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MINERAL REGENERATION BY SERPULID POLYCHAETE WORMS [1]

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Serpulid polychaete worms live in tubes which are constructed of an admixture of crystalline calcium carbonate and a mucopolysaccharide matrix material. Two glandular tissues participate in the secretion of the mineral and organic material of the tube. These are the calcium-secreting glands and the ventral shield epithelium (Swan, 1950; Hedley, 1956a, b; Vovelle, 1956). There are two calcium-secreting glands situated one on each side of the mid-ventral line embedded in the subepithelial connective tissue of the anterior peristomium under the fold of the collar. The ventral shield is a glandular epithelium surrounding the openings of the calcium-secreting glands on the surface of the ventral peristomium.

Several investigators have shown that the growth of the tubes of several species of serpulids can be very rapid (Hargitt, 1906; Harms, 1912; Dons, 1927; Fischer-Piette, 1937; Hill, 1967). However, no estimates have yet been published of the rate of $CaCO_3$ secretion by the calcium-secreting glands.

If carefully removed from their tubes and placed in sea water, many species of serpulids will within a few hours begin to secrete concretions of calcium carbonate from the calcium-secreting glands (Robertson and Pantin, 1938; Thomas, 1940; Swan, 1950; Vuillemin, 1954; Vovelle, 1956). These concretions have been called the mineral regenerate in the present investigation. The ventral shield epithelium probably does not contribute much to the formation of the mineral regenerate. Thus, the mineral regenerate can be used as a relatively precise indicator of the secretory activity of the calcium-secreting glands.

Two species of serpulids, *Hydroides brachyacantha* and *Eupomatus dianthus*, were used in the studies reported here, to examine (1) the ability of worms to secrete mineral regenerate at different salinities; (2) the rate of mineral regenerate production at different salinities; (3) the effect of the size (age) of the worm on the rate of mineral regenerate production; and (4) the relationship between the concentration of calcium in the tissues of the worm and the rate of mineral regenerate production.

[1] This work represents part of a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Duke University. The experiments with *Hydroides brachyacantha* were conducted at the Instituto Oceanographico of the Universidade de São Paulo, at São Paulo and Ubatuba, Brazil. The experiments with *Eupomatus dianthus* were conducted at the Duke University Marine Laboratory at Beaufort, North Carolina. I wish to express my sincere thanks to Dr. Karl M. Wilbur, Department of Zoology, Duke University, and Dr. Edmundo Nonato, Instituto Oceanographico, Universidade de São Paulo, for their advice and encouragement during this study. This study was supported by Public Health Service Grants 5TI DE 92-05 and DE 02668 from the National Institutes of Health.

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Materials and Methods

Hydroides brachyacantha Rioja was collected from reef-like colonies of the sabellarid polychaete *Phragmatopoma lapidosa* Kinberg near the low-water mark of the exposed sandy beach, Praia do Tenorio, near Ubatuba in the state of São Paulo, Brazil. Small pieces of the reef containing serpulid tubes were removed and placed in 2–5 gallon aquaria filled with sea water. The aquaria were maintained at room temperature (18–23° C) and were aerated. The sea water in the aquaria was changed and dead animals removed every few days. Under these conditions serpulids could be kept alive for several weeks. However, all quantitative experimental studies with *H. brachyacantha* were conducted with worms that had been collected no more than one week previously.

Eupomatus dianthus (Verrill) was collected on shells and rocks lying just below the low water mark at Pivers Island, Beaufort, North Carolina. The worms in their tubes were placed in large wooden tanks with running sea water. The mortality of worms maintained in this way was very low and they could be kept in healthy condition indefinitely.

Studies of mineral regeneration

Recently collected serpulids were removed from their tubes in such a way as to minimize damage to their delicate abdominal and collar tissues. This was done in the following manner. The posterior end of the tube was carefully broken with a fine dissecting needle. The bristles of a fine camel's hair artist's brush were then inserted through the hole in the posterior end of the tube in order to push the worm forward in the tube. Then a little more of the posterior end of the tube was broken away and the bristles inserted again. This was continued until only a short length of the anterior part of the tube remained and the worm was forced to extend its branchial crown from the anterior end of the tube. Finally, the worm was pushed back into the tube and out the broken posterior end with the artist's brush.

When removed from their tubes, the worms usually released eggs and sperm into the water. After this had stopped, uninjured worms were placed in petri dishes containing 50–100 ml full strength sea water (salinity 33–34‰), dilute sea water, or hypercalcium sea water. Dilute sea water was prepared by mixing distilled water with full strength sea water. Hypercalcium sea water was prepared by adding reagent grade $CaCl_2$ to standard sea water. The salinity of all sea-water samples was determined by the hydrometer method.

From 10 to 20 worms, all about the same size, were placed in each petri dish. The worms were allowed to regenerate mineral for 24 hours. At the end of 24 hours, the worms were examined under a dissection microscope and the relative amount of mineral regenerate which had accumulated under the collar of each worm was recorded. The mineral regenerate was carefully removed with a pair of fine forceps, rinsed in dilute NaOH solution (pH 7.5–8.5), and placed in 12-ml conical Pyrex centrifuge tubes. The mineral regenerate material from the 10–20 worms of the same size in each petri dish was pooled for each calcium determination. The worms were then removed from the sea water, blotted on absorbent ash-free filter paper, and weighed on an analytical balance to the nearest 0.1 mg. The pooled worms were then placed in 16 × 125 mm high temperature Pyrex ignition tubes

(Corning #9880) and ashed in a muffle furnace at 550–600° C for 5–8 hours. Samples of the sea water from the petri dishes were also collected for determination of calcium.

Calcium determinations

Both the mineral regenerate and the ash were dissolved in 1 ml of 1 N HCl and then brought to neutral pH by the addition of an equivalent amount of 1 N NaOH. The samples were then buffered at pH 5.6 with 1 ml of 0.2 M acetate buffer. Calcium was determined by the chloranilate method of Ferro and Ham (1957). The calcium concentration in duplicate 0.5–1.0-ml aliquots of the sea water samples was determined by the same technique without prior addition of HCl, NaOH or acetate buffer.

