Age determination of larval strombid gastropods by means of growth increment counts in statoliths

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The queen conch, *Strombus gigas* Linnaeus, and the milk conch, *S. costatus* Gmelin, are important gastropods of the Caribbean region (Appeldoorn and Rodríguez, 1994). To age strombid larvae by means of their statoliths would be useful in order to study aspects of their larval life histories and ecology. Statoliths, like fish otoliths, are formed of aragonitic calcium carbonate deposited on a protein matrix and exhibit periodic growth increments (Radtke, 1983). Research on statolith microstructure has been limited primarily to age determination of commercially important cephalopods (e.g. Jackson, 1994). D'Asaro (1965) observed statocysts that appeared in four-day-old embryos of *S. gigas* and that were fully functional by day six; he also noted growth increments or rings on these structures. These increments were confirmed by Salley (1986). Our objective is to validate the use of statolith microstructure in *S. gigas* and *S. costatus* to provide information on age, growth, and length of larval life.

Materials and methods

Egg masses from eight *Strombus costatus* and one *S. gigas* were collected from ovidepositing females at a site 7 km south of La Parguera, southwest coast of Puerto Rico (17.92°N, 67.05°W). Egg masses were held in 75-L aquaria subjected to the natural light-dark cycle. Culture methods of Ballantine and Appeldoorn (1983) were used. Aquaria were cleaned daily, after which one liter of Tahitian *Isochrysis* (10^6 cells/mL) was added. A minimum daily sample of ten individuals was removed from each aquarium and preserved in 70% ethanol. We examined the statolith microstructure of larvae from the longest surviving cultures. Since no veligers reared in our laboratory developed through metamorphosis, we obtained preserved (5% buffered formalin, pH 8.0) *S. gigas* veligers and juveniles of known age from the Trade Wind Industries' hatchery in the Turks and Caicos Islands.

Preserved veligers were examined with a dissecting microscope, their larval shell length (apex to siphonal canal) was measured, and their shell removed. A drop of 60% solution of alizarin red in glycerin was added to increase contrast between stained soft tissues and the unstained statolith. Coverslips were added, sealed with Permount, and samples were inspected under a compound scope at 1,000×. Statolith diameters were measured with an ocular micrometer. Increments on statoliths were counted by focusing up and down through the statolith. For each day of age, counts were made from one statolith from each of 20 veligers; all counts were made by the same reader in a blind manner. General physical structure was observed and described, with emphasis placed on the periods preceding and following hatching, and, for *S. gigas* juveniles, preceding and following metamorphosis to determine the presence and nature of any transitional marks associated with these events.

Statolith diameter, number of growth increments, and shell length were averaged for each day and arranged with age (in days after hatching). Linear least-squares regressions were calculated to determine the relations among these three variables. To determine the precision (or reproducibility) of counts of increments (i.e. verification, Wilson et al., 1983), repeated counts for both statoliths were made on subsamples. The number of increments in these representative samples were counted three separate times (double-blind), and the results were averaged for individual veligers. Standard deviations (SD) were calculated for each individual; standard error of the means (SE) was calculated as appropriate. A nested analysis of variance (ANOVA) was done for each species to determine if variability in incremental counts was due to errors in measurement or to natural variability in increment deposition (Sokal and Rohlf, 1981). Data were grouped at four levels: 1) all

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individuals across age groups, 2) between individuals within age groups, 3) between statoliths within individuals, and 4) within statoliths.

For one culture of eight-day-old veligers of *S. costatus*, feeding was suspended for two consecutive days to induce change in statolith structure and to determine if changes in feeding regimen affected increment deposition. Statolith-increment numbers and statolith diameters (n=10) were compared with those of nonstarved larvae (n=10) of equivalent age with two-sample t-tests (Sokal and Rohlf, 1981).

Results

Statoliths from *Strombus costatus* and *S. gigas* veligers were similar in size, shape, and pattern of increment formation and were translucent in appearance. They have either a circular or elliptical appearance from a longitudinal view and a biconvex structure in transverse section. On the basis of changes in size and shape, three regions are apparent (Fig. 1). At the very center is a primordial granule, around which all other increments grow; newly hatched veligers show five increments (including the primordial granule). This five-increment region (region 1) is quite distinct because of its lighter color, greater width, and seemingly dome-like nature. Prehatching increments in *S. costatus* and *S. gigas* had mean widths of 1.11 µm and 1.22 µm, respectively. Deposited around region 1 is a second, slightly darker, region. Thinner and smaller in width, this region (region 2) is composed of increments formed between hatching and completion of metamorphosis. Increment widths corresponding to the first day after hatching averaged 0.33 µm for both *S. costatus* and *S. gigas*; over the first six days after hatching the average increment width was 0.24 µm for both species. A third region (region 3), observed only in juveniles of *S. gigas*, appears immediately after completion of metamorphosis. A darker band visible at the outer edge of region 2 results from the even smaller spacings between five or six increments deposited just before metamorphosis. Increments corresponding to the last day before metamorphosis (age 20 days) measured 0.09 µm on average, whereas increments corresponding to the first day after metamorphosis had a mean of 0.43 µm. Region 3 appears lighter than region 2, because of the wider increments.

