question when examining the re-evolution of morphologi-

cal features is how would we identify a reacquired feature? When the larval body is distinct from the juvenile body, as it is in groups with maximally indirect development, like echinoderms, it may be relatively easy to identify secondarily evolved larvae (Strathmann 1978a, b). For example, the sea star *Pteraster tessellatus* is interpreted as having secondarily derived planktonic development based on the absence of characteristic larval features (brachiolar arms, attachment disk and bilateral symmetry), as well as precocious development of juvenile features (McEdward 1992). Identification of secondarily derived planktotrophs may be trickier in groups where the larvae and juveniles share many of the same organ systems

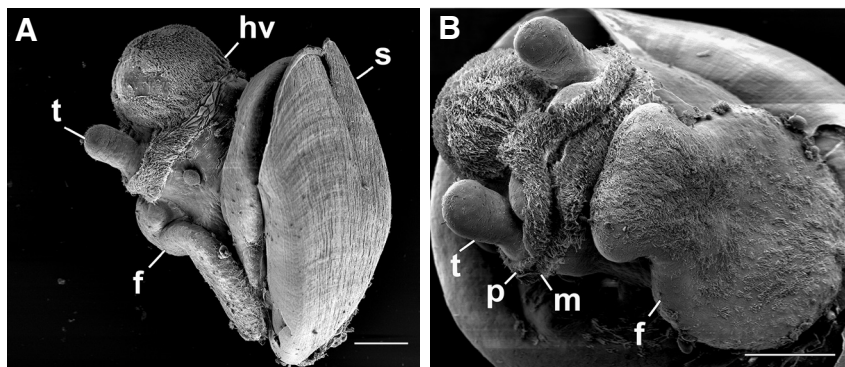


Fig. 10. Scanning electron micrographs of the embryo of *Crepidula williamsi*, a lecithotrophic direct developer that lacks a distinct velum. The ciliary band at the base of the tentacles is not differentiated into distinct bands dorsally (A), but a groove is visible ventrally as the band approaches the mouth in an embryo from the same capsule that appears to be slightly more developed (B). Scale bar, 100 μ m. f, foot; m, metatroch; p, prototroch; s, shell; t, tentacle; v, velum. Images by B. Pernet.

and body parts, like molluscs and most polychaetes. In a growing number of cases there is evidence that complete prototroch, metatroch and food groove systems are retained in non-feeding polychaete larvae (Pernet 2003, 2020), and in direct developing muricid gastropod embryos (Hookham and Page 2016). It seems possible that if feeding larvae were to evolve in these groups, the general design of the feeding structures and function of the ciliary band would not provide evidence of this secondarily derived planktotrophy. However, detailed behavioral observations like those of Strathmann *et al.*, (2019) may provide evidence of independent evolutionary derivation, despite similar form and function, as could differences in cell lineages of the three trochal bands (Hejnal *et al.*, 2007; Gharbiah *et al.*, 2013; Lyons *et al.*, 2015).

No obvious morphological differences provide additional evidence of secondary derivation of planktotrophy in *B. calyptraeiformis* and *C. fecunda*. The larvae are indistinguishable from other calyptraeid planktotrophs based on gross morphological observations of pre-hatching embryos or larval stages. There were no morphometric differences prior to hatching between the primarily planktotrophic and putatively secondarily planktotrophic species, with the allometry of velum perimeter, cilia length and food groove width showing no significant differences across the planktotrophic species measured. There were also no clear-cut differences in the sizes of spheres that were ingested. If the phylogeny reconstruction is accurate, this leaves us very much where we started with phylogenetic patterns suggesting that either (1) the loss of feeding larvae is extremely common in *Crepidatella* and *Bostrycapulus*, resulting in only a single remaining planktotrophic species in each clade (see Fig. 1), (2) differential extinction has created this pattern suggestive of the re-evolution of planktotrophy, or (3) there may have been true evolutionary reversals with secondarily planktotrophic larvae appearing indistinguishable from the closely related primary planktotrophs. While the structure of the velum appears to be stable, our understanding of the gene regulatory networks producing this structure is limited. Fine-grained comparisons across the calyptraeids may provide clues to understanding the evolution of MOD, as would the application of a more explicitly evolutionary approach to these kinds of evo-devo data (Sanger and Rajakumar 2019; Church and Extavour 2020). A robust resolution of this quandary will likely require an approach that truly integrates comparative embryology, analysis of genetic regulatory networks, and phylogenomics all combined with increased taxon sampling.