Mineralogy of tubes and mineral regenerate

Clean serpulid tubes free of adhering foreign matter were selected for examination by x-ray diffraction techniques. Mineral regenerate material was collected as described above for x-ray diffraction analysis. The mineral samples were rinsed

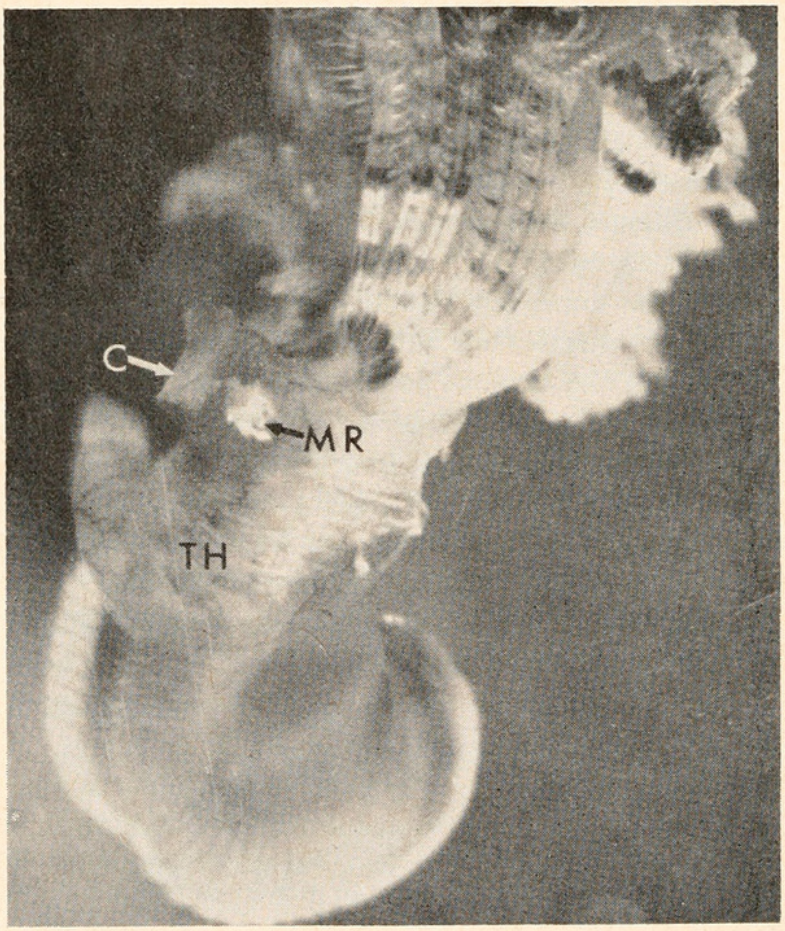

FIGURE 1. *E. dianthus* removed from its tube and allowed to regenerate mineral for about 4 hours. Side view. A small mineral concretion, the mineral regenerate (MR), lies under the fold of the collar and directly over the opening of the left calcium-secreting gland. Another concretion, not seen in this photograph, lies over the other calcium-secreting gland on the right side of the ventral peristomium. 20 ×. C, collar; MR, mineral regenerate; TH, thoracic membrane.

