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Comparative Ontogenetic Changes in Enzyme Activity During Embryonic Development of Calyptraeid Gastropods

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Abstract. A modification of a semi-quantitative color-based enzyme assay was used to quantify the activity of 19 enzymes (5 peptidases, 3 lipases, 3 phosphotases, and 8 carbohydrases) during five stages of development in eight species of calyptraeid gastropods. Sixteen of the 19 enzymes showed a significant effect of mode of development on the concentration of the reaction product after incubation of homogenates standardized for protein content. The overall pattern was that planktotrophs showed the highest activities, followed by adelphophages, and nonfeeding embryos, which had the lowest enzyme activities. Thirteen enzymes showed significant differences across developmental stages. Of these, eight showed a clear increase during development. Only one of the enzymes showed a sudden jump in activity between the unfed, pre-hatching stage and post-hatching stages that were fed *Isochrysis galbana*. In three cases, ANOVA identified two exclusive, significantly different groups of species. In naphthol-AS-BI-phosphohydrolase, the measured absorbance of *Crucibulum spinosum* samples was significantly higher than in all of the other species. The activity of α-fucosidase in *Crepipatella occulta* was significantly greater than in the other seven species. Finally, the activity of β-galactosidase was significantly higher in *C. occulta*, *Crucibulum spinosum*, and *Bostrycapulus calyptraeformis* than in the four *Crepidula* species. This is the only enzyme for which there is an indication of a phylogenetic effect. Relative enzyme activities were similar to those reported for other herbivorous gastropods, with the three phosphohydrolases, four carbohydrases (β-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, and α-fucosidase), and leucine arylamidase showing high activities.

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Introduction

From the perspective of energetics, marine invertebrates can be roughly divided into those that have feeding, planktonic larvae (planktotrophic development) and those that do not (referred to here as direct development) (see Strathmann, 1978a, b, 1985, for reviews; Allen and Pernet, 2007, and Collin, 2012, for discussion of intermediates). After hatching, planktotrophic larvae capture phytoplankton, or in some cases zooplankton, which fuel their continued development and growth for several days, months, or even years (Strathmann, 1985; Strathmann and Strathmann, 2007). In contrast, direct development 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. Direct development can be fueled entirely from endogenous yolk from the oocyte, consumption of nutritive eggs or embryos (oophagy or adelphophagy), or absorption of intracapsular fluid or maternal secretions (Thorson, 1950; Spight, 1976; Rivest, 1986; Collin and Spangler, 2012).

Many families or genera of marine invertebrates include species with different modes of development. In general, the larval structures used for feeding and swimming are reduced or lost in species with nonfeeding larval development and encapsulated development compared to their planktotrophic relatives (Strathmann, 1978a, b; Wray and Raff, 1991; Collin, 2004). If the alimentary system follows the same evolutionary patterns as the structures used for feeding and swimming, gut development and enzyme expression would also be expected to be delayed or reduced in species without feeding larvae. However, the reduction of feeding structures and digestive function may not be coincident. For example, some lecithotrophic gastropod larvae retain the ability to

Table 1

Calyptraeid species used in this study, the collection localities and mode of development

Species	Location	Mode of Development	Egg Size (μm)	References
Crepidula incurva	Playa Venado, Panama 8°53′N, 79°35'W	Planktotrophy	153–161	Collin, 2012
Crepidula cf. *marginalis*	Playa Venado, Panama 8°53′N, 79°35′W	Planktotrophy	158–177	Collin, 2012
Crepidula atrasolea	Near Fort Pierce, Florida 27°27′N, 80°18′W	Nonfeeding, direct development	330–344	Collin, 2000; Collin and Salazar, 2010
Crepidula ustulatulina	Near Fort Pierce, Florida 27°27′N, 80°18′W	Nonfeeding, lecithotrophic larvae	289–306	Collin and Salazar, 2010
Crepidula cf. *onyx*	Playa Venado, Panama 8°53′N, 79°35′W	Adelphophagy	157–159	Collin, 2003
Bostrycapulus calyptraeformis	Playa Venado, Panama 8°53′N, 79°35′W	Planktotrophy	180	Collin, 2005
Crucibulum spinosum	Playa Venado, Panama 8°53′N, 79°35′W	Planktotrophy	280–325	Collin, 2003
Crepipatella occulta	Totoralillo beach, Chile 30° 05′S; 71° 22′W	Adelphophagy	190–230	Véliz *et al.,* 2012

capture particles but have lost the ability to digest them (Kempf and Todd, 1989).

With the exception of some well-studied crustacean larvae that are of interest in aquaculture (*e.g.,* Biesiot and Capuzzo, 1990; Saborowski *et al.*, 2006; Rotllant *et al.*, 2008), very little is known about developmental changes of digestion and enzyme expression of marine invertebrate larvae (but see the comparative data set on crustacean larvae in Jones *et al.*, 1997). Here we describe a modification of a semi-quantitative color-based enzyme assay (the API-ZYM system) to quantify the activity of 19 enzymes, and use it to survey patterns of enzyme activity during the development of eight species of calyptraeid gastropods. The API-ZYM system shows good agreement with quantitative determinations of enzyme activities in juvenile abalone (Garcia-Esquivel and Felbeck, 2006). Our aims were to determine if the API-ZYM system can be used to detect statistically significant differences in enzyme activities; to document common ontogenetic patterns of enzyme activities; and to identify differences in enzyme expression that may be associated with different modes of development.

