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Concentrations of metals in *Patella intermedia*, *Patella rustica*, *Patella ulyssiponensis* and *Patella vulgata* shells along the Portuguese continental coast

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Abstract

Specimens of *Patella intermedia*, *Patella rustica*, *Patella ulyssiponensis*, and *Patella vulgata*, were collected at 13 sampling sites along the Portuguese continental coast, and analyzed for shell (size, form and Calcium, Iron, Potassium, Sodium and Strontium content) and radula characteristics. The main aims of the study were to assess if the concentration of these metals, (a) are reliable taxonomic characters for these limpet species, (b) were different in the studied limpets, at each sampling site. Whereas the shell calcium content was not significantly different between sites and species, the concentration of the other metals was generally significantly different between sites (for each species), and between species (at each site). Generally, at each site, *P. ulyssiponensis* or *P. rustica* had the highest iron shell content, and *P. intermedia* or *P. vulgata*, the lowest. An inverse situation was found for potassium and sodium. Several significant correlations were found in the concentration of the assayed metals in each of the species. However, no significant correlation was found between the concentration of the assayed metals in *P. intermedia* and *P. ulyssiponensis* shells and seawater temperature. The addition of the shell metal content variables to shell size or form and radula size variables, in Canonical Discriminant Analysis, increased the number of correctly identified specimens. The variables with higher loadings were radula variables followed by shell iron and potassium content. It was concluded that the concentrations of iron, potassium, sodium and strontium in *P. intermedia*, *P. rustica*, *P. ulyssiponensis* and *P. vulgata* shells was generally not constant along the Portuguese continental coast, and therefore are not reliable taxonomic characters for these species. However, on a local basis, the radula size together with the shell metal content could discriminate the studied limpet species.

Riassunto

Lungo la costa portoghese sono stati raccolti esemplari di *Patella intermedia*, *Patella rustica*, *Patella ulyssiponensis* e *Patella vulgata* e sono state analizzate le caratteristiche della conchiglia (forma, dimensioni e contenuto di calcio, ferro, potassio, sodio e stronzio) e della radula. Gli obiettivi principali dello studio erano quelli di valutare se la concentrazione di questi metalli fosse una caratteristica tassonomica attendibile per queste specie di *Patella*, o fosse variabile a seconda dei luoghi di provenienza degli esemplari.

Il contenuto di calcio nella conchiglia è risultato molto simile per tutti i luoghi di prelevamento e per le diverse specie. Al contrario, la concentrazione della maggior parte degli altri metalli, è per quasi tutte le specie variabile nei diversi luoghi di campionatura. Nonostante lungo la costa la concentrazione di ferro, potassio e sodio risulti variabile, tuttavia questi metalli hanno mostrato una trend comune nelle varie specie di *Patella* studiate nella maggioranza dei luoghi di campionatura. Nelle aree settentrionali della costa, le conchiglie con maggiore concentrazione di ferro sono quelle di *P. ulyssiponensis*, nelle aree meridionali quelle di *P. rustica.* Situazione inversa è stata osservata per il contenuto di potassio e di sodio. La concentrazione di stronzio è molto variabile lungo tutta la costa. Per la maggior parte delle combinazioni luoghi/metalli, si è osservata una differenza significativa nel contenuto di metalli nelle conchiglie delle diverse specie. Per tutte le aree, almeno un metallo mostra una concentrazione nella conchiglia diversa fra le varie specie. Le due specie con meno differenza nel contenuto dei metalli nella conchiglia sono *P. intermedia* e *P. vulgata.*

I dati di letteratura mostrano che in alcuni molluschi esiste una relazione tra la concentrazione di alcuni metalli nella conchiglia e la temperatura delle acque marine. Con riferimento a questi lavori si cercato di verificare, per *P. intermedia* e *P. ulyssiponensis* e per i metalli che presentavano una concentrazione significativamente differente nei diversi luoghi di campionatura, se esiste una correlazione significativa tra il contenuto di metallo nella conchiglia e la temperatura dell'acqua. In nessun caso è stata registrata una relazione significativa.

Allo scopo di confrontare la concentrazione dei metalli della conchiglia con le variabili generalmente utilizzate per distinguere le specie di *Patella* – dimensioni e forma della conchiglia e dimensioni della radula – è stata eseguita l'Analisi Discriminante Canonica. I risultati migliori per l'individuazione delle varie specie sono stati ottenuti usando contemporaneamente le variabili relative alle dimensioni conchigliari, al contenuto di metalli della conchiglia e alle dimensioni della radula. Tutti gli esemplari, tranne uno, sono stati identificati correttamente *a posteriori* a livello di specie. Le variabili con maggiore influenza sono state quelle relative alla radula, seguite dal contenuto in ferro e potassio.

