STRONTIUM IS REQUIRED FOR STATOLITH DEVELOPMENT AND THUS NORMAL SWIMMING BEHAVIOUR OF HATCHLING CEPHALOPODS

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Summary

When cephalopod eggs were incubated in artificial sea water it was found that they sometimes resulted in hatchlings with defects of the statocyst suprastructure, leading to the severe behavioural defect of uncontrolled swimming. Experiments in defined media (seven basic salts mixed in deionized water) with seven species of cephalopods demonstrated clearly that there is 100 % normal development of the aragonite statoliths when strontium levels were $8\,mg\,l^{-1}$. Conversely, statoliths did not develop when strontium was absent. In cuttlefish, the growth of the cuttlebone was also affected adversely when strontium was absent. In mariculture production tanks, supplementing commercial artificial sea water with strontium to normal levels of $8\,mg\,l^{-1}$ almost eliminated the occurrence of abnormal hatchlings. Circumstantial evidence indicates that there is a critical window in development during which strontium is required for normal development. The role of strontium in biomineralization during embryogenesis is unknown, but it appears to be important in the Mollusca.

Introduction

In this paper we demonstrate a correlation between levels of the element strontium (Sr) in sea water and embryonic development of statoliths. These mineralized aragonite structures lie within the statocyst organs, which control balance for activities such as swimming and attack. Hatchling octopuses, squids and cuttlefish reared in our recirculating seawater systems since 1980 often showed behavioural defects characterized by swimming in a spinning, somersaulting or corkscrew motion; they were termed spinners (Colmers *et al.* 1984). Examination of spinners at the gross anatomical level revealed abnormal statocyst develop-
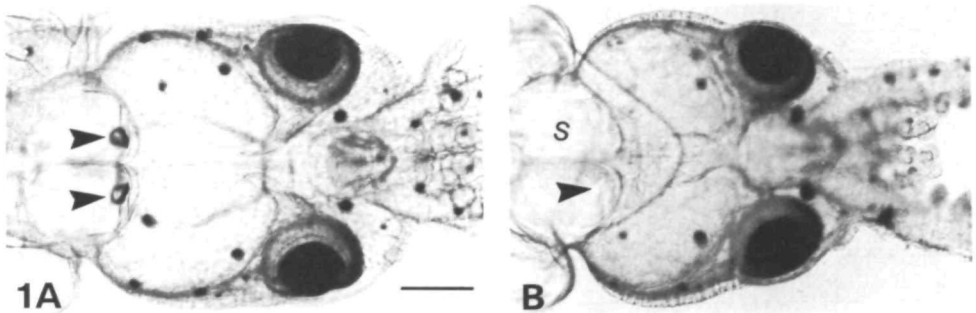
Fig. 1. Living normal (A) and spinner (B) hatchlings of the squid *Loligo pealei*. Statoliths (arrowheads) are visible on the macula statica princeps in the statocysts (*s*) of the normal animal only. Scale bar, 200 μm.

ment, namely a reduction or absence of the statoliths (Fig. 1). We were unable to identify the agent responsible, but we speculated that environmental parameters, particularly those involved in water quality, pH and temperature, might be responsible for the defects in statolith formation. In experiments since then, we noticed that the incidence of spinners was considerably higher in hatchlings reared in the commercial artificial sea water Instant Ocean® (Mentor, Ohio) than in those exposed to natural sea water (NSW) during embryogenesis. This was a significant concern since local sea water near Galveston was variable in quality and thus unsuitable for cephalopod mariculture. Strontium is a conservative element in ocean water and is present at a concentration of $90\,\mu mol\,kg^{-1}$ or about 8 p.p.m. (Bruland, 1983); recent Instant Ocean® products have lacked many trace elements, including Sr (Bidwell & Spotte, 1985).

In 1983, spinner larvae of the opisthobranch gastropod *Aplysia californica* were noted during laboratory culture at Woods Hole and it has since been reported that Sr is required in artificial sea water for normal embryonic development of both the shell and the statolith (Bidwell *et al.* 1986). Larvae cultured in the absence of Sr tumble through the water column or spin in place on the bottom. There is a dose response of mineralization to concentrations of Sr in the seawater medium as well as a critical window of exposure of this element during embryogenesis. Similarly, the bivalve molluscs *Mercenaria mercenaria* and *Bankia gouldi* require Sr for embryonic shell formation (Gallager *et al.* 1988).