Materials and Methods

Species of calyptraeid gastropods are diverse in their modes of development (Collin, 2003), and one species, *Crepidula fornicata* (L. 1758), has become a *de facto* model system for lophotrochozoan development (Henry *et al*., 2010). Development includes planktotrophic larvae (50% of species) and lecithotrophic larvae (5% of species). There are also two kinds of direct development: direct development in which large juveniles develop from large eggs (30% of species) and direct development with adelphophagy or oophagy (15% of species) (Collin, 2003; Collin and Spangler, 2012). In many cases, extant sister species have different modes of development, and species with different development often co-occur. Females of all species, regardless of mode of development, brood embryos in capsules, and development is synchronous within a brood.

Embryos and hatchlings of eight species (Table 1) were collected from females maintained in the laboratory under standard conditions (Collin *et al.*, 2005) or were collected directly from females brooding in the field. Adults of a single species, *Crepipatella occulta* Véliz *et al.,* 2013, were collected in Chile but maintained in incubators in Panama. Broods were collected at five ontogenetic stages (Fig. 1), transferred to a 1.5-ml Eppendorf tube containing UV-sterilized, filtered seawater, and frozen at −80 °C. Samples were not treated with antibiotics because the microbial gut fauna can contribute significantly to the digestive enzyme activity in some herbivorous snails (Erasmus *et al.*, 1997; Kusumoto *et al.*, 1997) and we were interested in the enzyme activity of the entire holobiont. A subsample of each brood was staged and photographed. Post-hatching veligers and juveniles were kept in 350-ml dishes of filtered seawater and fed *Isochrysis galbana ad libitum* for 3–4 days prior to preservation.

Extracts of eggs, embryos, and hatchlings were prepared by manually homogenizing a single brood for most species, or up to four broods for the very small-bodied species. The samples were brought to a final volume of 2 ml with API suspension medium (bioMérieux, France). Each sample was centrifuged briefly to remove large particles of tissue and shell. Samples were further diluted to a standardized protein concentration of 200 mg/ml by comparing them with a

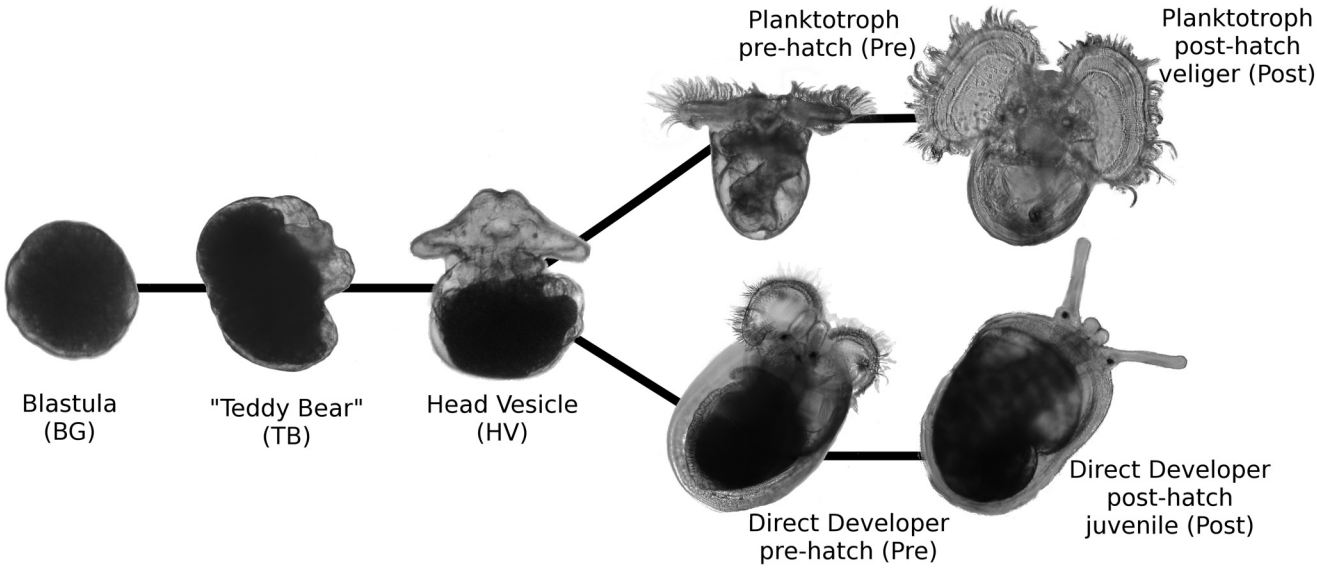

Figure 1. The five stages of development assayed during this study. Blastula/Gastrula (BG) appears under a dissecting microscope as a simple, unciliated ball of cells. Teddy Bear (TB) shows ciliation, stomodaeum, and the velum anlagen, before the development of eyespots or fluid-filled head vesicle. Head Vesicle (HV) has eyespots, small velum, and distinct head vesicle. Pre-Hatching (Pre) is a fully developed embryo with little remaining yolk and a fully developed velum in species with a velum. Post-Hatching (Post) is a veliger larvae or juvenile that was fed for 3 days prior to analysis.

bicinchoninic acid assay (BCA Sigma Aldrich) using 0%, 10%, 20%, 40%, 60%, 80%, and 100% bovine serum albumin protein standards.

