Hull flotation function in the eggs of *Mopalia ciliata* (Chitonida: Mopaliidae) and swimming of its larvae through ontogeny

Adriana P. Rebolledo[1,2]

Larval biology course

Summer 2014

[1]Friday Harbor Laboratories, University of Washington, Friday Harbor, WA, 98250

[2] Unidad de Investigación Pesquera y Acuicultura (UNIP) of the Centro de Investigación en Ciencias del Mar y Limnología (CIMAR), Universidad de Costa Rica, 11501-2060 San José, Costa Rica

Contact information:

Adriana P. Rebolledo: adriprn@gmail.com

Keywords: chitons, eggs, sinking rates, density, larvae, swimming, growth

Abstract

The free-spawning species of chitons produce eggs that are enclosed in a capsule known as the hull. Particularly in the group of Chitonida, this outer coat is formed by spine projections. Previous studies have identified the function of the hull as a "sperm guide" that facilitates the fertilization of the eggs, and one has suggested that this hull also serves to slow down the sinking rates of the eggs. In this study, I present concrete evidence showing that the hull does reduce the sinking rates of eggs in the chiton species *Mopalia ciliata*, acting as flotation device, as a consequence of its lower density compared to sea water and the egg without this structure. Additionally, changes during ontogeny in behavior, swimming velocities, and body shape of the larvae of this species were analyzed. Over time, the larvae decrease their tendency to swim up and prefer to stay in the bottom, and the body becomes elongated. These changes may be related to preparation for settle and metamorphosis into a juvenile. This study shows the importance of the hull structure in the sinking rates of the chitons eggs, and the ontogenetic changes in behavior and form that the larvae present, encouraging further studies to evaluate the ecological impact of these aspects in the persistence of the species.

Planctonic eggs of chitons are enclosed in an outer coating, known as the hull (Buckland-Nicks and Eernise 1993; Ituarte et al. 2010), which is secreted in the late vitellogenic stage of development (Barbosa et al. 2008). The morphology of the hull varies between groups (Liuzzi and Zelaya 2013); in Lepidopleurida the hull is a jelly-like coat perforated by a series of pores (Buckland and Schander 2008; Buckland-Nicks and Reunov 2009), while in Chitonida this structure consist of complex projections with spines-like forms (Buckland-Nicks and Reunov 2009; Ituarte et al. 2010) and narrow bases (Sirenko 1993).

In Chitonida species, several studies have shown that the hull projections direct sperm to specific areas of the egg (Buckland-Nicks 1993, 2006, 2008; Buckland-Nicks and Schander 2008; Buckland-Nicks and Brothers 2008), due to the close proximity of the spines, forcing the sperm to penetrate the egg between their polygonal bases (Buckland-Nicks 2006, 2008), and also facilitating fertilization since the pores in the bases gives the sperm access to the vitelline layer (Buckland-Nicks and Brothers 2008).

In addition to its function in aiding fertilization, Buckland-Nicks (1993) found evidence that the hull acts as a "parachute". He reported that hulled eggs of a free spawny species were the same density as those of a broader with a compact hull, but eggs of the former sank much more slowly. In order to specifically demonstrate the role of the hull in sinking, here I compare the sinking rates and densities of *Mopalia ciliata* eggs with and without the hull.

Regarding the trochophore chiton larvae, there has been only reported the swimming speed for the unidentified stage of development of *Tornicella marmorea* (Konstantinova 1966). The information about their swimming patterns and velocities through ontogeny is particularly important since these parameters determine the larvae distribution and the ability to reach suitable habits are determine (Kelman and Emlet 1999). Due to the lack of knowledge about this subject in chitons species, I also measure swimming velocities and larval size over time.

Methods

The free-spawning species *Mopalia ciliata* was collected from Argyle creek (48°31'12.36", -123°0'52.62"), San Juan Island, Washington, between August 7 and 14, 2014. Specimens were maintained at Friday Harbor Labs in separated containers on a seawater table at 17-18 °C. Females spawned over night the day after their collection.

- **Sinking rates**

Sinking speed was measured for ten eggs from each female. Intact eggs (with hulls) were placed individually in the middle of a cylindrical water column (high: 50 cm, diameter: 5cm), enclosed in a water bath to maintain near constant temperature (18-19 °C). Water column was stabilized by a week salinity gradient (32 ppt at the top and 35 ppt at the bottom). The time it took an egg to cross two marks separated by 5 cm was recorded. From eggs of 4 females, the hull was carefully removed using entomological pins, and sinking rates of these were also measured.

