Radula synthesis by three species of iron mineralizing molluscs: production rate and elemental demand

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A cold-shock technique was used to determine radula production rates for the chitons Acanthopleura hirtosa *and* Plaxiphora albida, *and for the limpet* Patelloida alticostata, *which replaced their radular teeth at rates of 0.40, 0.36 and 0.51 rows d^{-1}, respectively. These rates are far slower than those determined previously for non-iron-mineralizing molluscs, suggesting that the improved working life of the teeth afforded by iron-mineralization acts to significantly reduce replacement rates. In addition, inductively coupled plasma-atomic emission spectroscopy has been used to determine the quantity of iron and other elements comprising the radula of each species. These data, used in conjunction with the radula production rates, reveal that* A. hirtosa, Plaxiphora albida *and* Patelloida alticostata *have daily radula mineralization requirements for iron of 3.06, 4.12 and 0.55 μg, respectively. Such information is vital for continuing studies related to the cellular delivery of ions and subsequent biomineralization of the tooth cusps in chitons and limpets.*

INTRODUCTION

The radula is the principal feeding organ of many molluscan species. It comprises an elongated array of transverse tooth rows that are carried forwards in a conveyor belt manner (Lowenstam & Weiner, 1989). The teeth are prefabricated as an organic matrix by odontoblast cells at the posterior end of the radula, with the overlying epithelial tissue controlling the subsequent transportation and deposition of ions onto this matrix (Mann *et al.*, 1986; Kim *et al.*, 1989). Once mature, the teeth are progressively abraded and finally lost as a result of feeding (Runham, 1963; Runham & Isarankura, 1966).

In chitons (Mollusca: Polyplacophora), each transverse tooth row bears 17 teeth, including a pair of large major laterals whose cusps are hardened with iron and calcium biominerals. Magnetite (Fe_3O_4) is the principal mineral formed within the posterior cutting face and anterior surface of the tooth cusps, while at later stages of development the tooth core is infilled with either an apatitic calcium phosphate or limonite and lepidocrocite, depending on the species (Kim *et al.*, 1989; Lowenstam & Weiner, 1989; Macey *et al.*, 1994; Lee *et al.*, 2003a, b). Limpets (Mollusca: Patellogastropoda) possess fewer teeth per transverse row than chitons, and utilize the ferric oxide goethite (α-FeOOH) to mineralize their major lateral teeth, along with silica as hydrated amorphous opal ($SiO_2 \cdot nH_2O$) and calcium (Lowenstam, 1962; Runham *et al.*, 1969; Mann *et al.*, 1986; Liddiard *et al.*, 2004). As a consequence

of this mineralization, the teeth of chitons and limpets are well suited for rasping hard surfaces, and are commonly found grazing on hard substrata such as limestone where they rake or excavate the surface, ultimately ingesting the rock in order to extract the epi- or endolithic algal components (Steneck & Watling, 1982; Macey *et al.*, 1996; van der Wal *et al.*, 2000).

The rate of radula production has been determined for a limited number of molluscs, with most studies concentrating on prosobranch and pulmonate gastropod species (Runham, 1962, 1963; Runham & Isarankura, 1966; Isarankura & Runham, 1968; Padilla *et al.*, 1996). These studies, which have utilized a number of techniques, have reported radula production rates ranging between 1.0 and 6.4 tooth rows d^{-1}. Recently, a cold-shock technique was employed to determine the radula production rate (0.36 rows d^{-1}) of the chiton *Acanthopleura hirtosa* (Blainville, 1825) (Shaw *et al.*, 2002), which is considerably slower than rates reported previously.

In order to elucidate the relative disparity between the rate determined for *A. hirtosa* (Shaw *et al.*, 2002) and those determined for other molluscs, radula production has been re-examined in *A. hirtosa* and has been investigated in two sympatric iron-mineralizing mollusc species, the chiton *Plaxiphora albida* (Blainville, 1825) and the limpet *Patelloida alticostata* (Angas, 1865). These species are key inhabitants of intertidal communities in Western Australia (Black *et al.*, 1979, 1988), and have been the focus of numerous studies in relation to tooth biomineralization (Kim *et al.*, 1989; Macey *et al.*, 1996; Lee *et al.*, 2003a; Liddiard *et al.*, 2004). Further studies on the mineralization process require a detailed understanding of the demand for elemental precursors during radula development. As such, the quantitative elemental composition of radulae from each species has also been established.