Enzymatic activities were obtained for 19 enzymes (5 peptidases, 3 lipases, 3 phosphatases, and 8 carbohydrases) using an API-ZYM kit (bioMérieux). The ApiZym system is designed for use with unpurified samples and has previously been used successfully in assaying digestive enzymes for a variety of adult marine invertebrates (*e.g.,* periwinkles: Bärlocher *et al*., 1989; hydrothermal vent invertebrates: Boetius and Felbeck, 1995; giant keyhole limpets: Martin *et al*., 2011). It is a qualitative assay based on visual comparison of the colors produced by the enzymatic reactions with a printed color standard. We used a Nanodrop spectrophotometer to quantify the intensity and therefore the concentration of the reaction product. Following the manufacturers instructions, we inoculated each well in the API test strip with 65 μl of solution (13 μg protein content) and incubated the plate at 37 °C for 4.5 h. After incubation, reagents "ZymA" and "ZymB" were added to stop the reaction, and the resulting color was allowed to develop for 5 min. Each well was then exposed to intense fiber-optic light for 5 s to eliminate a yellow tint due to an excess of unreacted Fast Blue BB. The absorbance of each well was measured at the peak wavelength (Table 2) for that product, after using the control well from the test strip to calibrate the absorbance of the reagents at that wavelength without a reaction product. At least three broods were measured for each species at each stage. The effects of mode of development, developmental stage, and the interaction between the two on measured absorbance of the reaction products were analyzed using analysis of variance (ANOVA), with species nested within mode of development.

Results

The API-ZYM system is a useful way to get an overview of the activities of the most abundant enzymes in the tissues of adult invertebrates, embryos, and larvae. Our results reflect the wide range of enzyme expression in herbivorous gastropods (reviewed by Owen, 1966; Livingstone and de Zwaan, 1983). The quantitative spectrophotometric API-ZYM assay was reproducible, and we were able to detect statistically significant differences in concentrations of reaction product in all of the 19 enzymes assayed (Table 2). The high repeatability of the measures for replicate samples from different females collected at different times suggests that enzyme activity from potential environmental contaminants is negligible for most enzymes. Out of 2356 readings, 14 outliers identified by visual inspection of residuals were excluded from the statistical analyses.

Mode of development

Sixteen of the 19 enzymes showed a significant effect of mode of development on the concentration of the reaction product, as assayed by absorbance (Table 2; Fig. 2). Three enzymes (acid phosphatase, β-glucuronidase, N-acetyl-β-glucosaminidase) did not differ among the modes of devel-

Table 2

The 19 enzymes assayed by the API-ZYM test strips, the wavelength with maximum absorbance, and the statistical significance of each factor in the ANOVA analysis

Enzyme	Wavelength (λ)	MOD	Species [MOD]	Stage	MOD × stage
		Ester hydrolases			
Esterase (C4)	537	**<0.0001**	0.035	0.07*	0.176
Esterase lipase (C8)	537	**0.021**	0.052	**<0.0001**	0.075
Lipase (C14)	537	**0.001**	**0.035**	0.265	0.62
		Peptide hydrolases			
Leucine arylamidase	494	**<0.0001**	**0.0078**	**0.023**	0.854
Valine arylamidase	494	**0.0008**	**0.0004**	**0.0095**	0.46
Cystine arylamidase	494	**<0.0001**	0.37	**0.043**	0.091
Trypsin	494	**<0.0001**	0.099	0.42	0.204
α-Chymotrypsin	494	**<0.0001**	0.2108	0.4433	**0.0019**
		Phosphohydrolases			
Alkaline phosphatase	537	**<0.0001**	**<0.0001**	**<0.0001**	**0.0158**
Acid phosphatase	547	0.14	**0.033**	**<0.0001**	**0.0092**
Naphthol-AS-BI-phosphohydrolase	582	**0.0056**	**<0.0001**	**<0.0001**	0.2433
		Glycosidases			
α-Galactosidase	547	**0.039**	**<0.0001**	0.154	0.5033
β-Galactosidase	547	**0.0030**	**<0.0001**	0.0720	0.0233
β-Glucuronidase	582	0.4634	**<0.0001**	0.7577	**0.0016**
α-Glucosidase	537	**<0.0001**	**<0.0001**	**0.0005**	**0.0044**
β-Glucosidase	537	**<0.0001**	0.712	**<0.0001**	**0.0003**
N-Acetyl-β-glucosaminidase	**454**	0.0677	**<0.0001**	**<0.0001**	**0.0311**
α-Mannosidase	537	**0.0015**	0.459	**<0.0001**	**0.0002**
α-Fucosidase	537	**<0.0001**	**<0.0001**	**<0.0001**	**0.0097**

MOD, mode of development.
Entries in bold are statistically significant at the $P < 0.05$ level.
* Significant with removal of the nonsignificant interaction effect.