Materials and methods

In the present experiments, carried out from 1984 to 1986, experimental media included four seawater types: (A) defined basal medium composed of (in $g\,l^{-1}$): NaCl, 23·88; $MgCl_2.6H_2O$, 10·68; Na_2SO_4, 4·01; $CaCl_2.6H_2O$, 1·51; KCl, 0·725; $NaHCO_3$, 0·196; NaBr, 0·086; (B) basal medium plus strontium chloride added as $SrCl_2.6H_2O$ at $0\cdot024\,g\,l^{-1}$, which is approximately equivalent to $9\cdot1\times10^{-4}\,mol\,l^{-1}$ (see Bidwell *et al.* 1986 for further details); (C) Instant Ocean® and (D) natural

sea water. Water quality of experimental cultures was monitored by measurement of ammonia-, nitrite- and nitrate-nitrogen, pH and salinity. Experimental media were refreshed periodically to ensure that water quality standards for cephalopod culture were maintained (Hanlon, 1987). Strontium levels of the water in our recirculating systems were monitored using flame atomic absorption spectroscopy. All experiments were performed at room temperature (20°C).

For experiments with the defined media (i.e. basal medium at 0 p.p.m. Sr and basal medium plus $SrCl_2$ at 8 p.p.m. Sr) cephalopod eggs were collected from our recirculating systems or from the sea, their stage of development was recorded and they were then placed in 1-l jars filled with the test artificial sea water with continuous aeration and periodic water changes. Upon hatching, animals were scored for their swimming behaviour or for statolith development using light microscopy to assess the size and number of statoliths formed.

Test organisms for the defined media experiments included *Octopus maya*, *O. vulgaris*, *O. digueti*, the cuttlefish *Sepia officinalis* and the squid *Loligo vulgaris*, thus representing the three major orders of extant cephalopods. Eggs used for these experiments were collected from freshly laid broods in our laboratory and immediately transferred to the test sea waters. The *L. vulgaris* eggs were collected from the sea off Roscoff, France (15–16°C), shipped to Galveston and placed into our recirculating systems containing Instant Ocean®. These eggs had reached embryonic stage 16 (Arnold, 1965) when transferred to the 1-l jars filled with defined media.

The efficacy of Sr supplements to Instant Ocean® for reducing the incidence of spinners in our large recirculating systems was also tested. Strontium chloride was added to culture tanks containing about 2000 l of Instant Ocean® (see Hanlon & Forsythe, 1985; Yang *et al*. 1986, for descriptions of these systems). Test organisms for these studies included the squids *Loligo vulgaris* and *L. forbesi*. Eggs from *L. vulgaris* were collected as described above. Those of *L. forbesi* were collected from Tor Bay, England, shipped to Galveston and divided between two tanks filled with Instant Ocean® or natural sea water.

Results

Results in basal medium (0 p.p.m. Sr) were clear (Table 1). All octopuses were spinners whereas those cultured in the presence of Sr (8 p.p.m.) during embryonic development swam normally. Statoliths from the *Octopus vulgaris* spinners were inspected with light microscopy and found to be irregular in shape and considerably reduced in size compared with controls; other animals were missing one or both statoliths.

Cuttlefish (*Sepia officinalis*) reared in the absence of Sr during embryogenesis were all spinners. Animals swam in a loop-to-loop pattern and bumped into the bottom and walls of the container; many rested upside down on the bottom of the jar. Some cuttlefish learned to capture mysid shrimps by extending their tentacles and grabbing food without moving from the bottom, and we have reared such

Table 1. *Effects of strontium on swimming behaviour and statolith development*

		$8\,mg\,l^{-1}\,Sr*\dagger$ (basal medium + Sr)	$0\,mg\,l^{-1}\,Sr$ (basal medium)
Order Octopoda			
Octopus maya	Normal swimmer	14	0
	Spinner	0	19
O. vulgaris	Normal swimmer	20	0
	Spinner	0	20
O. digueti	Normal swimmer	201*	0
	Spinner	0	17
Order Sepioidea			
Sepia officinalis	Normal swimmer	–	0
	Spinner	–	33
Order Teuthoidea			
Loligo vulgaris	2 statoliths	159	0
	0 or 1 statoliths	0	155

* In this particular experiment eggs were brooded with the female in the control medium and we did not attempt to remove any eggs from this brood to equalize the experimental populations.

