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Published in:
Journal of Molluscan Studies

DOI:
10.1093/mollus/eyab009

Publication date:
2021

Journal of
Molluscan Studies

The Malacological Society of London

Journal of Molluscan Studies (2021) **87**: eyab009. doi:10.1093/mollus/eyab009
Published online 21 April 2021

RESEARCH NOTE

Statocyst content in Aeolidida (Nudibranchia) is an uninformative character for phylogenetic studies

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Morphological studies used to infer phylogenetic relationships rely on informative characters (Scotland, Olmstead & Bennett, 2003; Wiens, 2004). This means the characters should (1) carry some amount of phylogenetic information, (2) be specific for certain species, genera or families, and (3) not be randomly distributed. Statocysts were first described from heterobranchs in the 19th century (see review by Hoffmann, 1939) and have since been used in various morphological analyses (see Wägele & Willan, 2000). Statocysts have a spherical structure and the movement of the small, hard statoliths in these organs aids the animal's orientation in space (e.g. Pelseneer, 1894; Crofts, 1937; Hoffmann, 1939; D'Asaro, 1966). Following Wägele & Willan (2000), statoliths can be classified as otoliths (a statocyst containing a single statolith) or otoconia (statocysts containing multiple, small statoliths), as shown in Figure 1. While analysing histological slides of aeolid species, three character states were observed: the formerly used 'single otolith' and 'multiple otoconia' (Wägele & Willan, 2000), plus a third state, one characterized by both otoconia and otolith(s). Several studies have debated the usefulness of 'statocyst content' (SC) as an informative morphological character. A taxonomic pattern in the distribution of SC has not been observed (Pelseneer, 1894; Ponder & Lindberg, 1997; Wägele & Willan, 2000). Moreover, different character states of SC are known to occur even within the same genus. For these reasons, it is not clear whether SC is phylogenetically informative.

The distribution of SC may not be taxonomically correlated. It may instead simply depend on the size of the statocyst, which in turn may depend on the size of the animal, or at least the size of the head region. To test this hypothesis, we measured the diameters of statocysts and the size of the head region (i.e. head area in cross-section) of several cladobranch species. The length of the animal was not considered, as it varies strongly within families and does not directly impact the amount of space within the head that can be occupied by statocysts. Whole specimens from 34 species (see Table 1; 1 member of Proctonotoidea and 33 members of Aeolidida) were preserved in formaldehyde/seawater, embedded in hydroxyethyl methacrylate (Kulzer® 7100), cross-sectioned (2.5 μm) and stained with toluidine blue. Histological slides from the relevant area were investigated with a ZEISS Axio Imager Z2M microscope. Regions of interest were photographed with a Zeiss AxioCam HRc and the software ZEN 2012 (blue edition) provided by Carl Zeiss Microscopy GmbH (v. NT 6.1.7601 Service Pack 1, software v. 1.1.2.0). Horizontal and vertical diameters of the head region were measured using ImageJ, an open-source image-processing program (Schneider, Rasband & Eliceiri, 2012). SC was determined from the slide series. From the cross-sections, the size of the head region was estimated by calculating the area of an oval (area = $\pi \times$ ½ horizontal diameter × ½ vertical diameter). Comparing statocyst diameter to the type of statoliths it contains (otoconia, otolith and otoconia + otolith) was achieved via a Kruskal–Wallis H test followed by a Dunn's test with Benjamini–Hochberg false-discovery-rate adjustment for post-hoc analysis since the population lacks normal distribution (Q–Q plot: $r^2 = 0.68$).

Figure 2 summarizes the results for statocyst size in comparison with SC (see definition above). The Kruskal–Wallis H test indicates that at least one of the groups does have a significantly different distribution ($H = 20.26$, df = 2, $n = 34$, $P = 0.00004$) and the post-hoc analysis shows that single otoliths are found in statocysts that are significantly smaller than those containing otoconia ($P = 0.0001$) and otoconia + otoliths ($P = 0.0001$). A significant size difference between the statocysts containing multiple otoconia *vs* those with otoconia and otolith(s) could not be established ($P = 0.81$). This suggests that single otoliths only appear in the smallest statocysts with diameters ranging from 19 μm (*Embletonia pulchra*) to 37 μm (*Cuthona* sp.) (Table 1). In larger statocysts (<92 μm in *Phidiana lottini*; Table 1), both multiple otoconia and otoconia and otolith(s) were observed. Head size varied from 0.1 mm^2 in *E. pulchra* to 58.8 mm^2 in *Hermissenda crassicornis* (Table 1).