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MATERIALS AND METHODS

Radula production rates

All mollusc specimens were collected from intertidal limestone rocks within the Perth metropolitan area of Western Australia (32°S 116°E). Adult animals, as determined by size, were subjected to a cold-shock treatment (Isarankura & Runham, 1968) within 30 minutes of collection. This procedure induces a constriction anomaly in the developing radula membrane, the progression of which can be tracked after the subsequent production of new radula material. For *Acanthopleura hirtosa*, the cold-shock procedure followed that described in Shaw *et al.* (2002), where freshly collected animals were immersed for 48 h in seawater that had been pre-cooled to 4°C. For *Plaxiphora albida* and *Patelloida alticostata*, the technique was modified to account for species variation in procedure tolerance. Optimum shock parameters (seawater temperature and duration) were determined to be 4°C for 24 h and 1°C for 48 h for *Plaxiphora albida* and *Patelloida alticostata*, respectively (data not shown).

Following the cold-shock, a natural feeding regime was promoted by maintaining animals within an artificial environment approximating that of their natural habitat. This consisted of twin open-air tanks containing limestone rocks that were kept moist and partially submerged in fresh seawater, which was delivered via an overhead reticulation system at a rate of ~6.5 l. m^{-1}. During the course of each study, air and water temperatures were recorded using minimum/maximum thermometers.

Every two days a single animal was removed from each tank for immediate dissection. Following excision, radulae were cleaned in 2.5% NaOCl before observation under an Olympus SZH10 stereo dissecting microscope, where both the total number of tooth rows and the number of tooth rows occurring posterior to the constriction anomaly were recorded. Radula production rate curves were generated by plotting the number of tooth rows formed against the number of hours that had elapsed between the end of the cold-shock and dissection. As the cold-shock treatment is likely to result in a temporary slow-down of radula production (Shaw *et al.*, 2002), animals in which zero radula production was observed were discounted and the post-shock effect in the remaining animals was accounted for by justifying data points through time zero. The radula production rates calculated for individuals of each species were subsequently compared using regression analysis. The radulae of ten individuals from each species were excised from animals taken straight from the wild for comparison to cold-shocked radulae.

Elemental analysis

The radulae were excised from ten freshly collected specimens of *A. hirtosa*, *Plaxiphora albida* and *Patelloida alticostata* and cleaned using a fine jet of water. The number of tooth rows in each radula was documented, following which they were dried at 60°C for 12 h, and the sample dry weight recorded. A nitric acid with peroxide digest was then used to dissolve the radulae, followed by quantitative elemental analyses for Fe, Ca, P, S, Mg, Na, Zn, K, Al, Cu and extractable Si using inductively coupled plasma-atomic emission spectroscopy (ICP-AES) (McDaniel, 1991). Results were calculated as total µg of each element per sample, which was then converted to µg.g tissue^{-1} using the radula dry weights. The concentrations of elements in the tissues of each species were compared statistically using analysis of variance. Daily requirements were estimated by dividing the total µg of each element by the total number of tooth rows and by the radula production rates determined for each species.

RESULTS

Radula production rates

No constrictions were observed in radulae dissected from non-cold-shocked animals taken directly from the wild, indicating that constriction anomalies are attributable to the cold-shock and are not a natural part of radula formation. Radula production rates in adult individuals of *Acanthopleura hirtosa*, *Plaxiphora albida* and *Patelloida alticostata* were calculated to be 0.40, 0.36 and 0.51 rows. d^{-1}, respectively (Table 1). Regression analyses demonstrated a high positive fit for individuals within each study, with r^2 values of 0.96 ($y = 0.02x - 3 \times 10^{-5}$), 0.91 ($y = 0.01x + 3 \times 10^{-5}$) and 0.90 ($y = 0.02x + 4 \times 10^{-6}$) for *A. hirtosa*, *Plaxiphora albida* and *Patelloida alticostata*, respectively (Figure 1). The two chiton species were similar in body length, although *A. hirtosa* possessed a greater number of tooth rows per radula compared to *Plaxiphora albida*. *Patelloida alticostata* was smaller in body length than either of the chiton species but possessed the greatest number of tooth rows. The temperatures recorded during each study were similar for all species.