Digital pictures of the eggs (Fig. 1A) were taken using a microscope (Nikon eclipse E600), equipped with a camera (Sony exwave HAD). Measurements of the diameter of the egg and the egg plus the hull were made using the Image J software. Eggs presented a sphere shape (Fig. 1B); therefore, their volume was obtained using the formula for spheres objects, Vol= $4/3\pi r^3$, where r is the radio.

Densities of the egg with and without the hull were calculated with the following formula:

$$\text{Density}_{(sphere)} = \text{Density}_{seawater} - \frac{6\pi * \text{radio} * \text{viscosity} * \text{sphere sinking rate}}{\text{sphere volume} * \text{gravity}}$$

The volume and mass of the hull were estimated subtracting the values of the egg without the hull from the volume and mass of egg with the hull.

- **Larva swimming velocities**

Eggs from two females were fertilized in the laboratory and kept in containers with seawater at 17-18 °C. Hatching started 32 hours after fertilization (Fig 1C, D). Upward swimming velocities were measured 24 and 48 hours after hatching by placing them individually in the same water column used for egg sinking trials and the time it took to cross 2 cm mark was recorded. Additionally, another set of larvae were deciliated by osmotic shock: the larvae were placed in seawater with a double concentration of salinity, and then they were placed back to ambient seawater (31ppt). The sinking rate of the deciliated larvae was measured following the same protocols with the eggs.

Seventy two hours after hatching, the larvae were placed individually in a petri dish (23 °C) and videos of them crossing an area of 6 mm (length) by 8 mm (width) were recorded. Net horizontal velocities were calculated by dividing their time for crossing the length or the width.

Images of the larvae were taken every 24 hours from hatching to the third day. Length and width was measured using the ImageJ software. Ratios of the length to the width were estimated over time, and the volume was calculated with the formula for prolate spheroids.

- **Statistical analyses**

Data were analyzed with the statistical software SPSS v.20.0. The assumption of normality of the data was tested using the Kolmogorov-Smirnov test; when data did not meet this assumption, differences among groups were assessed using the corresponding non-parametric test. A Student t-test was used to compare sinking rate of the eggs measured with and without the hull of each female. Differences between the densities of the eggs with and without the hull, and the hull were analyzed applying an One-way Analysis of Variance (ANOVA) and a Tukey's test. Regarding the larvae, a Mann-Whitney U-test was applied to compare over time the upward swimming and sinking rates. Differences of size and volume through age were analyzed with a Kruskall-Wallis test.

Results

- **Egg sinking rate.**

Data of egg sinking rates with and without the hull were normally distributed (Kolmorov-Smirnov test, $p>0.05$). Sinking rates of the eggs without the hulls is 1/3 or 1/4 of those with the hull ($p<0.05$, Table 1).

The volume of the hull represents a large portion (72.6 % ± 0.9) of the whole structure. Density of the eggs with the hull is significantly lower (Table 2) compare to the eggs without it. In all the analyzed cases the density of the hull was lower than the eggs with and without it (Tukey's test, $p<0.05$), and even lower than the density of seawater.

- **Larvae swimming velocities**

Although upward swimming velocities and sinking rates did not show significant differences over time (Table 3), a decreasing trend through age was observed. During the first 24 hours they swam in a straight vertical path, rotating around their anterior and posterior axis. The second day they started to show an up and down swimming behavior, and by the time they reached the third day most of the larvae did not present the upward swimming behavior; however, they actively swam on the bottom of the containers, and their horizontal velocity was significantly higher (Kruskall-Wallis H test, $H= 14.6$, $df= 2$, $p<0.05$) than the upward swimming. All of the deciliated larvae were negatively buoyant, and the sinking rates did not differ significantly with the swimming upward velocities.

Regarding volume, this increases significantly over time. Also, the length/width ratio increases through age, with the larvae getting longer and not changing noticeably in width (Table 4, Fig. 1E, F, G, H).

Discussion

Besides the hull function in the fertilization process of the eggs (Buckland-Nicks 1993, 2006, 2008), the results of this study clearly show that this structure does in fact have a "parachuting" effect, as a previous study suggested (Buckland-Nick 1993). This is possible due to the lower density of the hull in comparison to seawater (Table 2), which may be related to the its chemical composition: mucopolysaccharides (Ituarte et al. 2010). Density of the egg is drastically higher without the hull, therefore the hull acts as a flotation device that prevents fast sinking of the egg.

Additionally, the volume of this coat may contribute to lowering the eggs density. Considering the eggs of *M. ciliata*, the hull represents a major portion of the total egg volume. In this species, Buckland-Nick (1993) hypothesized that the hull causes a larger effective volume that traps a layer of seawater around the egg, reducing its effective density and slowing its sinking rate. The findings of this study seem to support his conclusion.