To investigate whether statocyst size correlates with head size (Fig. 3), a Wilcoxon signed-rank test was applied since the assumption of normality was violated (Q–Q plot: $r^2 = 0.78$). The results showed that statocyst size and head size do not share the same distribution; this is unsurprising, since statocysts are found inside the slug's head ($z = -5.09$, $P = 0.00001$). To evaluate possible correlation, a Spearman rank correlation test was performed

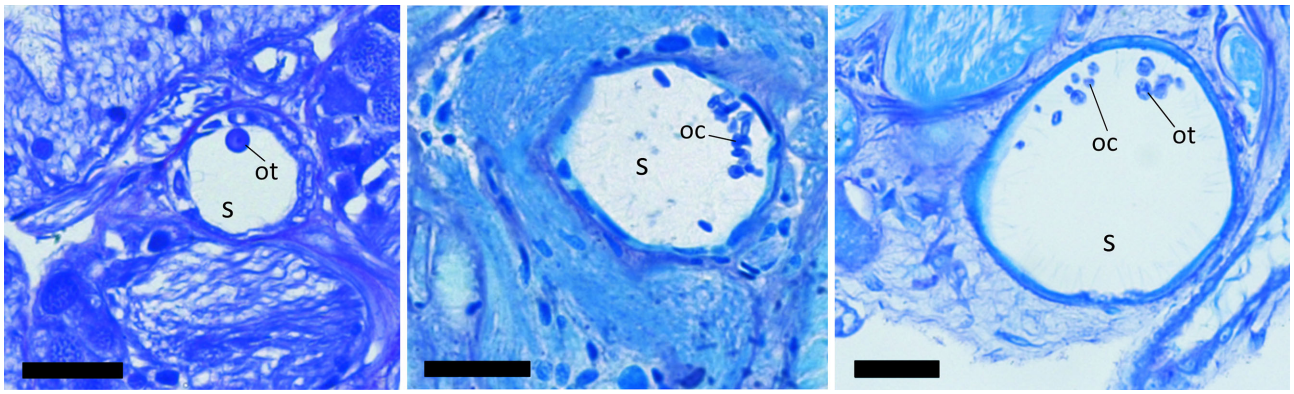

Figure 1. Histological sections of statocysts with different statolith contents. **A.** A statocyst with one otolith in *Trinchesia caerulea*. **B.** A statocyst with multiple otoconia in *Facelinopsis marioni*. **C.** A statocyst with both otoconia and otoliths in *Microchlamylla gracilis*. Abbreviations: oc, otoconia; ot, otolith(s); s, statocyst. Scale bars = 25 μm.

Table 1. Species analysed and associated measurements.