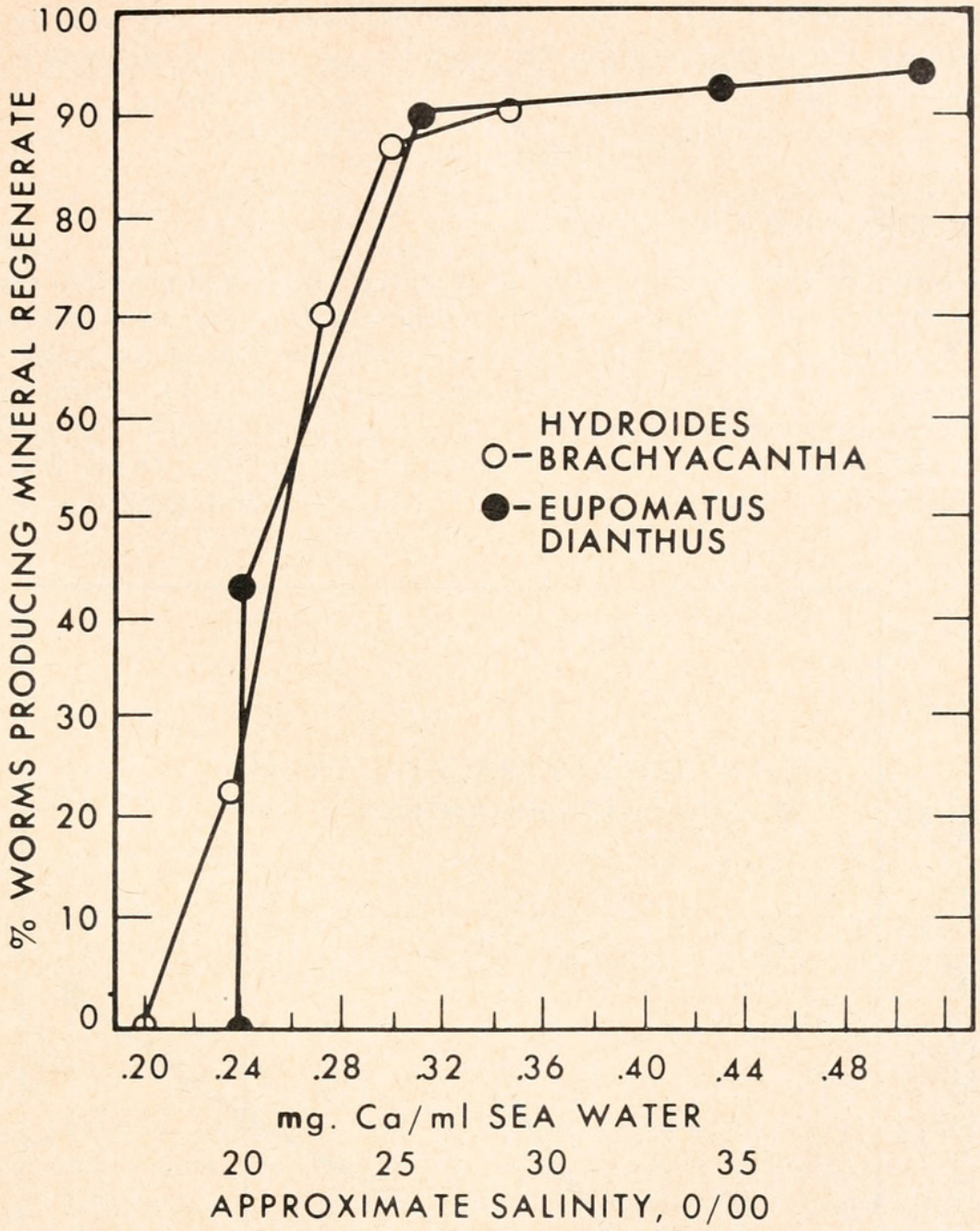

FIGURE 2. The effect of salinity on the ability of *H. brachyacantha* and *E. dianthus* to produce mineral regenerate. Regeneration ability is expressed as the percentage of worms which produced mineral regenerate within 24 hours after being removed from their tubes. In this and subsequent graphs, hypercalcium sea water (0.49 mg Ca/ml) prepared by adding $CaCl_2$ to standard sea water, has approximately the same salinity as standard sea water (34‰, 0.43 mg Ca/ml).

in several changes of distilled water and allowed to dry at room temperature. They were then ground to a fine powder with an agate mortar and pestle and placed in 0.3-mm (o.d.) lithium-borate glass capillary tubes (Lindemann Co., West Germany). X-ray diffraction pictures were taken with a Norelco 11.46 cm Debye-Scherrer x-ray diffraction camera. The x-ray source was a Norelco x-ray machine with Cu Kα radiation at 35 KV and 20 ma. Exposure time was 2–5 hours.

The presence of substituted magnesium in the calcite lattice (high Mg calcite) was estimated by the downward displacement of the 104 "d" line in the diffraction

pattern as described by Chave (1952). However, no attempt was made to quantitatively estimate the amount of Mg in the calcite or the ratios of calcite to aragonite in the mineral samples.

Results

Formation of the mineral regenerate

When serpulids were carefully removed from their tubes and placed in sea water, small concretions of mineral, the mineral regenerate, began to form within a few hours. The mineral regenerate first appeared under the fold of the collar directly over the openings of the two calcium-secreting glands (Fig. 1). With time, the mineral regenerate increased in size, first spreading laterally to fill the area under the fold of the collar and later extending posteriorly in the form of one or two thin sheets. At the end of 24 hours the mineral regenerate often extended to the poste-

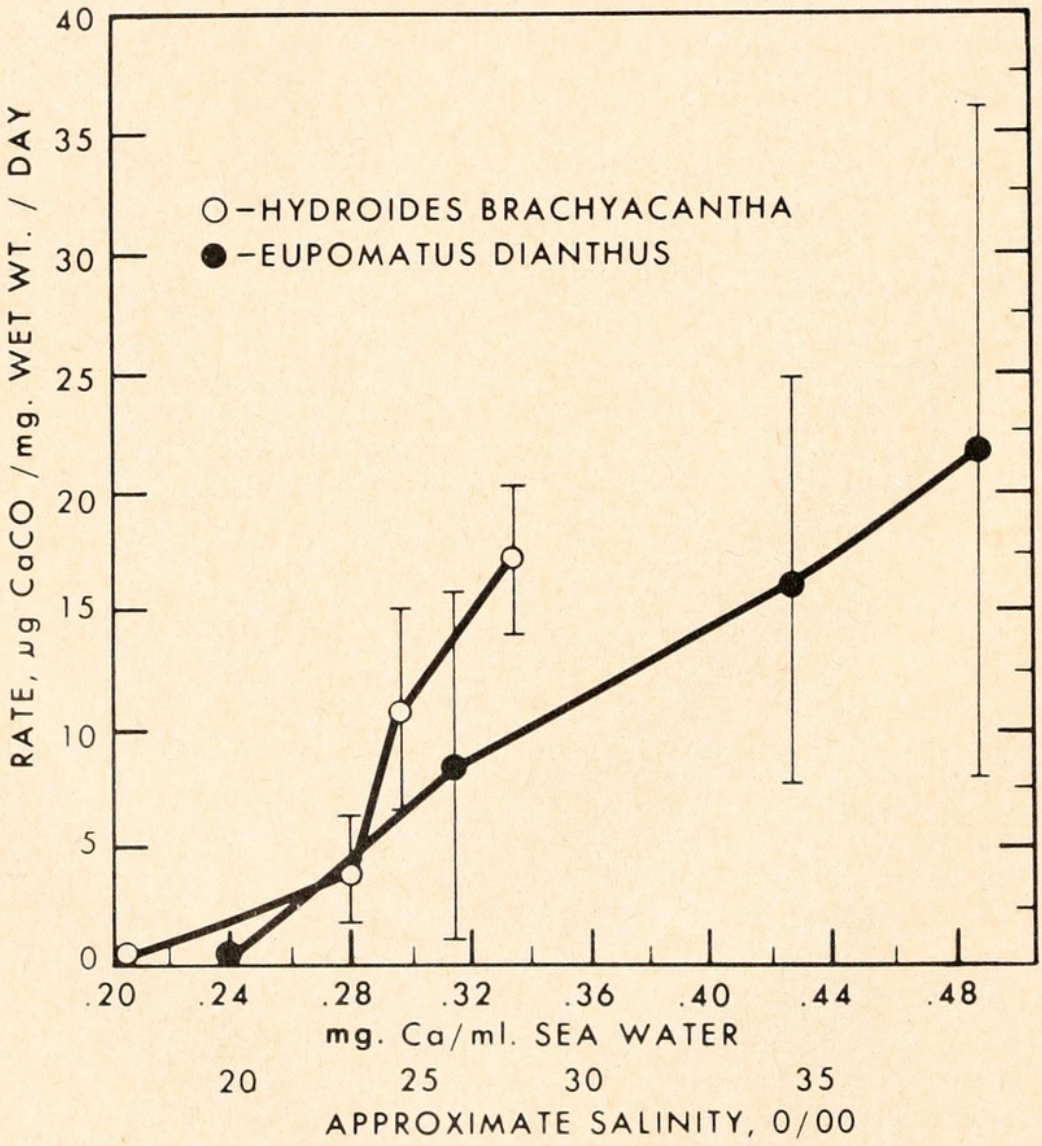

Figure 3. The effect of salinity on the relative rate of mineral regenerate production by *H. brachyacantha* and *E. dianthus*. Each point is the mean of 7 to 16 determinations on pooled samples of 10 to 20 animals each. The ranges indicate the sample standard deviations.