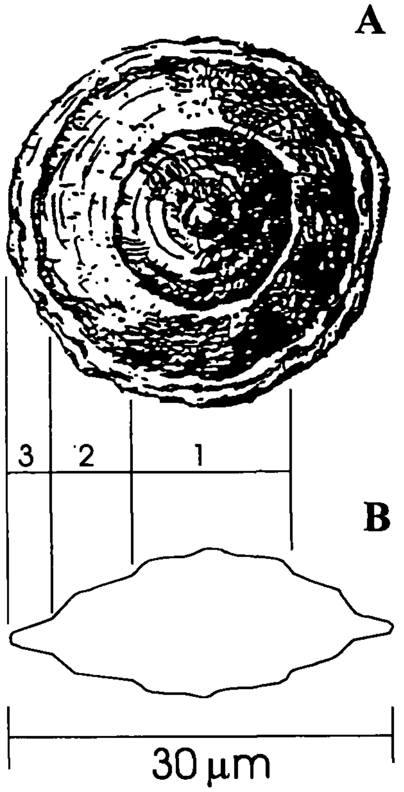

Figure 1

Generalized structure of *Strombus* statoliths: (**A**) longitudinal view; (**B**) inferred transverse view. Numbers identify the three morphological regions: 1 = prehatching, 2 = planktonic larva, and 3 = benthic postmetamorphic juvenile.

In both species, most veligers hatched on the sixth day after egg mass deposition, defined as age 0. The mean number of increments on the first day after hatching was 6.00 for *S. costatus*, 6.70 for *S. gigas* (Table 1). Statoliths in *S. costatus* show a deposition pattern of 1.11 increments/day (SE=0.59) over days 0–11; in *S. gigas* the deposition rate was 1.13 increments/day (SE=0.59) over days 0–9 (Table 1). In *S. gigas* this pattern continued until metamorphosis. For the day preceding metamorphosis (age 20 days), mean number of increments was 26.05; two days after metamorphosis (age 23 days) mean increment number was 29.40 (Table 1).

Significant regressions ($P<0.05$) were found between age (A, in days) and mean increment number (I) (*S. costatus*: $I = 6.08 + 0.986A$, r^2=0.98; *S. gigas*: $I = 6.17 + 0.984A$, r^2=0.99; data from Table 1). For analysis of *S. gigas*, we pooled locally-reared larvae with those brought from the Turks and Caicos. Separate regressions for these two sets of larvae were not significantly different. In the regression equations for both species, the slope did not differ from 1 significantly and the intercept did not significantly differ from 6 (t-test, $P<0.05$). Thus, the equations can be generalized to predict strombid veliger age from the number of statolith increments: $A = I - 6$.

Table 1

Mean statolith diameter (μm ± SD), mean number of increments (± SD) and mean shell length (μm ± SE) by age (days after hatching) for *Strombus costatus* and *S. gigas*. n = 20 for each day. META = day of metamorphosis, data not available.

	Strombus costatus			*Strombus gigas*		
Age (d)	Statolith diameter	Number of increments	Shell length	Statolith diameter	Number of increments	Shell length
0	13.35 ± 0.48	6.00 ± 0.65	345.00 ± 32.60	14.65 ± 0.80	6.70 ± 0.73	325.00 ± 35.36
1	14.05 ± 0.22	7.06 ± 0.64	390.00 ± 13.69	15.05 ± 0.59	7.30 ± 0.66	375.00 ± 40.82
2	15.30 ± 0.56	8.80 ± 0.70	440.00 ± 13.69	15.70 ± 0.71	7.25 ± 0.79	387.50 ± 17.68
3	15.50 ± 0.50	8.75 ± 0.50	470.00 ± 18.71	16.45 ± 0.50	8.00 ± 1.03	408.25 ± 14.43
4	15.93 ± 0.46	9.57 ± 1.28	625.12 ± 17.83	—	—	—
5	—	—		16.35 ± 0.51	10.72 ± 0.46	443.00 ± 18.32
6	16.14 ± 0.64	11.71 ± 0.73	625.80 ± 21.77	17.40 ± 0.97	12.35 ± 1.26	458.25 ± 28.87
7	15.65 ± 0.91	12.65 ± 1.04	635.00 ± 15.69	17.15 ± 0.57	13.45 ± 1.28	461.35 ± 15.22
8	16.50 ± 0.67	14.85 ± 0.99	641.75 ± 30.28	—	—	—
9	—	—		18.10 ± 0.30	15.55 ± 2.63	466.75 ± 14.43
10	16.35 ± 0.57	15.15 ± 2.03	650.00 ± 14.42	17.93 ± 0.46	17.07 ± 1.59	475.80 ± 13.67
12	17.33 ± 1.55	18.33 ± 1.15	700.67 ± 24.32			
19				27.60 ± 0.82	24.35 ± 0.75	1160.00 ± 31.75
20				27.90 ± 0.55	26.05 ± 0.83	1177.50 ± 40.00
21				META	META	META
22				28.15 ± 1.39	26.75 ± 4.28	1340.00 ± 44.50
23				30.45 ± 1.10	29.40 ± 1.10	1655.00 ± 83.25