Family	Species	SC	SD (μm)	HS (mm^2)
Aeolidiidae	*Berghia stephanieae* (Valdés, 2005)	2	55	5.2
	*Bulbaeolidia alba** (Risbec, 1928)	1	29	1.3
	*Cerberilla ambonensis** Bergh, 1905	2	65	27.6
	Limenandra nodosa Haefelfinger & Stamm, 1958	2	37	1.1
	Spurilla neapolitana (Delle Chiaje, 1841)	3	63	12.8
Calmidae	*Calma glaucoides* (Alder & Hancock, 1854)	1	31	6.9
Coryphellidae	*Fjordia lineata** (Lovén, 1846)	2	64	8.6
	Microchlamylla gracilis (Alder & Hancock, 1844)	3	75	7.8
Embletoniidae	*Embletonia* cf. *gracilis** Risbec, 1928	1	23	0.3
	Embletonia pulchra (Alder & Hancock, 1844)	1	**19**	**0.1**
Eubranchidae	*Eubranchus exiguus* (Alder & Hancock, 1848)	1	30	2.0
Facelinidae	*Austraeolis ornata* (Angas, 1864)	3	68	38.5
	Caloria elegans (Alder & Hancock, 1845)	2	33	3.6
	Caloria indica (Bergh, 1896)	3	51	11.0
	Facelina rubrovittata (Costa A., 1866)	3	61	3.1
	Facelinopsis marioni (Vayssière, 1888)	2	49	2.7
	Favorinus branchialis (Rathke, 1806)	2	55	2.7
	*Moridilla jobeli** Schillo & Wägele in Schillo et al., 2019	2	58	12.7
	*Phidiana lottini** (Lesson, 1831)	2	**92**	19.6
	Pteraeolidia semperi (Angas, 1864)	3	69	11.9
Flabellinidae	*Calmella cavolini* (Vérany, 1846)	1	26	1.8
	Edmundsella pedata (Montagu, 1816)	2	60	4.3
	Flabellina affinis (Gmelin, 1791)	2	91	9.5
Glaucidae	*Glaucus atlanticus* Forster, 1777	2	39	33.6
Myrrhinidae	*Godiva quadricolor* (Barnard, 1927)	3	90	30.7
	Hermissenda crassicornis (Eschscholtz, 1831)	3	79	**58.8**
	Phyllodesmium colemani Rudman, 1991	2	75	12.4
Proctonotidae	*Janolus mokohinau* M.C. Miller & Willan, 1986	3	46	14.7
Samlidae	*Luisella babai* (Schmekel, 1972)	2	89	9.9
Tergipedidae	*Tergipes antarcticus* Pelseneer, 1903	1	30	0.6
	Tergipes tergipes (Forsskål in Niebuhr, 1775)	1	26	1.2
Trinchesiidae	*Trinchesia caerulea* (Montagu, 1804)	1	30	3.4
'Cuthonidae'	*Cuthona* sp.	1	37	8.6

An asterisk indicates juvenile individuals (identified by the state of development of the genital organs**)**. SC is represented by three states: 1, one otolith; 2, multiple otoconia; and 3, both otoconia and otolith(s). Largest and smallest SD (statocyst diameter) and HS (size of head region: rough approximation based on calculating surface area of oval) are highlighted in bold.

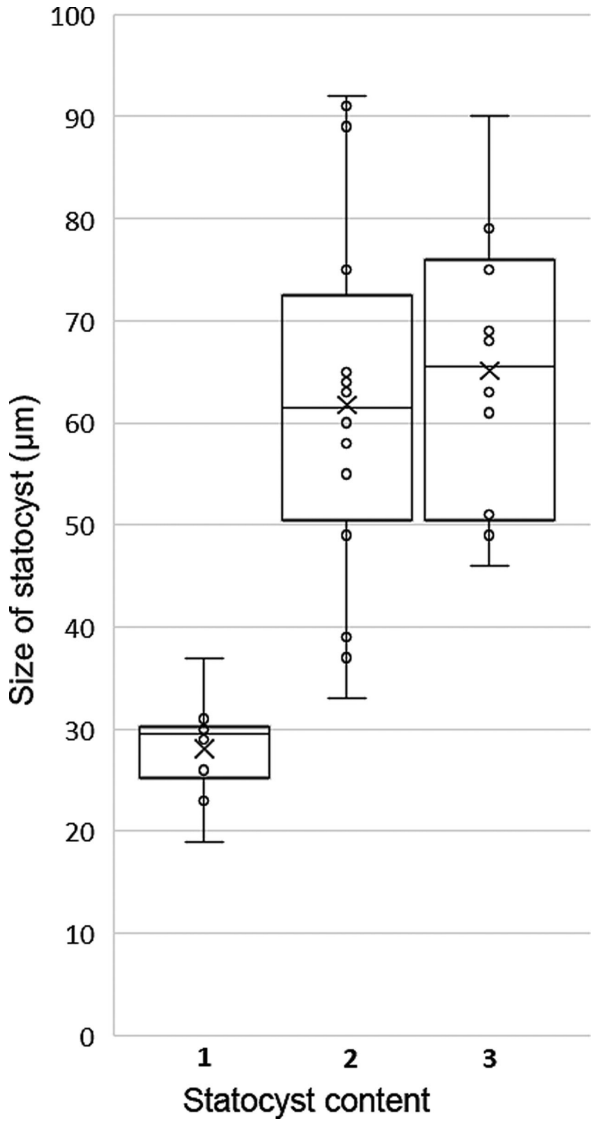

Figure 2. Boxplot of SC and statocyst size (diameter). Character states and associated sampling sizes: 1 (one otolith), $n = 10$; 2 (multiple otoconia), $n = 13$; 3 (both otoconia and otolith(s)), $n = 10$.