† Artificial sea water prepared volumetrically, $8\,mg\,l^{-1}$, approximately 8 p.p.m.
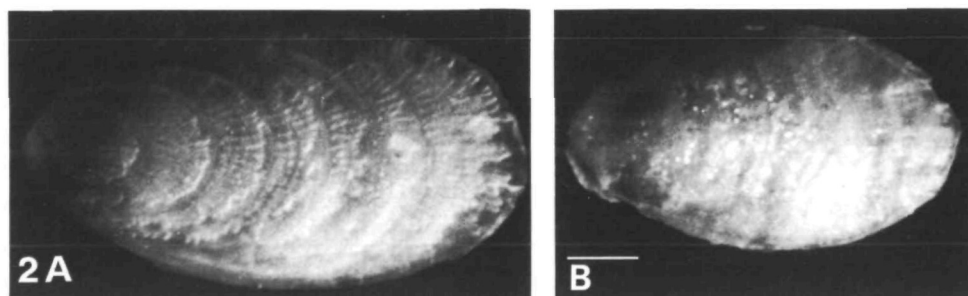

Fig. 2. Normal (A) and abnormal (B) cuttlebone of a hatchling cuttlefish *Sepia officinalis*. See text for details. Scale bar, 1 mm.

animals into the juvenile stages. All spinner cuttlefish lacked statoliths, and it is likely that the statoconia were absent also, since Budelmann (1975) showed in *Sepia* that the absence of statoliths but presence of the statoconia resulted in fairly normal compensatory eye movements needed for swimming. Each cuttlebone was also incompletely developed (Fig. 2). This structure was reduced in size compared with those from normal animals, and although the dorsal shield was formed, the flotation chambers were absent, corresponding to stage 25 of embryonic development. At the time these experiments were conducted, we lacked a sufficient number of eggs for controls of basal medium plus Sr, but comparisons have been

made with hundreds of normal hatchlings since reared in our recirculating tanks containing Instant Ocean® supplemented with Sr to 7–8 p.p.m.

Hatchlings of *Loligo vulgaris* reared in defined media were not scored on behaviour but were checked for statolith development (Table 1). Those reared in the presence of Sr during development had two normal statoliths, but all animals from the basal medium without Sr had only one statolith or lacked them altogether. Table 2 outlines the results of supplementing Instant Ocean® in our recirculating systems with strontium chloride. The majority of animals from these tanks had two normal statoliths, and the *Loligo forbesi* checked for swimming behaviour were normal.

Discussion

Our results demonstrate an irrefutable correlation between Sr in the culture medium during embryogenesis and both swimming behaviour and mineralized tissue development in coleoid cephalopods. The experiments with the defined media show that the absence of Sr results in 100 % of the test organisms being afflicted with the spinner syndrome and that the presence of this element during embryogenesis eliminates this structural and behavioural abnormality.

Supplementation of our recirculating systems containing Instant Ocean® with strontium chloride has greatly reduced the occurrence of spinners in our mariculture operation, whereas previously we had variable and typically large numbers of these defective hatchlings in our tanks (26–95 % of the initial brood, Colmers *et al.* 1984). The apparently random occurrence of large numbers of spinners in past cultures appears to have been related to the fluctuating background Sr contamination of the Instant Ocean® sea salts; levels varied widely from 0·1 to 3·7 p.p.m. but in all cases were less than 8 p.p.m. Nevertheless, other undefined factors may play a role in statolith development since spinners are occasionally still found in both our supplemented Instant Ocean® and natural seawater cultures (see Table 2; see also Hanlon *et al.* 1988). We cannot yet explain these results. It was particularly surprising that 28 of 170 (or 16 %) of the *Loligo forbesi* hatchlings (Table 2) in natural sea water were abnormal. However, it is known that standard biological, chemical and physical filtration processes in recirculating systems can remove or deplete various elements (Spotte, 1979). This line of inquiry deserves future attention.