At an ecological level, the development of this complex coat may have great implications in the persistence of the free-spawning species of chitons. Besides its function as a "sperm guide" (Buckland-Nicks and Schander 2008; Buckland-Nicks and Brothers 2009), the role of the hull in decreasing sinking rates of the eggs allows an increase in the duration of the gamete in the water column, therefore, increasing its probability of encountering sperm; and also reduce its likelihood to settle on the bottom where it may be consumed by benthic suspensions feeders or others predators.

Regarding the larvae of *M. ciliata,* this species change its swimming behavior over time, decreasing the actively upward swimming tendency and its velocity. However, the third day they are still capable of swim but prefer to stay in the bottom. In addition to these behavioral variations, they also modify the proportion of their body plan through ontogeny; as seen in other trochophore larvae (Wanninger and Haszprunar 2002; Lord 2010). The larvae become more elongated and the dorso-ventral axis flattens. All of these changes in behavior and form could be related to their preparation for metamorphosis into a juvenile (Wanninger and Haszprunar 2002; Hadfield and Koehl 2004; Lord 2010).

This study confirms the importance of the hull structure in relation to the sinking rates of the chiton eggs, and encourages further studies to evaluate the ecological impact of this structure on aiding the fertilization of this species. With regard to *M. ciliata* larvae, I present the first insight into changes in swimming patterns, and its possible relation with the variation of the body form through ontogeny.

Acknowledgments

I am grateful to Friday Harbor Laboratories for allowing me to participate in the Larval Biology course. Also, I am highly grateful to Danny Grünbaum for his help in the preparation of the experiments, to Leah Sloan for helping me in the collection of specimens, and to Melissa Romero for her review of the manuscript. A special thanks goes to Richard Emlet for his enthusiasm and support in all of the aspects of this project.

References

Barbosa, S.S., Byrne, M., and Kelaher, B. P. 2009. Reproductive periodicity of the tropical intertidal chiton *Acanthopleura gemmata* at One Tree Island, Great Barrier Reef, near its southern latitudinal limit. J. Mar. Biol. Assoc. 89:, 405-411.

Buckland-Nicks, J. 1993. Hull cupules of chiton eggs: parachute structures and sperm focusing devices? Biol Bull. 184: 269–276.

Buckland-Nicks, J. 2006. Fertilization in chitons: morphological clues to phylogeny. Venus 65: 51–69.

Buckland-Nicks, J. 2008. Fertilization biology and the evolution of chitons. Am. Malaco. Bull 25:, 97-111.

Buckland-Nicks, J., and Brothers, E. 2008. On fertilization in *Chaetopleura apiculata* and selected Chitonida. Biol. Bull 214: 184-193.

Buckland-Nicks, J., and Schander, C. 2008. Fertilization in the white chiton, *Stenosemus albus,* with implications for its phylogenetic position. Can. J. Zoo. 86:, 1024-1030.

Buckland-Nicks, J., and Reunov, A. 2009. Ultrastructure of hull formation during oogenesis in *Rhyssoplax tulipa* (= Chiton tulipa) (Chitonidae: Chitoninae).Invertebr. Reprod. Dev. 53: 165-174.

Hadfield, M. G., and Koehl, M.A.R. 2004. Rapid behavioral responses of an invertebrate larva to dissolved settlement cue. Biol. Bull. 207: 28-43.

Ituarte, C., Liuzzi, M., and Centurión, R. 2010. Egg hull morphology in two chitons (Polyplacophora) from the southwestern Atlantic Ocean. Malacologia 53: 167-174.

Kelman, D., and Emlet, R.B. 1999. Swimming and buoyancy in ontogenetic stages of the cushion star *Pteraster tesselatus* (Echinodermata: Asteroidea) and their implications for distribution and movement. Biol. Bull.197: 309-314.

Konstantinova, I . 1966. Characteristics of movement of pelagic larvae of marine invertebrates. Dokl. Akad. Nauk. SSSR, 170: 726-729.

Liuzzi, M. G., and Zelaya, D.G. 2013. Egg-hull ultrastructure of *Ischnochiton stramineus* (Sowerby, 1832), a South American brooding chiton (Chitonina: Ischnochitonidae). J. Mollusc. Stud. 79:, 372-377.

Lord, J.P. 2011. Larval development, metamorphosis and early growth of the gumboot chiton *Cryptochiton stelleri* (Middendorff, 1847) (Polyplacophora: Mopaliidae) on the Oregon coast. J. of Mollus. Stud. *77*:, 182-188.