TABLE I

Statistical analysis of the relationship between the relative rate of mineral regenerate production by H. brachyacantha *and* E. dianthus. *"P" values are based on "student T test" for significance between means (Snedecor, 1956)*

Species	mg Ca/ml sea water (salinity)	Sample size	Mean rate μg Ca/mg wet wt/day	Sample standard deviation	*P* value
Hydroides brachyacantha	0.270 (23‰)	7	1.70	0.75	<0.01
	0.285 (25‰)	9	4.33	1.63	<0.01
	0.330 (28‰)	16	6.86	1.29	
Eupomatus dianthus	0.318 (26‰)	7	3.30	3.15	0.05
	0.430 (34‰)	16	6.30	3.17	0.25
	0.490 (34‰)	6	8.70	5.44	

rior border of the thorax. However, the only points of attachment between the worms and the mineral regenerate were at the openings of the calcium-secreting glands.

Minerology of the tubes and mineral regenerate

The tubes of *H. brachyacantha* and *E. dianthus* contained large amounts of two polymorphs of calcium carbonate, high magnesium calcite and aragonite. The tubes sometimes also contained a small amount of α-quartz which probably represented inclusions of sand grains in the mineral material of the tube. Most samples of mineral regenerate material contained only aragonite. However, a few also contained traces of high magnesium calcite. Low magnesium calcite and vaterite were not detected in the tubes or mineral regenerate material of either species.

Mineral regenerate production and salinity

The ability of serpulids which have been removed from their tubes to produce mineral regenerate is influenced by several factors. One is salinity. The ability of two species of serpulids, *Hydroides brachyacantha* and *Eupomatus dianthus,* to produce mineral regenerate at different salinities is summarized in Figure 2. Mineral regeneration ability was expressed as the percentage of individuals which produced mineral regenerate within 24 hours after being removed from their tubes. A total of 540 *H. brachyacantha* and 670 *E. dianthus* was used for these determinations. Both species of serpulids responded similarly to changes in the salinity with respect

to their ability to produce mineral regenerate. The relative success of both species in producing mineral regenerate rose steeply between salinities of about 20‰ and 25‰. At salinities above about 25‰ (corresponding to a sea water calcium concentration of about 0.30 mg Ca/ml), 88% or more of *H. brachyacantha* and *E. dianthus* produced mineral regenerate. Ninety-five per cent of *E. dianthus* in hypercalcium sea water (0.49 mg Ca/ml) produced mineral regenerate within 24 hours.

The rate of mineral regenerate production was affected by the salinity and possibly also by the calcium concentration of the sea water. The relationship between the rate of mineral regenerate production by *H. brachyacantha* and *E. dianthus* and the salinity, expressed as the calcium concentration of sea water, is shown in Figure 3. In both species, the rate of mineral regenerate production per unit wet weight of worms rose with increasing salinity. There was a significant increase in the rate of mineral regenerate production by *E. dianthus* between salinities of 26‰ (0.32 mg Ca/ml sea water) and 34‰ (0.43 mg Ca/ml sea water) ($P = 0.05$), but not between 34‰ normal sea water and hypercalcium sea water (0.49 mg Ca/ml sea water) ($P = 0.25$). In the range of salinities in which mineral regenerate production by *H. brachyacantha* was observed, the observed increases in the rate of mineral regenerate production with increasing salinity were significant ($P < 0.01$). Statistical data are summarized in Table I.