Elemental analysis

Iron formed the major elemental component in the radulae of each of the three species examined, with those of *A. hirtosa* and *Plaxiphora albida* containing approximately 88,500 and 98,000 µg. g^{-1} of Fe, respectively. That of *Patelloida alticostata* contained significantly less Fe at 65,000 µg. g^{-1} ($P = 0.03$ and 0.01 for *A. hirtosa* and *Plaxiphora albida*, respectively, $\alpha = 0.05$) (Table 2). Relative to the two other species, the radula of *A. hirtosa* contained significantly higher concentrations of Ca, P, Mg and Zn ($P < 0.01$, $\alpha = 0.05$ in all cases). Notably, high concentrations of S were detected from all species, although the radula of *Patelloida alticostata* contained significantly less than that of the two chitons ($P < 0.01$, $\alpha = 0.05$ in all cases). *Patelloida alticostata* also had significantly higher concentrations of Al and extractable Si than either *A. hirtosa* or *Plaxiphora albida* ($P < 0.01$, $\alpha = 0.05$ in all cases).

The daily requirements for elements involved in radula synthesis were calculated using the radula production rates determined for each species in the previous section (Table 2). The limpet *Patelloida alticostata* was shown to require 0.55 µg Fe. d^{-1}, while *A. hirtosa* and *Plaxiphora albida* required 3.06 and 4.12 µg Fe. d^{-1} for radula mineralization. A substantial daily demand for Ca, P, Mg and S was also established, especially in *A. hirtosa*. All remaining elements were typically required in amounts <0.1 µg. d^{-1}.

DISCUSSION

Radula production rates

The radula production rates of 0.40, 0.36 and 0.51 rows per day determined for *Acanthopleura hirtosa*, *Plaxiphora albida* and

Table 1. Data (± 1 SE) for *Acanthopleura hirtosa*, *Plaxiphora albida* and *Patelloida alticostata* maintained within an artificial habitat after a cold-shock treatment.

Species	Average radula production rate (rows d^{-1})	Average adult size (maximum length mm)	Average total number of tooth rows	Average seawater temperature (°C)
Acanthopleura hirtosa	0.40 ± 0.01 (N = 34)	54 ± 0.6 (N = 34)	81 ± 0.8 (N = 34)	24 ± 0.3 (N = 20)
Plaxiphora albida	0.36 ± 0.01 (N = 31)	48 ± 1.7 (N = 31)	52 ± 0.8 (N = 31)	21 ± 0.1 (N = 18)
Patelloida alticostata	0.51 ± 0.02 (N = 35)	26 ± 0.5 (N = 35)	88 ± 1.0 (N = 35)	24 ± 0.4 (N = 20)

SE, standard error.

Patelloida alticostata are among the slowest reported to date for any mollusc. Rates have been determined for 21 mollusc species (including the current study), with five being species that incorporate significant amounts of iron into their radula teeth. In a comprehensive study by Isarankura & Runham (1968), radula production rates were determined for eight prosobranch and seven pulmonate gastropod species, with production rates ranging from 1 to 6.4 rows per day, and averaging approximately 3.3 rows per day across all species. Relatively fast radula production rates have also been determined for the prosobranch molluscs *Lacuna vincta* and *L. variegata*, which produce 2.94 and 2.97 rows per day, respectively (Padilla *et al.*, 1996).

A major distinction between the species investigated in the current study, and most of those observed by previous researchers, is that *A. hirtosa*, *Plaxiphora albida* and *Patelloida alticostata* each incorporate iron biominerals into their tooth cusps. The only iron-mineralizing species investigated by Isarankura & Runham (1968) was the limpet *Patella vulgata*, which had a replacement rate of ~1.5 rows per day, comparatively slow relative to the remaining species documented in their study. Further evidence for slow rates in iron-mineralizing species has been provided by Nesson (1969), who reported a rate of between 0.4 and 0.8 rows per day for the chiton *Mopalia muscosa*, comparable to the rates determined for the species in the present study.

Research by van der Wal *et al.* (2000) on the mechanical properties of chiton and limpet teeth demonstrated that tooth hardness was dependent on the relative amounts of mineral and organic matter present in the tooth. As such, the inclusion of iron minerals in the teeth of chitons and limpets acts to prolong the working life of the teeth when

feeding on hard substrata such as calcareous limestone (Runham *et al.*, 1969; Bullock, 1989; Lowenstam & Weiner, 1989). The results determined for *A. hirtosa*, *Plaxiphora albida* and *Patelloida alticostata* suggest that the extended functional life of the teeth, attributable to iron-mineralization, translates to a slower rate of radula replacement.