Individual statolith increment counts and the magnitude of corresponding standard deviations (Tables 2 and 3) indicate that observed variability may be due to errors in measurement, not to variability in increment deposition. Nested ANOVA of subsamples of each species supported this hypothesis (Table 4), showing significant variability only at the level of readings between age groups.

Within each species, the relation between age (A) and statolith diameter (D, μm) was more variable than that between age and increment count (*S. costatus*: $D = 14.21 + 0.26A$, $r^2 = 0.80$; *S. gigas*: $D = 14.93 + 0.34A$, $r^2 = 0.93$). Similarly, the relation between age and shell length (L, μm) was more variable than that between age and increment count but was similar to that between statolith diameter and age (*S. costatus*: $L = 397.6 + 29.2A$, $r^2=0.83$; *S. gigas*: $L = 356.7 + 13.7A$, $r^2=0.89$).

Significant regressions occurred between shell length and statolith diameter (*S. costatus*: $L = -1015 + 100.4D$, $r^2=0.85$; *S. gigas*: $L = -237 + 39.9D$, $r^2=0.90$). Data for *S. gigas* obtained from the Turks and Caicos hatchery did not, however, fit this relation, suggesting that 1) environmental or genetic factors influence the relative growth of these structures, or 2) the relation is not linear over the entire larval and postmetamorphic period.

Larvae of *S. costatus* subjected to starvation showed no unusual pattern in region 2; mean number of increments between seven-day-old starved (12.09 ± 0.74 SD) and nonstarved (12.65 ± 1.04 SD) larvae were not significantly different ($t_{18}=0.262$; $P>0.05$). Mean statolith diameter was also similar in the two groups (15.65 ± 0.91 SD μm for nonstarved larvae; 14.83 ±0.75 SD μm for starved ones) ($t_{18}=1.414$; $P>0.05$).

Discussion

Statoliths of strombid larvae have a distinctly recognizable structure at hatching. Region 1 consists of a primordial granule surrounded by four increments. Three regions within the statolith result from changes in density of increments caused by abrupt changes in increment width at times of hatching and metamorphosis. Large transitions in incremental width may be caused by differences in metabolism during normal larval growth and development, including variable mineral deposition in the larval shell (Maeda-Martínez, 1987).

In both *S. costatus* and *S. gigas*, the rate of increment formation is constant after hatching. Variability found in incremental deposition was due largely to errors in measurement, not variation in depositional rate. A two-day period of starvation did not produce any noticeable structural change or precise mark. Starved veligers may have continued growing on stored energy reserves (Rodríguez Gil, 1995), negating differences between treatments. That starved veligers still produced statolith rings without structural change implies that age estimates of veligers from statolith increment counts are robust. Periodicity of statolith growth in larval *S. costatus* and *S. gigas* is sufficiently reliable to be considered a better tool for age determination than diameter of statolith or measurement of shell length. Counts of increments were measurably less variable with age than were measurements of statolith diameter, or shell length.

In fishes, otoliths have been used to address questions regarding larval dispersal (Thresher and Brothers, 1985); settlement dynamics (Victor, 1983); growth rates (Radtke and Dean, 1982); mortality rates (Essig and Cole, 1986); and larval patch-size estimation (Victor, 1984). Statolith-based age and growth determination, however, has not been used in early life history studies of mollusks; characteristics such as size, shell structure, and distance offshore have been used to infer relative age or length of planktonic life (e.g. Scheltema, 1978; Jablonski and Lutz, 1980, 1983; Pechenik et al., 1984; Pechenik, 1986). Our results here indicate a more highly quantitative method for ageing larvae.

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Table 2

Variability in statolith increment counts within laboratory-reared larval *Strombus costatus*. Age = days after hatching.