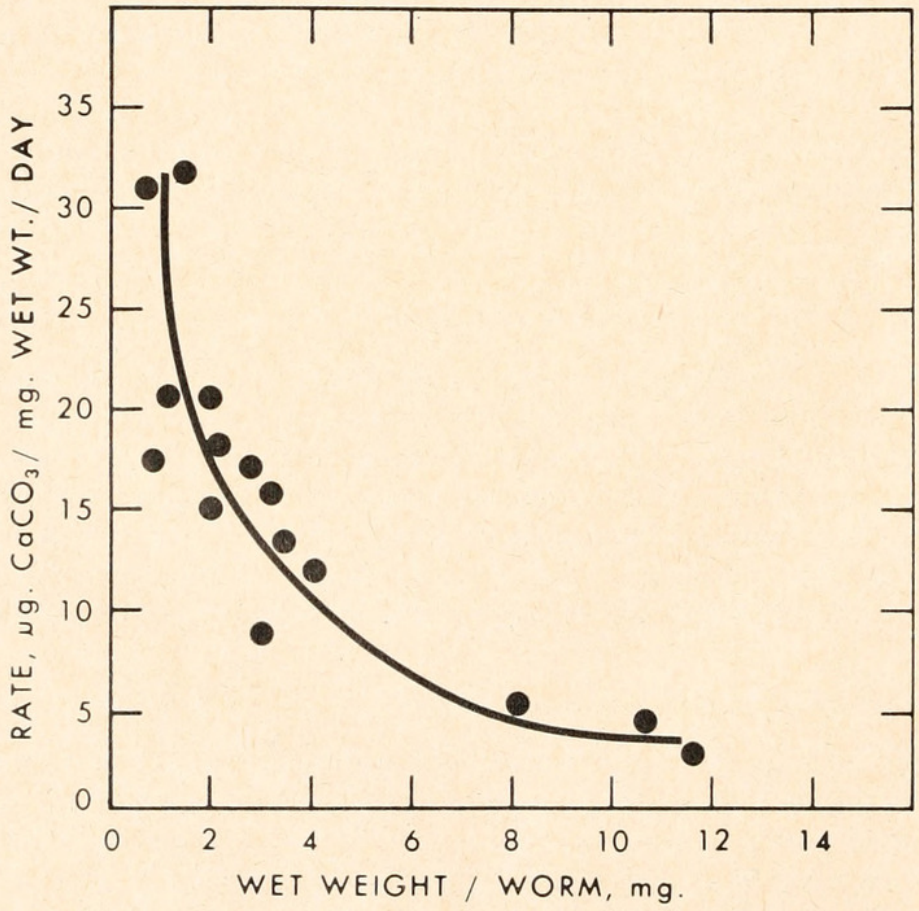

FIGURE 4. The relative rate of mineral regenerate production as a function of size in *E. dianthus* at a salinity of 34‰ (0.43 mg Ca/ml sea water). Each point represents a pooled sample of 10 to 20 individuals of approximately the same size. The average curve was fitted by inspection and approximates an exponential function.

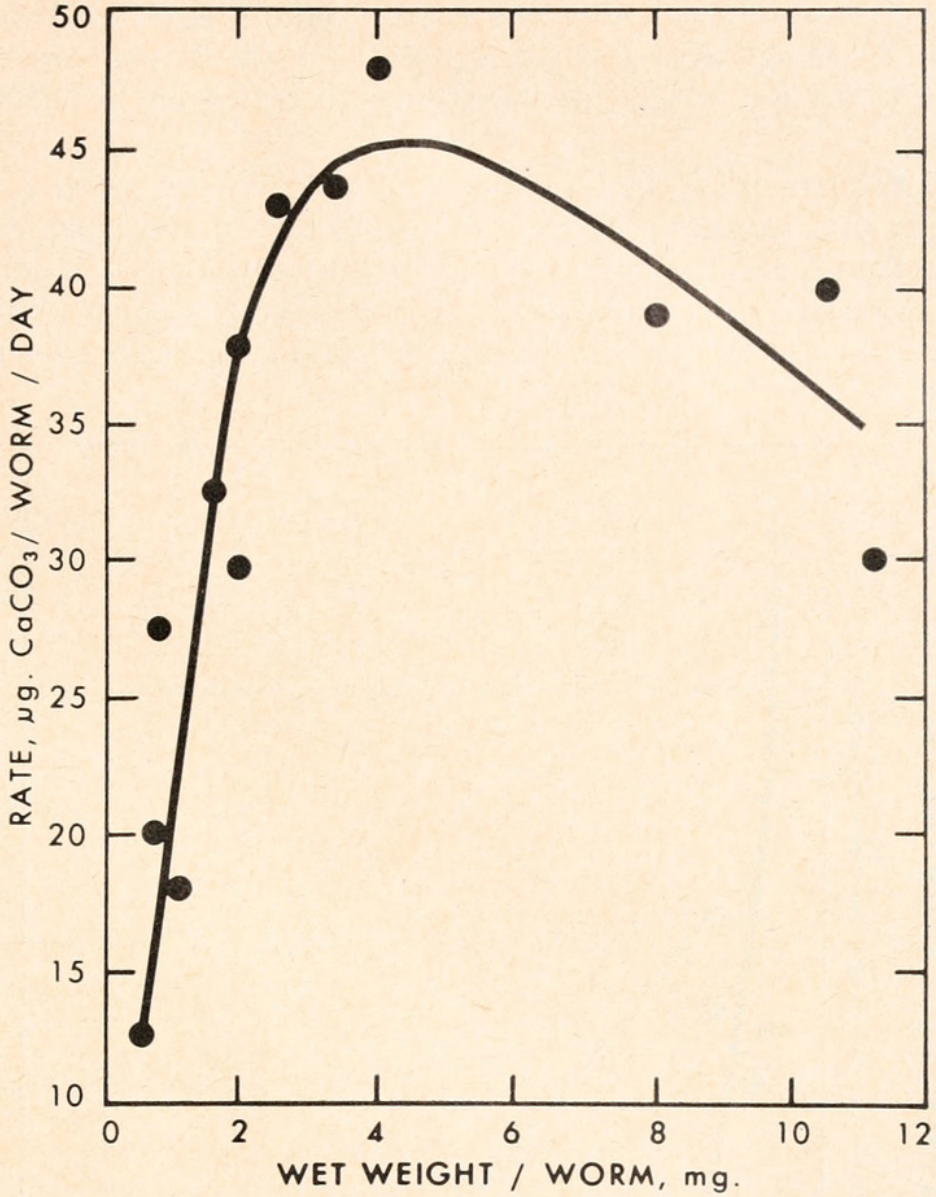

FIGURE 5. The effect of size of *E. dianthus* on the rate of mineral regenerate production per worm at a salinity of 34‰ (0.43 mg Ca/ml sea water). Each point represents a pooled sample of 10 to 20 individuals of approximately the same size. The average curve was fitted by inspection.

At each salinity there was a large variation in the rate of mineral regenerate production by both species. This variability was expressed as the sample standard deviation (Snedecor, 1956) in Table I. The rate of mineral regenerate production by *H. brachyacantha* was less variable than that by *E. dianthus* at each salinity.

Size and the rate of mineral regenerate production

Much of the observed variability in the rate of mineral regenerate production at different salinities can be attributed to differences in the rate of mineral regenerate production by worms of different sizes. Figure 4 shows the effect of size on the relative rate of mineral regenerate production by *E. dianthus* in normal sea water. The rate decreased approximately exponentially with increasing size of individuals.

Thus, the smallest worms with an average wet weight of less than 1 mg produced a maximum of 30–33 μg $CaCO_3$/mg wet wt/day, while the largest worms examined, averaging 10–12 mg wet weight, produced mineral regenerate at about one tenth this rate.

However, the rate of mineral regenerate production per worm (Fig. 5) increased sharply with increasing size and passed through a maximum at 4 mg. In worms larger than 4 mg, the rate of mineral regenerate production per worm decreased with increasing size of individuals. Worms averaging 4 mg wet weight produced nearly 50 μg $CaCO_3$/day, equivalent to about 1.25% of their body weight.