opment but did show a significant interaction between mode of development and stage (Table 1). The overall pattern for the other 16 enzymes is that planktotrophs showed the highest activities, followed by adelphophages, and then by nonfeeding embryos. For five enzymes (esterase (C4), cystine arylamidase, trypsin, α-chymotrypsin, and β-glucosidase), planktotrophs showed significantly greater activities than both adelphophages and nonfeeding embryos (Fig. 2). For two peptidases and a carbohydrase (leucine arylamidase, valine arylamidase, and β-galactosidase) nonfeeding developers showed significantly lower activities than the planktotrophs and adelphophages, which did not differ from each other (Fig. 2). In two carbohydrases (α-glucosidase, α-fucosidase) the adelphophages had significantly higher expression than the other two modes of development, and β-galactosidase showed a trend toward the same pattern (Fig. 2). However, the unusually high values for *Crepipatella occulta* for both α-glucosidase and α-fucosidase (see Discussion) may be driving this pattern.

Developmental stage

There was a significant overall effect of stage on the activities of six enzymes and both an effect of stage and the interaction between stage and mode of development (stage × development) on seven enzymes (Table 2; Fig. 3). Three enzymes (α-chymotrypsin, β-galactosidase, and β-glucuronidase) showed a significant interaction but no overall effect of stage (Fig. 3). Three other enzymes (lipase (C14), trypsin, α-galactosidase) showed no significant effect of stage and no significant interaction between mode of development and stage. In these species the values were low and included a number of samples with 0 absorbance. Reaction product concentrations of these three enzymes may be near the limit of detection for this assay.

Of the 13 enzymes with significant differences across developmental stages, 8 showed a clear increase during development. One, α-glucosidase, showed a clear decrease in adelphophages and planktotrophs but low and flat expression in nonfeeders. Much of this pattern is driven by the high activities and unusual pattern of α-glucosidase in *C. occulta* (see Discussion). Two others had a humped pattern of activity through development. Alpha-mannosidase had peaks at different stages for the three different modes of development, but in all three a humped pattern was evident. In α-fucosidase, in contrast, the humped pattern is, again, largely driven by *C. occulta* (Fig. 3).