However, it is acknowledged that numerous factors combine to influence the given radula production rate, therefore making it difficult to make direct comparisons between any particular species. Previous studies have proposed that tooth size and shape, the mode of feeding, the properties of the food source and tooth hardness all contribute to tooth wear qualities (Isarankura & Runham, 1968; Steneck & Watling, 1982; Padilla, 1985; Okoshi & Ishii, 1996). In addition, both Isarankura & Runham (1968) and Padilla *et al.* (1996) have reported slower rates in larger animals, proposing that this is due to the slower metabolic rates of large individuals compared to small ones. It is likely that rates would be faster in juvenile *A. hirtosa*, *Plaxiphora albida* or *Patelloida alticostata* compared to their adult counterparts, even though size may not be useful for making rate comparisons between adults of various species.

During the course of the study, animals were observed to be feeding, as evidenced by the deposition of faecal pellets and the maintenance of limited algal coverage on the rock near each individual. Although grazing effort, and therefore radula wear and replacement, may have been influenced by the lack of a tidal system in the tanks, the continual supply of reticulated seawater is likely to have promoted opportunities for feeding rather than reduce them. As such, the rates determined for each of the species examined may be slight overestimates compared to those of animals in the wild. However, given the extremely small tidal amplitudes (Pattiaratchi *et al.*, 1997) and narrow patterns of zonation experienced along the Perth coastline (Black *et al.*, 1979) the rates observed for the chitons and limpet are likely to be representative of those in their natural habitat.

The rate of radula production determined for *A. hirtosa* in the current study was slightly higher compared to the rate determined previously for this species by Shaw *et al.* (2002) (0.40 rows per day compared with 0.36). Notably, a slightly higher average water temperature was also recorded in the current study (24°C compared to 22°C). Although temperature has been shown to influence radula production rate (see, for example Isarankura & Runham, 1968; Padilla *et al.*, 1996), its effect is likely to be small for animals acclimatized/acclimated within their natural metabolic range. The higher rate observed in the current study is likely to be attributable to the correction made to the post cold-shock slowdown of radula production, which has a short-term affect on all animals during the early stages of the study, and which, if not corrected for, will result in a slight depression of the
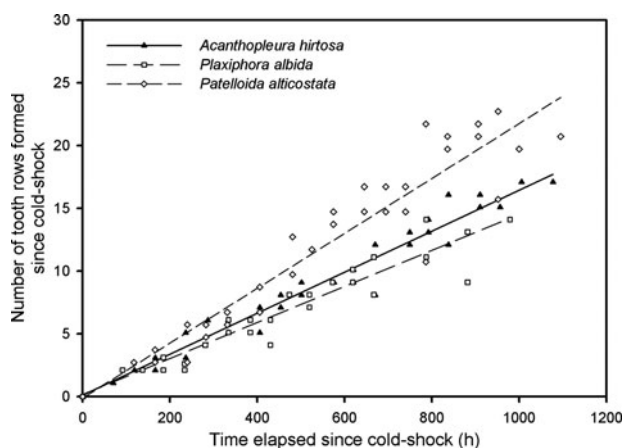

Fig. 1. Radula production rates for *Acanthopleura hirtosa*, *Plaxiphora albida* and *Patelloida alticostata*. Points represent the number of tooth rows produced after a cold-shock treatment. Data and regression lines have been justified through time zero to account for post-shock effects.

Table 2. Composition and daily requirement of elements for the process of radula formation in *Acanthopleura hirtosa*, *Plaxiphora albida* and *Patelloida alticostata*.

Species	A. hirtosa			P. albida			P. alticostata		
Sample size (N)	10			10			10		
Number of tooth rows (± 1 SE)	74 ± 1.1			54 ± 0.7			84 ± 2.0		
Radula dry weight (mg) (± 1 SE)	6.6 ± 0.4			6.5 ± 0.5			1.5 ± 0.1		
Element	$\mu g\ g\ tissue^{-1}$	%	$\mu g\ d^{-1}$	$\mu g\ g\ tissue^{-1}$	%	$\mu g\ d^{-1}$	$\mu g\ g\ tissue^{-1}$	%	$\mu g\ d^{-1}$
Fe	88457	59.2	3.06	98087	86.6	4.12	64731	83.5	0.55
Ca	28304	18.9	1.00	2433	2.1	0.11	1672	2.2	0.01
P	19238	12.9	0.68	5169	4.6	0.22	828	1.1	0.01
Mg	5082	3.4	0.18	1033	0.9	0.05	470	0.6	0.00
S	4395	2.9	0.16	4629	4.1	0.20	3274	4.2	0.03
Na	2003	1.3	0.07	693	0.6	0.03	1324	1.7	0.01
Zn	993	0.7	0.03	101	0.1	0.00	53	0.1	0.00
K	256	0.2	0.01	271	0.2	0.01	BDL		
Al	242	0.2	0.01	252	0.2	0.01	1201	1.5	0.01
Cu	149	0.1	0.01	110	0.1	0.00	62	0.1	0.00
Si	346	0.2	0.01	424	0.4	0.02	3924	5.1	0.03

Note: The nitric acid with peroxide digest cannot fully dissolve Si, and as such the results only provide an indication of the relative extractable Si for each species. SE, standard error; BDL, below detectable limits.

actual rate. This lag effect was acknowledged by Shaw *et al.* (2002), but not accounted for in the final determination of radula production rate in that study.