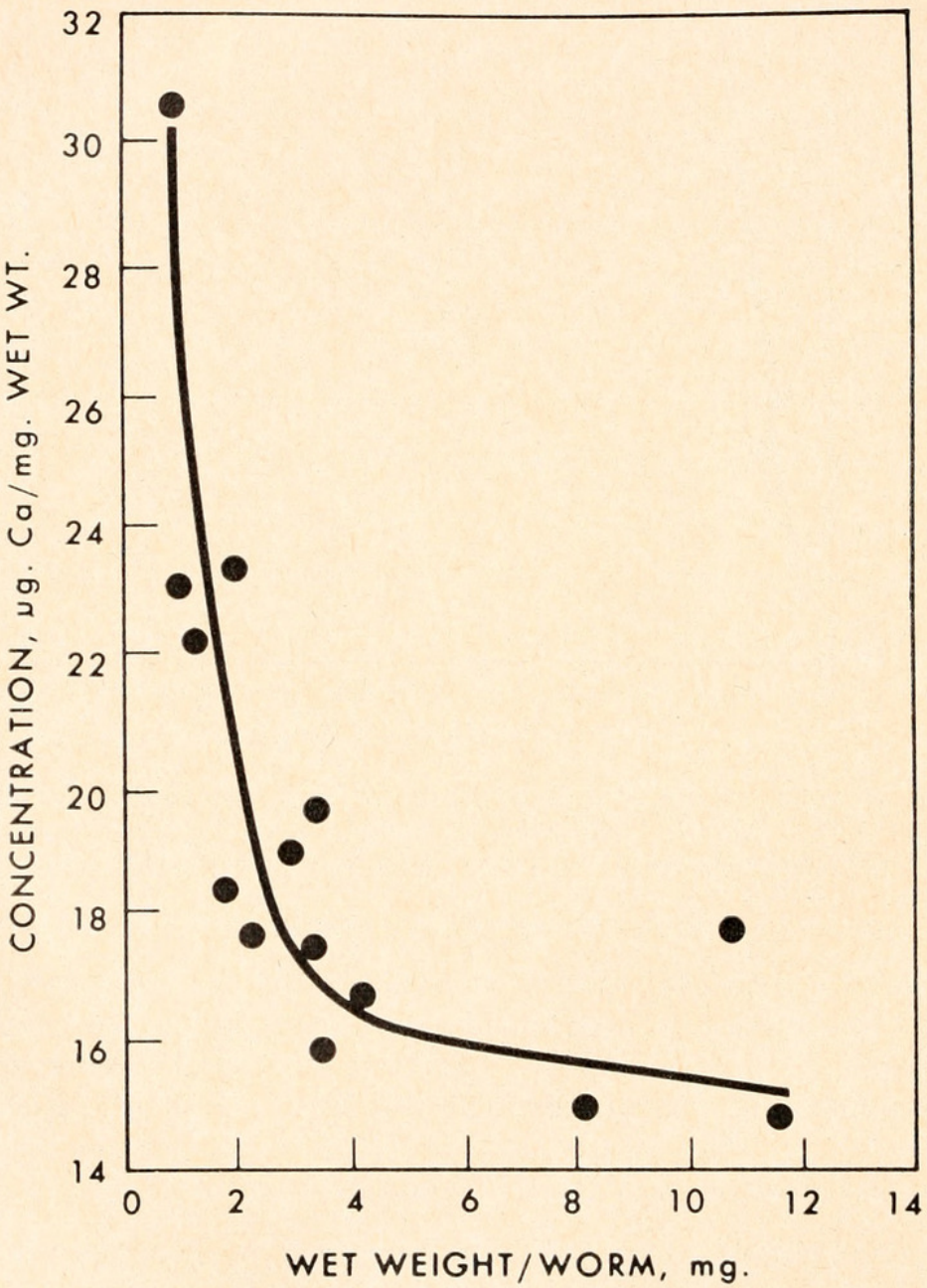

FIGURE 6. The relationship between the concentration of tissue calcium of *E. dianthus* and size. Salinity, 34‰ (0.43 mg Ca/ml sea water). Each point represents a pooled sample of 10 to 20 individuals of approximately the same size. The average curve was fitted by inspection and approximates an exponential function.

Tissue calcium in Eupomatus dianthus

There was an inverse exponential relationship between the concentration of tissue calcium and the size of the worms (Fig. 6). The concentration of tissue calcium of whole worms fell from about 3.0 μg Ca/mg wet wt tissue (75 mM Ca/kg) in the smallest worms to about 1.5 μg Ca/mg wet wt tissue (37.5 mM Ca/kg) in the largest. These concentrations of tissue calcium were very high as compared with the concentration of calcium in the tissues of most other marine invertebrates. The concentration of tissue calcium in other marine invertebrates is rarely higher than about 17 mM/kg (Prosser, 1961).

Tissue calcium and mineral regenerate production

The rate of mineral regenerate production by *E. dianthus* was related not only to the salinity of the sea water and the relative size of the worms, but also to the concentration of tissue calcium (Fig. 7). There was a linear relationship between