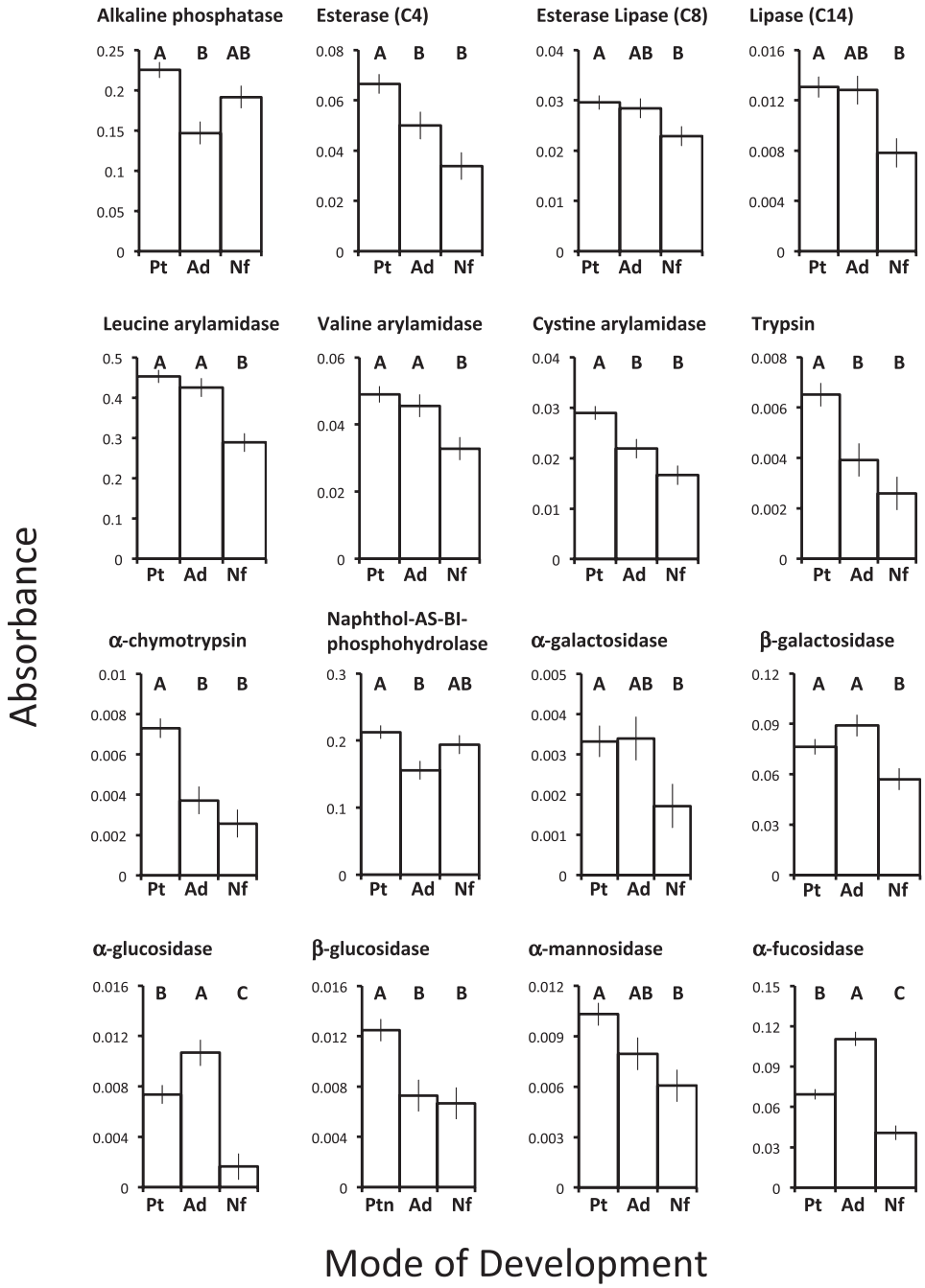

Figure 2. Least-square means of absorbance of enzymatic reaction products for each mode of development for the 16 enzymes for which there was a significant effect of mode of development. Bars linked by the same letter do not differ significantly. Planktotrophic (Pt) development proceeds from small eggs to hatching veliger larvae; nonfeeding (Nf) proceeds from large eggs to hatching juveniles (*Crepidula atrasolea*) or ephemeral, nonfeeding lecithotrophic larvae (*Crepidula ustulatulina*); and adelphophagic development (Ad) proceeds from small eggs that produce hatching juveniles because embryos consume each other. Error bars represent standard error of the mean.

Only one of the enzymes showed a sudden jump in activity between the pre- and post-hatching stages, contrary to what would be expected if increased expression or activity was associated with exposure to exogenous food. In β-glucosidase, activity increased in planktotrophs and nonfeeders, while enzyme activity in the adelphophages dropped between the pre- and post-hatching stages. In fact, adelphophages showed this distinct drop in activity between