($r_s = -0.1174$, $P = 0.50838$). This showed that although statocyst size varies considerably and appears to increase as head size increases (Fig. 3), a correlation between these factors is not evident in this dataset. The smallest statocyst (19 µm diameter) belongs to *E. pulchra* (0.1 mm^2 head area). *Bulbaeolidia alba* and *Tergipes tergipes* show a similarly small head size (Table 1, Fig. 3).

Descriptions of nudibranch statocysts are rare, so data from the literature were deemed too inconsistent to include in this analysis. However, a few authors discuss SC as a distinguishing character to define or delimit taxonomic groups. While Risbec (1928) was the first to suggest that Aeolidiidae show statocysts with one otolith, whereas Facelinidae show statocysts with multiple otoconia, this statement is not confirmed in his descriptions. Hoffmann (1939) mentioned that within Goniodorididae (Nudibranchia) and Aeolidiidae, SC reportedly varied even within the same genus. The families Calmidae, Eubranchidae, Tergipedidae, Cuthonidae and Trinchesiidae were united in one clade in the genetic analyses of Korshunova *et al.* (2017). In our observations for this study, all specimens of these families exhibited one otolith. This is the only indication that SC might be an informative character for this clade and correlated to phylogeny; more data are needed to confirm this. We note, however, that these families consist mostly of small species, so the single otolith may also be due to the size of the animal.

The character state detailed here as 'containing both otoconia and otolith(s)' had only been described for non-cladobranch species prior to this investigation, that is two members of the Goniodorididae (Nudibranchia) (*Goniodoris castanea* and *G. joubini*; Pelseneer, 1894; Risbec, 1928), both of which are relatively large (up to 40 and 18 mm long, respectively) compared to the specimens included in our analysis (<*c.* 20 mm long).

Our results provide strong evidence that SC depends on the diameter of the statocyst. Only minute statocysts show a single calcareous element (otolith) (Risbec, 1928; Wägele & Willan, 2000), while multiple otoconia or a combination of otoconia and otoliths were found only in larger specimens. As our results indicate, SC appears to be related to head size. It is an unreliable character for phylogenetic studies because the age and life history of most specimens are unknown. Therefore, this character should ideally not be included in future morphological analyses, or if included, utmost caution should be taken and large sampling sizes used.

ACKNOWLEDGEMENT

We would like to thank the Alexander-Koenig-Gesellschaft for funding this study.

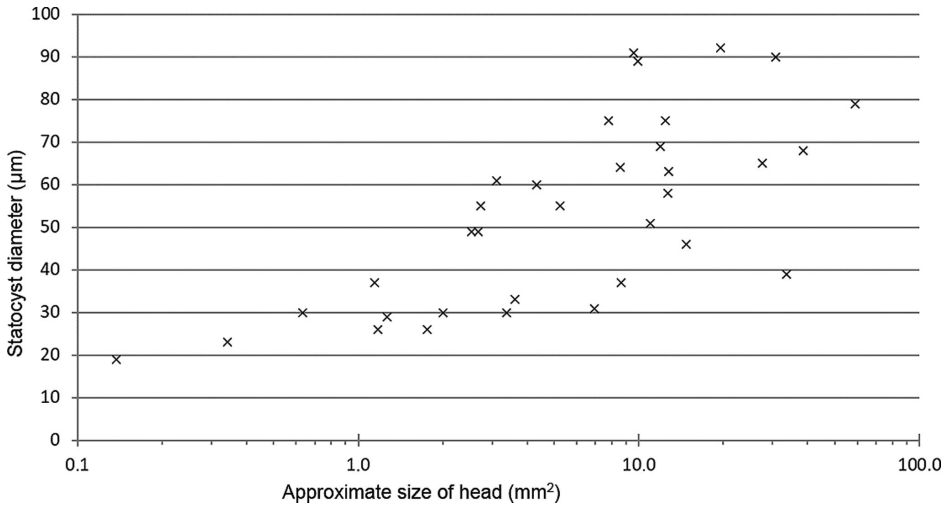

Figure 3. Comparison of statocyst size (diameter) and head size. Note the logarithmic scale of the *x*-axis.

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