Wanninger, A., and Haszprunar, G. 2002. Chiton myogenesis: perspectives for the development and evolution of larval and adult muscle systems in molluscs. J. Morphol. 251: 103-113.

Table 1. Mean (± S.D) egg sinking velocities of *Mopalia ciliata* with and without the hull. N= 10 eggs per female

Female	Sinking velocity (mm/s)		Velocity ratio= $V_{Egg+Hull}/ V_{without\ hull}$	Stastistical significance
	Egg with hull	Egg without hull		
1	0.18 ± 0.04	0.65 ± 1.5	0.3	t= -9.4, df= 18, p= 0.000
2	0.14 ± 0.02	0.59 ± 2.2	0.2	t= -6.5, df= 18, p= 0.000
3	0.20 ± 0.05	0.64 ± 1.4	0.3	t= -9.3, df= 18, p= 0.000
4	0.16 ± 0.02	0.52 ± 1.2	0.3	t= -8.9, df= 18, p= 0.000
5	0.19 ± 0.05	-	-	-

Table 2. Mean (± S.D) volume, mass and density of *Mopalia ciliata* eggs with and without the hull. N= 10 eggs per female

Female	Egg with hull			Egg without hull			Hull			Statistical significance of density
	Volume (10^{-12} m^3)	Mass (10^{-9} Kg)	Density (kg/m^3)	Volume (10^{-12} m^3)	Mass (10^{-9} Kg)	Density (kg/m^3)	Volume (10^{-12} m^3)	Mass (10^{-9} Kg)	Density (kg/m^3)	
1	18.4 ± 0.8	18.9 ± 0.1	1026.5 ± 0.1	5.1 ± 0.1	5.4 ± 1.0	1054.2 ± 0.7	13.3 ± 0.7	13.5 ± 0.7	1015.9 ± 0.4	F= 18613.7, df= 2, p= 0.000
2	20.0 ± 0.6	20.5 ± 0.6	1025.5 ± 0.1	5.1 ± 0.2	5.4 ± 2.1	1050.9 ± 0.8	14.8 ± 0.6	15.1 ± 0.6	1016.8 ± 0.3	F= 13383.8, df= 2, p= 0.000
3	18.3 ± 0.5	18.8 ± 0.6	1026.8 ± 0.1	5.1 ± 0.1	5.4 ± 1.0	1053.5 ± 0.4	13.2 ± 0.6	13.4 ± 0.6	1016.5 ± 0.3	F= 38278.8, df= 2, p= 0.000
4	18.6 ± 1.1	19.1 ± 1.2	1026.1 ± 0.1	5.2 ± 0.1	5.5 ± 1.2	1047.4 ± 0.4	13.4 ± 1.1	13.7 ± 0.1	1017.9 ± 0.4	F= 20414.5, df= 2, p= 0.000
5	18.6 ± 0.6	19.1 ± 0.6	1026.7 ± 0.1	5.3 ± 0.1	-	-	-	-	-	-

Table 3. Mean (± S.D) swimming velocities of *Mopalia ciliata* larvae over time. N= 10 larvae per female

Female	Swimming velocities (mm/s)					Statistical significance			
	Upward		Sinking		Horizontal	Through age (24 and 48 hours)		Upward swimming vs sinking	
	24 hours	48 hours	24 hours	48 hours	72 hours	Upward	Sinking	24 hours	48 hours
1	0.6 ± 0.4	0.5 ± 0.2	0.8 ± 0.2	0.7 ± 0.2	1.2 ± 0.2 (N= 3)	U= 41, Z= -0.7, p>0.05	U= 39.5, Z= -0.8, p>0.05	U= 36.5, Z= -1.0, p>0.05	U= 33.0, Z= -1.3, p>0.05
2	0.8 ± 0.3	0.6 ± 0.3	0.8 ± 0.2	0.7 ± 0.1	1.6 ± 0.7 (N= 9)	U= 32, Z= -1.4, p>0.05	U= 27, Z= -1.8, p>0.05	U= 45, Z= -0.4, p>0.05	U= 34.5, Z= -1.2, p>0.05

Table 4. Size and volume of the *Mopalia ciliata* larvae over time.