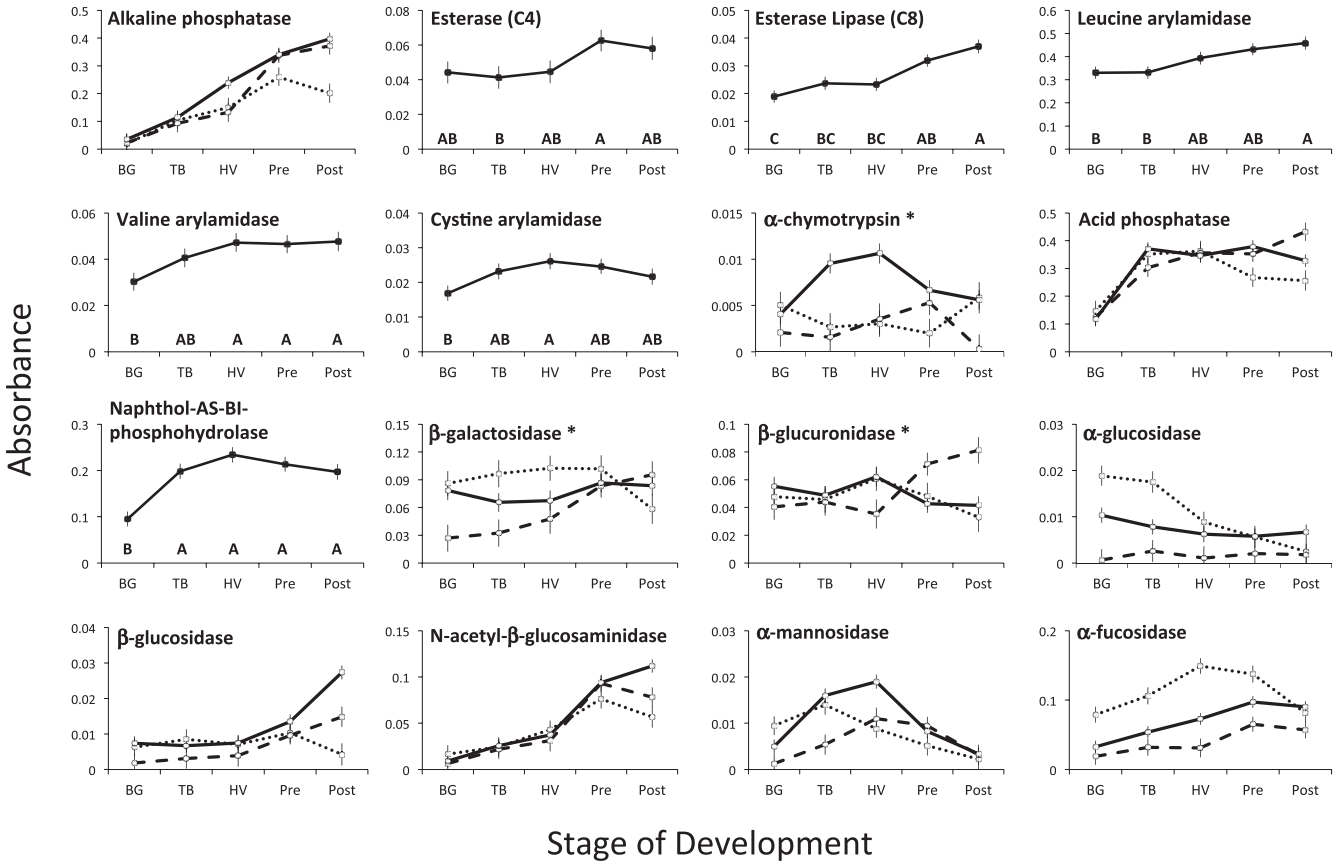

Figure 3. Least-square means of absorbance of enzymatic reaction products for each stage in development. The six enzymes for which there was a significant effect of stage but no significant interaction between stage and mode of development (MOD) are shown as a single line of the mean for each developmental stage. Stages linked by the same letter do not differ significantly. The 10 enzymes for which there was a significant interaction between stage and MOD are shown as means for each mode of development. * indicates species for which there was no overall significant effect of stage. Solid line = planktotrophs; dotted line = adelphophages; dashed line = nonfeeders. Stages: Blastula/Gastrula (BG), Teddy Bear (TB), Head Vesicle (HV), Pre-Hatching (Pre), and Post-Hatching (Post). Error bars represent standard error of the mean.

the pre- and post-hatching stages in six enzymes (alkaline phosphatase, β-galactosidase, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase, and α-fucosidase). This drop is not evident in the other modes of development.

Species

Thirteen enzymes showed a significant effect of species nested within mode of development. The general effects of species were in line with the effect of mode of development and usually involved only a few significant pairwise differences between the species with the most extreme means. However, in three cases ANOVA identified three exclusive, significantly different groups of species. For naphthol-AS-BI-phosphohydrolase the measured absorbance of *Crucibulum spinosum* (Sowerby 1824) samples were significantly higher than all of the other species, which did not differ from each other (Fig. 4). The activity of α-fucosidase in *C. occulta* was significantly greater than in the other seven species (Fig. 4) and is the cause of the significantly high absorbances for adelphophages compared to the other species (Fig. 2). Finally, activity of β-galactosidase was significantly higher in *Crepipatella occulta*, *Crucibulum spinosum*, and *Bostrycapulus calyptraeformis* (Desheyes, 1830) than in the four *Crepidula* species (Fig. 4). This is the only enzyme for which there was a clear indication of a phylogenetic effect. Visual inspection of interaction graphs also showed that *C. occulta* had a different pattern of activity compared to the other species for alkaline phosphatase and α-glucosidase (Fig. 4). In these cases, *post-hoc* comparisons show that *C. occulta* was significantly different from most of the other species, although two distinct significantly different groups were not recovered. Similarly, *Crepidula ustulatulina* Collin, 2002, showed particularly low levels of leucine arylamidase.

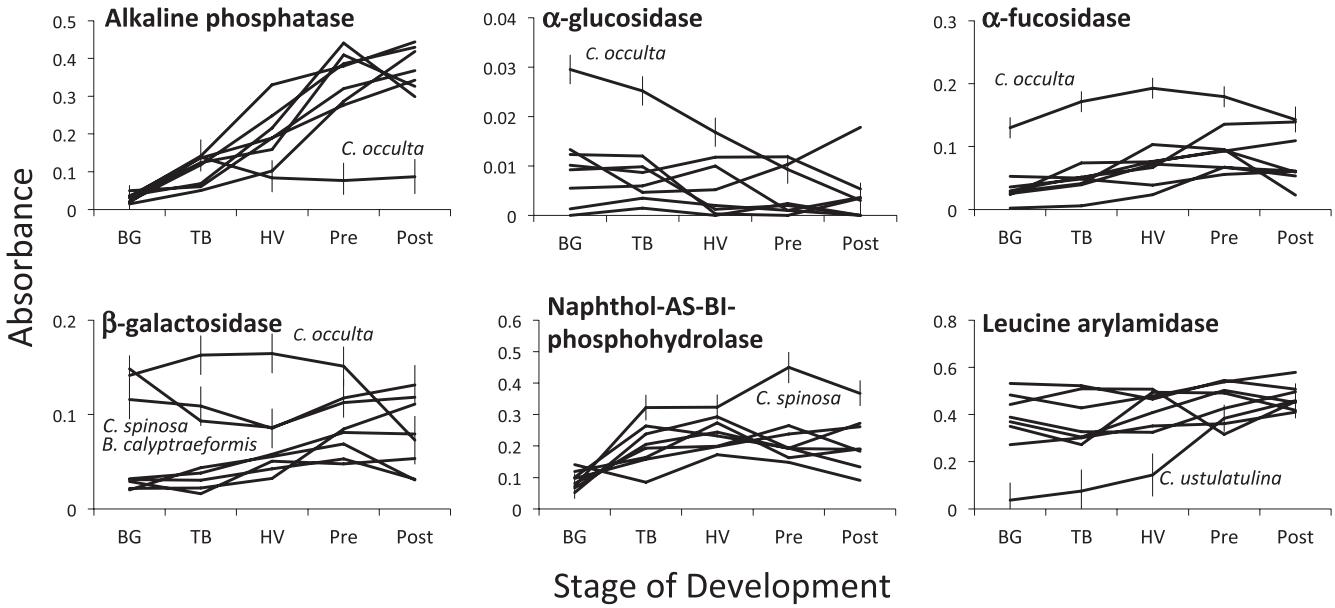

Figure 4. Changes in enzyme activities during development for six enzymes in which there were significant differences among the species, independent of mode of development. Stages: Blastula/Gastrula (BG), Teddy Bear (TB), Head Vesicle (HV), Pre-Hatching (Pre), and Post-Hatching (Post). Error bars represent standard error of the mean.