Although they are similar in overall size, the major lateral teeth of *A. hirtosa* are unicuspid, while those of *Plaxiphora albida* are tricuspid. As these two species are sympatric, it is likely that they are exploiting different niches on the same substratum. This is supported by the composition of their faeces, which contained significantly different amounts of inorganic material, at 88% and 70% for *A. hirtosa* and *P. albida* respectively (Macey *et al.*, 1996). It is possible that *A. hirtosa* is targeting the endolithic algal component of the rock, whilst *P. albida* targets the epilithic component and, thereby, interspecific differences in tooth structure are balanced out.

Elemental analysis

A number of studies have determined the elemental composition of chiton and limpet radulae (Nesson, 1969; Kim *et al.*, 1986; Okoshi & Ishii, 1996). The concentration of elements, especially iron, determined in these earlier studies compares closely with those established in the present study. A single exception is the radula of *Mopalia muscosa*, which contains 80 μg of iron per tooth row (Nesson, 1969); eight times higher than the value determined for the chitons in the present study. The reasons for the difference are unclear, as *M. muscosa* is similar in body size and radula morphology to *P. albida* (Nesson, 1969).

The difference in the amount of iron in *P. albida* compared to that of *A. hirtosa* is likely due to differences in the distribution of this element within the major lateral teeth of these two species. In *P. albida*, magnetite covers the posterior cutting face of the cusp and coats the anterior tooth surface, thus enveloping the tooth's limonite/lepidocrocite core (Figure 2). In *A. hirtosa*, the extent of magnetite coverage is primarily limited to the cutting face of the cusp and the apatitic calcium phosphate core region contains no iron (Macey *et al.*, 1996; Lee *et al.*, 2003a). Similarly, the low amount of iron observed in the limpet *Patelloida alticostata* is likely to derive from its reliance on the iron oxide goethite with silica

in the form of hydrated amorphous opal to strengthen and cushion its teeth (Runham *et al.*, 1969; Lowenstam, 1971; Mann *et al.*, 1986; Liddiard *et al.*, 2004).

The process of biomineralization in chiton and limpet teeth continues to draw interest within the emerging fields of biomimetics and nanotechnology, with the ultimate aim of controlling the formation of nanocomposite structures. One of the remaining objectives in this discipline is to elucidate the mechanisms that control the cellular transport and deposition of ions responsible for tooth mineralization. Such studies will require an accurate understanding of the demand for elemental precursors in radula mineralization in order to facilitate accurate dosing with reagents for molecular and immunological studies. In addition, an understanding of the physiological demand for iron in radula synthesis may assist further studies pertaining to the supply of this element, which is likely to be derived from a combination of sources, such as algae, substratum, seawater and even tooth recycling.
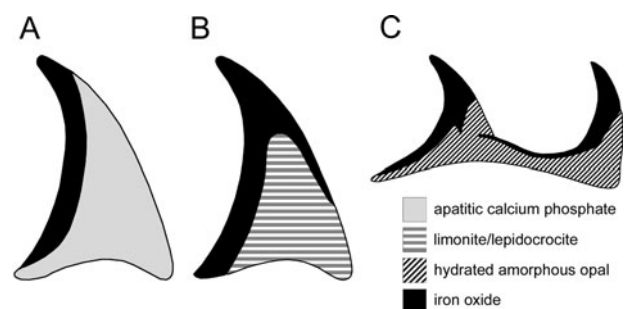
Fig. 2. Diagrammatic representation of cross-sections through the posterior cutting surface and tooth core, showing the distribution of magnetite in (A) *Acanthopleura hirtosa*; (B) *Plaxiphora albida*; and goethite and silica in (C) *Patelloida alticostata*. (Adapted from Liddiard *et al.* (2004).)

Legend:
- apatitic calcium phosphate
- limonite/lepidocrocite
- hydrated amorphous opal
- iron oxide

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