Female	Measurements	Age (hours)								Statistical significance	
		1		24		48		72		Length/ width	Volume
		Mean ± S.D	N	Mean ± S.D	N	Mean ± S.D	N	Mean ± S.D	N		
1	Length (10^{-5} mm)	2.31 ± 0.04	5	2.61 ± 0.10	5	3.12 ± 0.10	5	2.85	1	H= 8.7, df= 2, p<0.05	H=10.5 df=2 p<0.05
	Width (10^{-5} mm)	2.02 ± 0.01		2.00 ± 0.10		2.24 ± 0.07		1.90			
	Length/width	1.14 ± 0.02		1.31 ± 0.15		1.40 ± 0.05		1.50			
	Volume (10^{-1} mm^3)	4.96 ± 0.50		5.47 ± 0.70		8.21 ± 0.80		5.44			
2	Length (10^{-5} mm)	2.41 ± 0.07	5	2.93 ± 0.09	5	3.03 ± 0.10	7	3.35 ± 0.40	6	F= 23.0, df= 3, p<0.05	H= 10,4 df= 3, p<0.05
	Width (10^{-5} mm)	2.02 ± 0.03		2.01 ± 0.08		2.02 ± 0.06		1.94 ± 0.09			
	Length/width	1.19 ± 0.03		1.46 ± 0.03		1.50 ± 0.05		1.71 ± 0.18			
	Volume (10^{-1} mm^3)	5.14 ± 0.30		6.25 ± 0.70		6.47 ± 0.5		6.42 ± 1.07			

Figures

Fig. 1. *Mopalia ciliata*. A) Egg, B) Egg without the hull, C) Larva hatching, D) Hull after larva hatched, E) Larva after hatching, F) Larva 24 hours after hatching, G) Larva 48 hours after hatching, H) Larva 72 hours after hatching. Scale bars: 100 µm

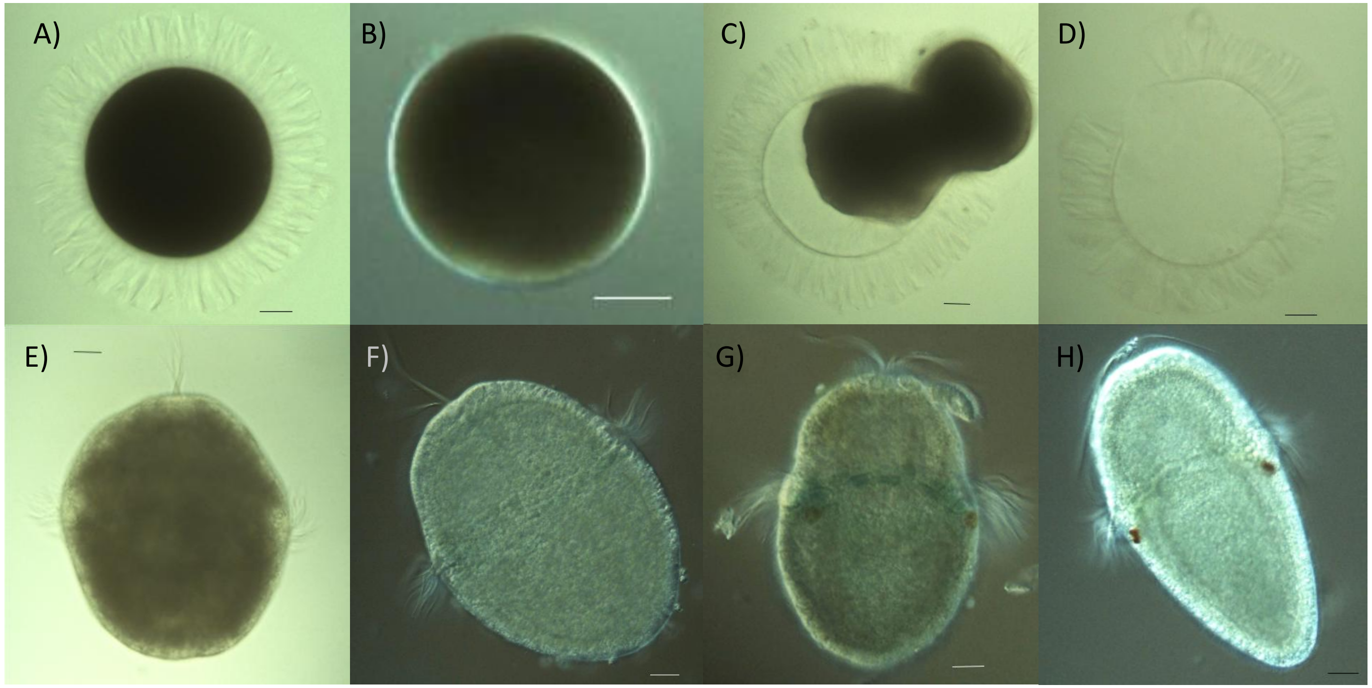

Fig. 1