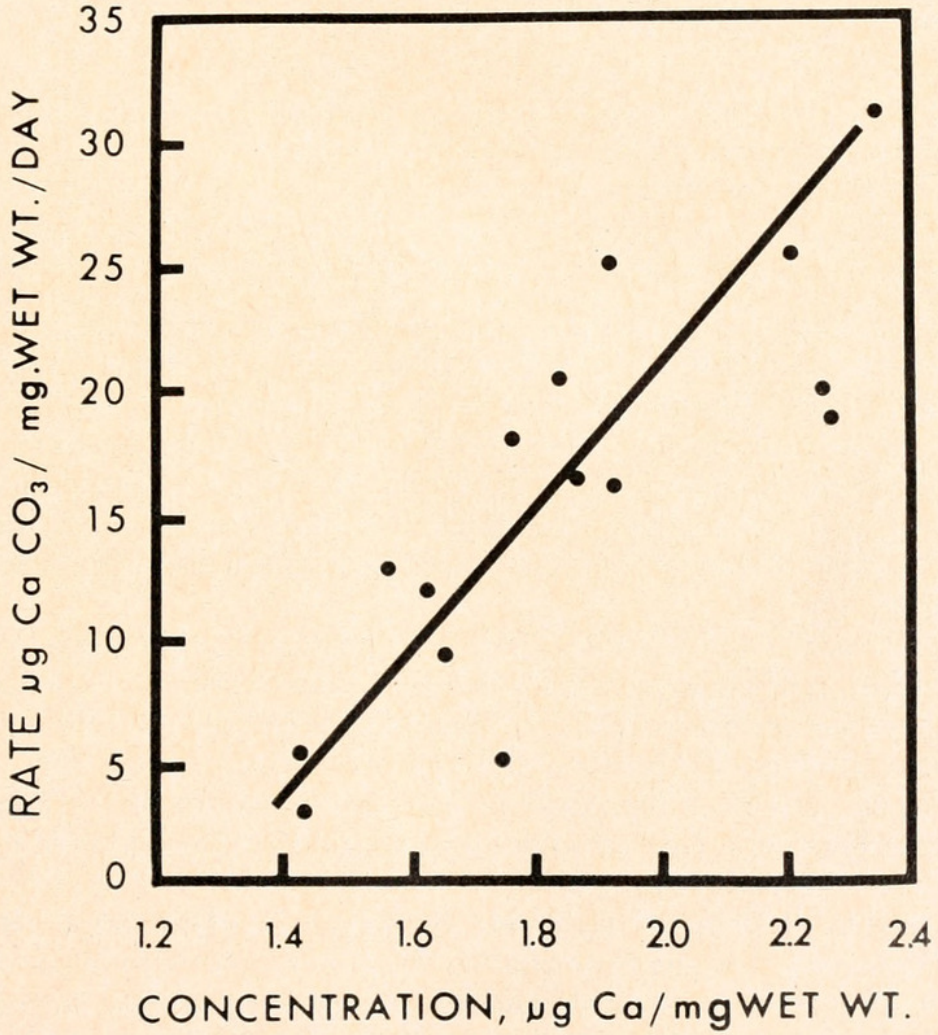

FIGURE 7. The relationship between the tissue calcium concentration and the relative rate of mineral regenerate production of *E. dianthus* at a salinity of 34‰ (0.43 mg Ca/ml sea water). Each point represents the results of a determination of both parameters on a single pooled sample of 10 to 20 worms of approximately the same size. The average curve was fitted by inspection.

tissue calcium concentration and the relative rate of mineral regenerate production. The coefficient of correlation (Snedecor, 1956) for the relationship between the relative rate of mineral production and the tissue calcium concentration was high (0.86) in worms allowed to regenerate mineral in normal sea water (0.43 mg Ca/ml sea water) (Table II). At lower and higher levels of environmental calcium, the coefficient of correlation was lower.

The ratio of the rate of mineral regenerate production per unit wet weight of worms to the concentration of tissue calcium varied between 3.34 and 4.33 (Table II) over the range of environmental calcium concentrations studied. That is, the worms secreted three to four times the amount of tissue calcium per day.

TABLE II

The relationship between the tissue calcium concentration and the relative rate of mineral regenerate production by E. dianthus *at different salinities and environmental calcium concentrations*

Calcium concentration of the sea water, mg Ca/ml	Ratio of rate of mineral production to tissue calcium concentration	Coefficient of correlation, R	Number of samples used. 10–20 worms per sample
0.318	4.33	0.64	7
0.427	3.39	0.86	11
0.490	3.34	0.68	8

DISCUSSION

The x-ray diffraction data indicate that the mineral regenerate represents only a part of the material found in the normal tube. Muzii (personal communication) found that the tube of *E. dianthus* was composed of three distinct mineral layers, an outer aragonitic layer and two inner calcitic layers. The observations of mineral regenerate production strongly suggest that the mineral regenerate material is secreted by the calcium-secreting glands alone. This conclusion is supported by the observation that the lumen of the calcium-secreting gland sometimes contained aragonite (Neff, 1967).

Hedley (1956a, b) and Vovelle (1956) found that the columnar mucocytes of the ventral shield epithelium contained a high concentration of calcium and suggested that they may secrete part of the mineral destined for the tube. The ventral shield may secrete the calcitic layers of the tubes of *E. dianthus* and *H. brachyacantha*. However, it apparently does not participate in the formation of the mineral regenerate. Therefore, mineral regeneration may be used as an indicator of the secretory activity of the calcium-secreting glands.

The production of mineral regenerate by serpulids which have been removed from their tubes may represent an attempt by the worm to produce a new tube. However, the amount of mineral regenerate produced by worms during 24 hours varied tremendously from one worm to another and not all worms produced mineral regenerate within this length of time. Vovelle (1956) noted that, in the serpulid *Pomatoceros triqueter,* the time before the first appearance of mineral regenerate varied from less than 2 to more than 24 hours. He suggested that the calcium-secreting glands underwent cyclical changes in secretory activity and the

ability of the worms to produce mineral regenerate depended on the activity stage of the calcium-secreting glands at the time the worms were removed from their tubes. Furthermore, he observed that many *P. triqueter* stopped producing mineral regenerate after about 48 hours and concluded that the calcium-secreting glands had become exhausted. However, Faouzi (1931) reported that some specimens of *P. triqueter* continued to produce mineral regenerate for as long as 40 days.

Robertson and Pantin (1938) found that *P. triqueter* was unable to produce mineral regenerate in artificial sea water containing less than 50% of the normal concentration of calcium. More extensive experiments on the relationship between sea water calcium concentration and the rate of mineral regenerate production have been presented in the present investigation.