Discussion

The quantification of the API-ZYM assay allowed us to statistically reject the null hypotheses that embryos of each mode of development express similar enzyme activities, and that all enzyme activity increases as development progresses. Instead we found that the levels of enzyme activities varied with mode of development and different enzymes showed different patterns of change with development. Overall, planktotrophic embryos showed higher enzyme activities per microgram of total protein, adelphophagic embryos showed intermediate levels, and species with large eggs had the lowest enzyme activities. This could be due to differences in enzyme concentrations or different enzyme kinetics in the different species. Large invertebrate eggs are very yolky and have greater mass-specific energy content compared to eggs of planktotrophic species (Jaeckle, 1995; Moran and McAlister, 2009). This is generally the result of higher lipid and lower protein concentrations in large eggs (Jaeckle, 1995). Although the constituents of calyptraeid eggs have not been analyzed biochemically, if they follow the patterns evident in other invertebrates, the planktotrophic eggs we used probably had higher protein concentrations than the large eggs. Because we diluted the homogenates to a standard protein concentration, our results should not reflect this greater protein concentration in small eggs. It is, however, possible that large eggs also contain higher concentrations of yolk protein, resulting in lower concentrations of other proteins when total protein is standardized. This could explain the lower measured activities of enzymes from species with large eggs. However, in many cases the differences were also evident in the pre- and post-hatching stages, which do not retain much, if any, visible yolk.

Early development is primarily fueled by lipids stored in the egg, and later, post-hatching development is fueled by the digestion of microalgae. We therefore expected that lipases would show their highest activity early in development while carbohydrases would increase toward hatching. However, this pattern was not realized, as most enzymes assayed here increased in activity over the course of development. The two most active lipases, esterase (C4) and esterase lipase (C8), both showed slight increases in activity through development for all the species, whereas lipase (C12) showed no effect of stage. Similarly, carbohydrases tended to show an increase with development, but in most cases we detected significant interactions between mode of development and stage (Figs. 3 and 4), indicating that different kinds of development or different species showed different patterns of activity. In the following cases, activities did not increase with development: α-glucosidase for all species except *C. occulta*; β-galactosidase for three non-*Crepidula* but increasing for the four *Crepidula* species; β-glucuronidase for planktotrophs and adelphophages, but increasing for species with large eggs.

The different levels of carbohydrase activity may provide some information about the diet of hatchling calyptraeids.

Table 3

Summary of API assays of other gastropods compared to the overall values for calyptraeids hatchlings

Enzyme	Calyptraeids (average absorbance of post-hatching stage)	*Littorina* stomach	*Littorina* digestive gland	*Haliotis* stomach	*Haliotis* digestive gland	*Megathura* style sac	*Megathura* digestive gland	*Aplysia* digestive gland
			Ester hydrolases					
Esterase (C4)	**0.063**	1.4	1	**3**	**3**	**1.8**	2.2	0
Esterase Lipase (C8)	0.037	**3.2**	**2.4**	2	2	1	**2.8**	0
Lipase (C14)	0.012	1	1.4	2	1	1	1	0
			Peptide hydrolases					
Leucine arylamidase	**0.472**	**5**	**2.8**	**5**	**4**	**4.6**	**4**	3
Valine arylamidase	0.049	1.4	**2.8**	4	3	3.8	2	3
Cystine arylamidase	0.023	1	1	3	2	0.8	1.2	3
Trypsin	0.005	1.4	1	0	0	0.3	0.5	3
α-Chymotrypsin	0.004	1	1	2	0	3.9	1	2
			Phosphohydrolases					
Alkaline phosphatase	**0.339**	**5**	**5**	4	3	4	1.8	**3**
Acid phosphatase	**0.339**	**5**	**5**	4	**5**	4.4	**4.3**	**3**
Naphthol-AS-BI-phosphohydrolase	0.211	**5**	**5**	**5**	**5**	**4.7**	2.8	2
			Glycosidases					
α-Galactosidase	0.002	1.4	1	1	2	0	2.9	0
β-Galactosidase	**0.079**	**5**	**4.2**	**4**	**5**	3.3	**4.8**	**3**
β-Glucuronidase	**0.050**	**5**	3	**5**	**5**	3	**4.6**	**3**
α-Glucosidase	0.004	3	3.4	3	2	1.8	3.9	3
β-glucosidase	0.019	2	2.4	1	2	0.3	2.8	0
N-Acetyl-β-glucosaminidase	**0.090**	2.2	**3.8**	4	4	**3.5**	3.2	0
α-Mannosidase	0.003	0.8	1.2	3	4	0.7	2.8	0
α-Fucosidase	**0.081**	1.2	2.2	4	**5**	**3.5**	**4.6**	**3**

Data from *Aplysia punctata* (Taïeb, 2001), *Haliotis rufescens* (Garcia-Esquivel and Felbeck, 2006), *Megathura crenulata* (Martin *et al.,* 2011), and *Littorina irrorata* (Bärlocher *et al.,* 1989). Entries in bold are the highest activities in each enzyme category.