Materials and Methods

Gastropod larvae use two ciliated flaps of tissue, the velum, to both swim in the water and to capture algal particles. The velum is edged with a band of long, preoral prototrochal, compound cilia and a band of smaller post-oral metotrochal cilia (Fig. 2). Between the two trochal bands the food groove, lined with a lawn of short cilia, moves particles toward the mouth after they are captured (Strathmann and Leise 1979; Romero *et al.*, 2010; Strathmann *et al.*, 2019). Loss of feeding larvae is thought to be irreversible due to loss or modification of the velum in non-planktotrophic species. Here, we document velar morphology and function in pre-hatching individuals to understand the extent of velar reduction. Pre-hatching stages must be observed to compare species without feeding larvae to those with planktotrophic development. All pre-hatching individuals are referred to here as embryos, irrespective of the extent of development. Embryos were compared at 3 similar developmental stages.

We collected 16 calyptraeid species from the intertidal or shallow subtidal (Table 2), representing four modes of development. Within 4 days of collec-

tion we gently pried females from the substrate and removed brooded egg capsules. In 11 species we measured the developing velum and the shell. Embryos were staged (see below) and the shell length, velum perimeter, cilium length, and food groove width were measured for 20 embryos from each brood under the compound microscope. Food groove measurements are approximate (15%), as it was quite difficult to get clear views of the margins in the very yolky direct developers. For a subset of taxa and stages we also imaged the velum using scanning electron microscopy.

To assess the ability of embryos to use the velum to capture and ingest particles of different sizes, embryos from the same broods were placed into suspensions of plastic microspheres. Embryos were incubated in solutions of 2, 10, 25, 45, and 90 μm polystyrene beads (Duke Standards) and a cocktail containing all bead sizes (following protocol in Phillips and Pernet 1996). Stock solutions, whose concentrations were determined with 10 replicate counts for each size class either using a hemocytometer for the smaller or a Bogorov tray for large beads (90 μm) were sonicated for 6 minutes to reduce clumping and used to make working solutions with the following concentrations: 5447ml⁻¹ of 2 μm , 1350ml⁻¹ of 10 μm , 540ml⁻¹ of 25 μm , 300ml⁻¹ of 45 μm , and 150ml⁻¹ of 90 μm beads. Solutions used in Argentina and Chile had a final concentration of ~20% fewer 2 and 10 μm spheres. Microspheres were not flavored or coated in any way.

Whenever possible ~20 embryos from each brood were allocated into one of six replicate 5-mL glass vials. Groups of embryos from different broods at 3 stages of development (Early, Middle and Late) were incubated in each vial. 'Early' refers to an embryo showing initial development of the velar ridge and partial shell development. The 'Middle' stage includes embryos with a clear veliger morphology but with significant yolk reserves remaining and a large head vesicle. The 'Late Stage' includes embryos immediately prior to or at hatching with scant yolk reserves, reduced or absent head vesicle, and black pigment giving the brood an overall brown color. Since broods cannot be staged before removal from the mother, we obtained between 1 and 8 broods for each species x stage combination.

For the single size class trials we added 100 μl of one of the five sonicated working solutions to 4.5mL filtered seawater containing the embryos. For the mixed suspension we added 100 μl of each solution to a single vial. To maintain the suspension of the beads in the solution, the vials were strapped to a slowly rotating rotisserie (1 revolutions·m⁻¹). After one hour we recovered the vials, and killed the embryos immediately by adding 2 drops of dilute formalin. Individual embryos were mounted on a microscope slide and examined using a compound microscope, and all beads that were anywhere in the digestive tract of the embryo were counted and the size class was recorded. To locate the fluorescent 2 μm beads we used epifluorescence and a green excitation filter block with excitation wavelengths of 530-560 nanometers and emission wavelengths of 590-650 nanometers and squashed the embryos under the coverslip to ensure all beads were counted. This approach cannot be used to estimate clearance rates, as we do not know the gut passage time of these embryos, but evidence from larvae suggest that it is less than an hour (Mapstone 1970). Likewise, failure of embryos to consume particles is not evidence of the inability of the embryos to capture and ingest particles, as individuals may not ingest particles for any number of reasons, but presence of particles in the gut is evidence that the embryos are able to capture and consume some particles.

We took video footage of embryos of 10 species at the stages with well-developed velums capturing black 10 μm particles to compare the particle capture and ingestion with the typical particle capture mechanism reported for gastropod larvae (Strathmann and Leise 1979; Romero *et al.*, 2010). Our goal was to determine if the particles were moved along the food groove to the mouth, as is typical of planktotrophic species. The exact mechanism involved in particle interception and capture by the cilia could not be determined as we did not take high-speed video at high magnification. But overall capture and transport along the velum was visible. To increase the rate of particle capture for visualization, we used more concentrated particle solutions. These solutions were not quantified, and therefore videos of particle capture cannot be used to quantify particle capture rates.

Rather than quantifying capture or clearance rates, our primary focus was to determine that the particles were captured on the velum and transported to the mouth, rather than, for example, directly engulfed by the mouth or entrained by the foot. Archived video footage is available at: <https://doi.org/10.25573/data.c.4961189.v1>

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