It was beyond the scope of this study to characterize the response of cephalopod embryonic mineralization to varying Sr levels or to determine whether there is a critical window of exposure to this element. The gastropod *Aplysia californica* and the bivalves *Mercenaria mercenaria* and *Bankia gouldi* have nearly identical dose–response curves to Sr, each species requiring a minimum of 4–5 p.p.m. for normal mineralization (Gallagher *et al.* 1988). Cephalopods probably have a similar requirement. Also, the critical developmental windows of the gastropod and the bivalves occur just prior to and at the beginning of the mineralization process of statolith and shell, and therefore we are tempted to speculate that a

Table 2. *Results of strontium supplementation of Instant Ocean sea water (IO) on statolith formation in squids*

Loligo forbesi (England*)

					Number of statoliths formed		
Date	8 Dec	12 Dec	21 Jan	28 Jan–11 Feb (Hatch)	2	1	0
Egg stage	9–10			Hatch			
Medium	IO						
Sr (mgl⁻¹)	7.97†	7.19	6.70	6.30			
Results					150	0	0
Medium	NSW						
Sr (mgl⁻¹)	7.36		7.70	7.40			
Results					142	12	16

Loligo vulgaris (France*)

					Number of statoliths formed		
Date	19 Jul	26 Jul	31 Jul	4–27 Aug (Hatch)	2	1	0
Egg stage	9–10	16	24	Hatch			
Medium	IO						
Sr (mgl⁻¹)	3.73‡	5.59	7.36†				
Results					673	2	27
Date				8–13 Aug (Hatch)			
Medium	IO						
Sr (mgl⁻¹)	3.73‡	3.52	7.96†				
Results					293	10	67

Time →

Natural sea water (NSW) was not supplemented with strontium.

Each of the four horizontal lines represents a separate recirculating seawater system with IO or NSW.

* Origins of eggs before placement in seawater media; these were large-scale production experiments.

† SrCl₂ supplemented on this date to result in level indicated; all numbers represent total Sr in the media in p.p.m.

‡ Sr levels present when sea salts were first mixed with deionized water.

similar window exists for cephalopods. This would occur around stages 17–22 (Arnold, 1965) when the primordium or *Anlage* of the statocyst organ appears, followed by the formation of the statoliths themselves at stage 22 (Naef, 1928; Meister, 1972; Segawa *et al.* 1988). Circumstantial evidence for this is that most embryos of *L. vulgaris* subjected to low levels of 3·73 p.p.m. Sr from stages 10 to 16, then 5·59 p.p.m. until hatching developed normal statoliths (Table 2).

Cephalopod statoliths and other mineralized hard parts contain less than 1 % Sr by weight, although Sr is a common component of other molluscan shells (Radtke, 1983; Lowenstam *et al.* 1984). Despite the wealth of data on Ca/Sr ratios in molluscan and other tissues (Lowenstam, 1964; Weiner *et al.* 1983), the physiological significance of the requirement for Sr during the biomineralization process remains to be elucidated. Strontium is obviously important and has been used to mark growth rings in squid statoliths (Hurley *et al.* 1985). A review of the pertinent literature on Sr in molluscs is given by Bidwell *et al.* (1986).

In our original report (Colmers *et al.* 1984) we noted the similarities in the behavioural as well as the anatomical deficits between cephalopods and manganese-deficient mice (Erway *et al.* 1986). Both organisms display ataxic behaviour along with abnormal statolith or otoconia formation. The more detailed work with *Aplysia californica* and Sr (Bidwell *et al.* 1986) indicates that there are parallels between the mammalian and invertebrate phenomena, particularly with respect to the timing of the critical windows of exposure and the dose responses to the respective metal ions (Hurley & Everson, 1963; Erway, 1984; Erway *et al.* 1986). Manganese is required for enzyme systems involved in mucopolysaccharide synthesis of the cartilage in both birds and mammals (Erway *et al.* 1986). Although the chemical composition of the molluscan organic matrix of shell and statoliths has not been described in detail, mucopolysaccharides are a component of molluscan tissue (Weiner *et al.* 1983). Perhaps the Sr system in marine invertebrates is similar to the manganese system operative in mammals.

Our finding that Sr supplementation of Instant Ocean® artificial sea water reduces the incidence of spinners removes a major impediment to our cephalopod mariculture operation. A primary objective of our programme is to culture large numbers of cephalopods under controlled conditions for biomedical research. Ironically, the discovery of a technique for producing a statolith-less cephalopod may prove useful to the biomedical community. We have reared the statolith-less cuttlefish and octopuses to the juvenile stage, and these organisms continue to manifest ataxic behaviour. Such experimental preparations have the potential of providing neurobiologists with useful comparative models.

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