Adult calyptraeids are suspension-feeders, which appear to feed non-selectively on a variety of phytoplankton (Riera *et al.*, 2002; Beninger *et al.*, 2007; Decottignies *et al.*, 2007). Larvae can be raised on the same diets as the adults, and several species can be raised for their entire life-cycle on *Isochrysis galbana* (R. Collin, pers. obs.). Early hatchlings are also known to graze on biofilms (Navarro and Chaparro, 2002). Of the eight carbohydrases assayed here, β-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, and α-fucosidase showed the highest activities, with absorbances an order of magnitude higher than the remaining three α-carbohydrases. This finding suggests that the natural diet may be rich in diatoms and brown algae, which are richer in carbohydrates with β-linkages than in green algae.

The relative enzyme activities obtained here for the post-hatching stages show some similarities to the activities of other herbivorous gastropods. We know of no previous studies of these enzymes in early developmental stages of marine gastropods. However, other herbivorous gastropods have been assayed with the qualitative API system. These include the digestive gland of adult *Aplysia* sea hares (Taïeb, 2001) and different parts of the digestive system of juvenile red abalone, *Haliotis rufescens* (Garcia-Esquivel and Felbeck, 2006); the giant keyhole limpet *Megathura* (Martin *et al.*, 2011); and the periwinkle *Littorina* (Bärlocher *et al.*, 1989). The values reported for *Aplysia* do not fit the 0–5 scale used by the other assays, and they are very different from the other published results (Table 3). Unfortunately, the assays are not discussed by Taïeb (2001). The enzyme activities of the post-hatching calyptraeids show some overall similarities with the qualitative assessment of the relative activities of the stomach and digestive glands of these other gastropods (Table 3). In the calyptraeids and the other gastropods, overall activity of ester hydrolases was low, but esterase (C4) and esterase lipase (C8) activity were consistently higher than lipase (C14) activity. The three phosphohydrolases showed uniformly high activities, and there were no consistent rankings among the species or parts of the digestive system. Four carbohydrases (β-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, and α-fucosidase) exhibited very high activities throughout the guts of the abalone and keyhole limpet (Garcia-Esquivel and Felbeck, 2006; Martin *et al.,* 2011) as well as in the calyptraeid hatchlings. β-galactosidase and β-glucuronidase were also high in the periwinkle, but α-fucosidase activity was low compared to the other species. Since none of these

studies were conducted on sterile samples, it is unknown if these assays reflect the activities of enzymes produced by the snails or, as has been demonstrated by previous work with abalone, by bacteria resident in the gut (Erasmus *et al.*, 1997; Kusumoto *et al.*, 1997). Leucine arylamidase is very active in most species and most parts of the digestive system, followed by valine arylamidase, which shows much lower levels but is consistently the second most active peptide hydrolase in the species assayed. Cysteine proteinases are the major enzymes involved in the degradation of yolk in several invertebrates (*Bombyx*: Kageyama *et al.*, 1981; *Artemia*: Perona and Vallejo, 1982; *Drosophila*: Medina and Vallejo, 1989; insects *Blattella:* Nordin *et al.*, 1991; sea urchins: Mallya *et al.*, 1992). Our enzyme assay showed no decrease in cystine arylamidase activity as yolk stores were depleted, and the relative activity of cystine arylamidase in calyptraeid hatchlings is similar to those reported for the other species (Table 3).

The information on enzyme activities obtained from this sample of eight species gives some important insights into the evolutionary transitions between different modes of development. None of the 19 enzymes assayed here showed a sudden jump in activity between the pre-hatching and post-hatching stages, showing that enzyme activity is not triggered by exposure to food after hatching. Since calyptraeid embryos commonly express digestive enzymes prior to hatching, a heterochronic shift in hatching time could result in earlier stages becoming planktotrophic larvae without any major change in enzyme regulation. Adelphophagic embryos that feed on nurse eggs or siblings have higher enzyme activity levels, more similar to those of planktotrophs, than do embryos from large eggs. This shows a similar pattern to the morphological modifications of the embryos, where adelphophages retain more of the structures characteristic of planktotrophs than do embryos developing from large eggs. The species that demonstrated the most distinct pattern of enzyme activities was the adelphophage *C. occulta*. For one enzyme, β-galactosidase, the high activities of *C. occulta* are part of what appears to be a phylogenetic signal in which the four *Crepidula* show significantly lower activities than the four species representing *Crepidula*'s sister clade. For this and the other three enzymes for which *C. occulta* was an outlier, the other adelphophage, *C.* cf. *onyx*, did not show the same patterns. *Crepipatella occulta* is the only representative of its genus in this study, and it is also the only temperate species. Since the significant differences between *C. occulta* and the other species appeared at only a few developmental stages in each enzyme, and since enzyme activities were significantly higher in two enzymes and lower in one, it is difficult to interpret these results with the current sampling. These significant differences between *C. occulta* and the other calyptraeids, as well as the other patterns detected here, could be investigated in more detail in future studies using more sensitive methods than employed in this broad survey.

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