The present observations that both species of serpulids failed to produce mineral regenerate below a salinity of about 20‰ and that above this salinity the rate of mineral regenerate production increased with increasing salinity and sea water calcium concentration lend support to the conclusion of Robertson and Pantin (1938) that serpulids utilize dissolved calcium for the construction of their tubes. There are two ways in which dissolved calcium could be utilized for the construction of the calcified tube. Calcium could be precipitated directly from sea water onto an organic matrix material after the latter is secreted by the worm. On the other hand, calcium could be taken up from the sea water by various tissues of the worm and transported to the calcium-secreting glands and ventral shield epithelium where it might be mixed with the organic matrix material before being secreted to form the tube. All the available evidence indicates that the latter scheme is the more likely. Swan (1950), using Sr^{85}, showed that at least part of the calcium destined for incorporation in the tube passed through the worm. Hedley (1956a, b) and Vovelle (1956) found high concentrations of calcium in the calcium-secreting glands and ventral shield epithelium, strongly indicating that these tissues secrete calcium as well as matrix material.

Recent studies of the ultrastructure of the calcium-secreting glands of *Pomatoceros caeruleus* (Neff, 1966, 1967) have shown that the primary secretory product of these glands in this species has the form of highly organized granules of crystalline calcite. In *Eupomatus dianthus,* microcrystals of aragonite have been observed in the upper part of the lumen of the calcium-secreting glands (Neff, 1967).

Potential sites for the uptake of calcium from the sea water have been identified in the epithelia of the anterior surface of the collar and base of the branchial crown of *P. caeruleus* (Neff, 1967, 1968). Swan (1950) and Vovelle (1956) described similar calcium-rich epithelia in the lining of the anterior gut of *Mercierella enigmatica* and *Pomatoceros triqueter* and suggested that they may function in the uptake and storage of calcium from the gut.

The non-linear relationship between salinity and both the ability of worms to produce mineral regenerate and the rate at which it is formed would be hard to explain in terms of precipitation of $CaCO_3$ directly from sea water onto the organic matrix. However, the possibility that some $CaCO_3$ is precipitated directly from the sea water onto the mineral-matrix material secreted by the worm cannot be completely discounted.

The highest rate of mineral regenerate production was observed in *E. dianthus* weighing about 4 mg. Worms of this size produced approximately 50 μg of arago-

nite per day. Hedley (1956b) described the morphology of the calcium-secreting glands of a closely related serpulid *Hydroides norvegica*. He indicated that the calcium-secreting glands were simple tubular organs with an average length of 200–250 μ and a diameter of 40–50 μ in worms probably somewhat larger than 4 mg (worms 15 mm long). Recent ultrastructural studies by the author (Neff, 1967) have shown that the calcium-secreting glands of *E. dianthus* have a similar morphology and roughly similar dimensions. The central oval gland duct has an average diameter of about 10 μ. Thus the total cell volume of each calcium-secreting gland is about $9.5 \times 10^5 \mu^3$. Each gland secretes approximately 1 μg aragonite/hour equivalent to a volume of $3.3 \times 10^5 \mu^3$. Thus, each gland secretes an amount of mineral equivalent to about $\frac{1}{3}$ its cell volume per hour.

It has been shown in the present investigation that the concentration of calcium in the tissues of *E. dianthus* was very high as compared with the concentration of calcium in the tissues of most other marine invertebrates. However, several species of marine molluscs (McCance and Shackleton, 1937) and the holothurian *Caudina* (Koizumi, 1935) have tissues with a calcium concentration as high or even higher than that in serpulids. Much of the calcium was concentrated in the tissues in the form of small mineral concretions or spicules (McCance and Masters, 1937; Koizumi, 1935). In serpulids also, much of the tissue calcium was apparently associated with discrete mineral concretions in various tissues. Hedley (1956a, 1958) observed that the secretory cells of the lumina of the calcium-secreting glands as well as the mucocytes of the ventral shield epithelium and the dorsal surface of the last few abdominal segments of *Pomatoceros triqueter* contained high concentrations of calcium. Swan (1950) and Vovelle (1956) identified calcium-storage tissues containing calcium-rich granules in the anterior intestinal epithelium, the nephridia, and the chloragosomes of *Mercierella enigmatica* and *Pomatoceros triqueter*. Granules of crystalline calcite have been identified in the secretory cells and ducts of the calcium-secreting glands of *Pomatoceros caeruleus* (Neff, 1966, 1967) and intracellular hydroxyapatite crystals have been found in the calcium-uptake tissues on the anterior surface of the collar and base of the branchial crown of *Pomatoceros caeruleus* (Neff, 1967, 1968). All these calcium-rich tissues in serpulids probably play a role in some phase of mineral secretion. Although allometric data concerning the relative rates of growth of different tissues in serpulids are completely lacking, the observed exponential decrease in the total tissue calcium concentration with increase in size of worms and the close relationship between the rate of mineral regenerate production and the concentration of tissue calcium suggest that the mass of these calcium-rich tissues does not increase as rapidly as the mass of the whole worm.

Summary

1. The tubes of *H. brachyacantha* and *E. dianthus* contained two polymorphs of calcium carbonate, high magnesium calcite and aragonite, whereas the mineral regenerate produced by both species contained only aragonite. The site of initial appearance of the mineral regenerate over the openings of the calcium-secreting glands and the presence of aragonite in the duct of the calcium-secreting gland of *E. dianthus* indicate that the aragonite of the mineral regenerate and probably also of the tube is secreted by the calcium-secreting glands. The high magnesium calcite

fraction of the tube is probably secreted by the ventral shield epithelium.

2. At all salinities in which worms were able to produce mineral regenerate the rate of mineral regenerate production was extremely variable.

3. Both species of serpulids failed to produce mineral regenerate below a salinity of about 20‰. Above this salinity the rate of mineral regenerate production increased with increasing salinity and environmental calcium concentration. However, there was not a significant increase in the rate of mineral production by *E. dianthus* between normal sea water (34‰, 0.430 mg Ca/ml) and hypercalcium sea water (0.490 mg Ca/ml).

4. *E. dianthus*, weighing about 4 mg, secreted up to 50 μg of $CaCO_3$ per day. Thus worms of this size secreted an amount of aragonite equivalent to about ⅓ of the cell volume of the calcium-secreting glands per hour.

5. There was an inverse exponential relationship between the size of worms and both the relative rate of mineral regenerate production and the concentration of calcium in the tissues of the worms, strongly suggesting that the mass of the tissues involved in mineral production did not increase in proportion to the increase in mass of the worm.

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