	Statolith A					Statolith B				
	Counts					Counts				
Age (d)	1	2	3	Mean	SE	1	2	3	Mean	SE
0	6	6	6	6.00	0.00	6	5	5	5.33	0.58
0	6	6	6	6.00	0.00	5	5	5	5.00	0.00
0	6	7	7	6.67	0.58	7	7	6	6.67	0.58
(average)				6.22	0.44				5.67	0.87
1	6	6	6	6.00	0.00	7	7	7	7.00	0.00
1	7	7	8	7.33	0.58	8	6	8	7.33	1.15
(average)				6.67	0.82				7.17	0.75
2	8	8	9	8.33	0.58	9	9	9	9.00	0.00
2	10	8	9	9.00	1.00	9	9	9	9.00	0.00
2	9	9	9	9.00	0.00	10	10	8	9.33	1.15
(average)				8.78	0.67				9.11	0.60
4	10	10	10	10.00	0.00	10	9	9	9.33	0.58
6	11	12	11	11.33	0.58	12	13	12	12.33	0.58
6	11	11	11	11.00	0.00	11	12	11	11.33	0.58
(average)				11.17	0.41				11.83	0.75
7	10	10	11	10.33	0.58	11	11	12	11.33	0.58
7	13	11	13	12.33	1.15	12	10	13	11.67	1.53
7	14	14	12	13.33	1.15	15	13	13	13.67	1.15
7	14	14	15	14.33	0.58	15	13	13	13.67	1.15
(average)				12.58	1.73				12.58	1.51
8	15	14	15	14.67	0.58	14	13	16	14.33	1.53
8	13	15	14	14.00	1.00	12	14	13	13.00	1.00
(average)				14.33	0.82				13.67	1.37
10	15	14	14	14.33	0.58	16	15	15	15.33	0.58
10	14	16	15	15.00	1.00	15	14	16	15.00	1.00
(average)				14.67	0.82				15.17	0.75

Table 3

Variability in statolith increment counts within laboratory-reared larval *Strombus gigas*. Age = days after hatching.

	Statolith A					Statolith B				
	Counts					Counts				
Age (d)	1	2	3	Mean	SE	1	2	3	Mean	SE
0	6	6	6	6.00	0.00	6	6	7	6.33	0.58
0	8	8	7	7.67	0.58	8	8	7	7.67	0.58
0	7	7	7	7.00	0.00	7	6	7	6.67	0.58
(average)				6.89	0.78				6.89	0.78
1	8	7	7	7.33	0.58	8	7	6	7.00	1.00
1	8	7	7	7.33	0.58	9	8	7	8.00	1.00
1	7	8	7	7.33	0.58	8	8	8	8.00	0.00
(average)				7.33	0.50				7.67	0.87
2	8	6	7	7.00	1.00	7	7	7	7.00	0.00
2	7	7	6	6.67	0.58	7	6	7	6.67	0.58
(average)				6.83	0.75				6.83	0.41
3	7	7	6	6.67	0.58	7	7	7	7.00	0.00
5	11	11	11	11.00	0.00	11	11	11	11.00	0.00
5	10	11	10	10.33	0.58	10	10	11	10.33	0.58
(average)				10.67	0.52				10.67	0.52
6	13	15	13	13.67	1.15	12	14	14	13.33	1.15
6	10	11	11	10.67	0.58	11	12	12	11.67	0.58
(average)				12.17	1.83				12.50	1.22
7	14	14	12	13.33	1.15	14	12	11	12.33	1.53
7	12	12	12	12.00	0.00	12	13	13	12.67	0.58
7	14	16	15	15.00	1.00	16	13	14	14.33	1.53
(average)				13.44	1.51				13.11	1.45
9	18	17	15	16.67	1.53	16	20	16	17.33	2.31
9	16	15	17	16.00	1.00	16	18	16	16.67	1.15
9	14	14	14	14.00	0.00	13	15	13	13.67	1.15
(average)				15.55	1.51				15.89	2.20
10	16	18	17	17.00	1.00	20	20	19	19.67	0.58

Table 4

Nested ANOVA of statolith increment counts for subsamples of laboratory-reared larval *Strombus costatus* and *S. gigas*. SS = sum of squares, df = degrees of freedom, MS = mean square, P = probability of error, S = $P \leq 0.05$, NS = $P > 0.05$.

Source	SS	df	MS	F-ratio	P
Strombus costatus:					
Among age groups	6,868.3	7	981.2	103.3	$P<0.05$ S
Among individuals	171.5	18	9.5	5.3	$P>0.05$ NS
Between statoliths	68.1	37	1.8	0.2	$P>0.05$ NS
Within statoliths	1,063.4	123	8.7		
Total	8,171.3	185	44.2		
Strombus gigas:					
Among age groups	6,732.5	8	841.6	195.7	$P<0.05$ S
Among individuals	81.7	19	4.3	10.8	$P>0.05$ NS
Between statoliths	14.1	39	0.4	0.7	$P>0.05$ NS
Within statoliths	74.8	119	0.6		
Total	6,885.2	185	37.2		

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