Si deduce che le concentrazioni di ferro, potassio, sodio e stronzio in *P. intermedia, P. rustica, P. ulyssiponensis* e *P. vulgata* in genere non sono costanti lungo la costa portoghese e di conseguenza, non rappresentano caratteristiche tassonomiche attendibili per queste specie di *Patella*, mentre, a scala locale, la dimensione della radula insieme al contenuto di metalli della conchiglia può differenziare in maniera soddisfacente le specie di *Patella* studiate. La concentrazione di calcio, ferro, potassio, sodio e stronzio in *P. intermedia* ed in *P. ulyssiponensis* non risulta essere in relazione semplice e diretta con la temperatura dell'acqua e dunque queste variabili non possono essere utilizzate per valutazioni precise delle paleotemperature.

Key Words

Limpets, *Patella*, metals, discriminant analysis, seawater temperature.

Introduction

In the European Atlantic coasts, the genus *Patella* Linné 1758 is represented by only four valid species: *Patella intermedia* Murray *in* Knapp 1857, *Patella rustica* Linné 1758, *Patella ulyssiponensis* Gmelin 1791, and *Patella vulgata* Linné 1758 (Christiaens, 1973; see also CLEMAM database). To each of these *Patella* species are associated, at present, a considerable number of synonyms (Christiaens, 1973; see also CLEMAM database), mostly new species proposed in the 19th century and beginning of the 20th century by eminent naturalists and malacologists. This proliferation of species in the European Atlantic limpets (actually in the genus) resulted from three main causes. (1) The definition and delimitation of a new *Patella* species based on conchological characters alone. (2) The appreciable variability in shell morphology and morphometry exhibited by most limpets. (3) The consideration that certain variations in the shell form, size or colour, were sufficient for the creation of a new species. Interestingly, the difficulties in the taxonomy of the European limpets stands out already in the writings by 19th century naturalists. Describing *P. vulgata* Linné 1758, Donovan (1804) wrote that «the shells vary exceedingly in colours, not only in the different stages of growth, but also in the adult stage (…) some authors have considered several varieties as distinct species». Also characterizing *P. vulgata* Linné 1758, Montagu (1803) described the «shell, subject to much variety in its degree of elevation, in the prominence and number of ridges, and in the indentations at the margin; so much (…) have occasioned it to multiplied into two or three different species (…) these varieties (if they are really such) have nothing to do with size or age, for we have found the depressed ones nearly as large as those of a conic shape», and we can read in Lamarck (1836) that «quiconque n'aurait qu'un exemplaire de cette coquille, pourrait se trouver fort embarrassé pour le rapporter à son espèce, tant celle-ci est variable; aussi les auters different ils beaucoup dans les descriptions et les figures qu'ils en donnent». It should be pointed out however that Montagu (1803) and Lamarck (1836) included in their description of *P. vulgata* Linné 1758, *P. depressa*, Pennant, 1777, fig. 146, now considered as a probable synonym of *P. intermedia* Murray *in* Knapp 1857 (Christiaens, 1967, 1973).

The systematics and taxonomy of the European limpets were therefore, in the beginning of the 20th century, very complicated and confused. Clarification began, when taxonomists started viewing species as biological entities and not as «things that look different» as in the typological species concept (Mayr, 1963, 1969), with the study of the soft-part morphology – foot and head colour, colour and arrangement of the cephalic and pallial tentacles, and specially, of the radula size and teeth morphology, with Fischer-Piette (1934, 1935) showing, for the first time, that radula teeth was much less variable then the shell, and afforded a sound *criterium* for the definition and delimition of the European limpets. Posterior work by this author and collaborators (Fisher-Piette, 1941, 1948; Fisher-Piette & Gaillard, 1959), and by Eslick (1940) and Evans (1947, 1953, 1958) fully confirmed Fisher-Piette's initial observations. Clarification continued in the second half of the 20th century with the study of the breeding behaviour (Orton & Southward, 1961), karyotic structure (Sella, Robotti & Biglione, 1993), sperm ultrastructure (Hodgson, Ridgway, Branco & Hawkins, 1996), and electrophoretic analysis of enzyme *loci* (Gafnney, 1980; Badino & Sella, 1980; Cretella, Scillitani, Toscano, Turella, & Picariello, 1990; Cretella, Scillitani, Toscano, Turella, Picariello, & Cataudo, 1994; Sella *et al.*, 1993; Corte-Real, Hawkins & Thorpe, 1996a, 1996b).

Physiological data can be used as reliable taxonomic characters provided they are constant and characteristic of a given taxon (Cuénot, 1932; Mayr, 1963, 1969; Mateus, 1989). With this notion in mind it was studied the concentration of calcium, iron, potassium, sodium and strontium in the shells of *P. intermedia*, *P. rustica*, *P. ulyssiponensis* and *P. vulgata* at 13 sampling sites along the Portuguese continental coast. These metals were chosen because they occurred at high or medium levels in the assayed shells, and their presence in the seawater is not directly linked to pollution sources.

In particular the following questions were raised:

- In any of these limpet species, was the concentration of assayed metals constant along the coast?
- Was the concentration of the assayed metals significantly different between species at each sampling site?
- Are the concentrations of the assayed metals reliable taxonomic characters for these species?
- What was the relative weight of the shell metal content in relation to shell form and shell and radula sizes, in the discrimination between these limpet species?

Material and methods

Sampling sites

Specimens were collected at 13 sites along the Portuguese continental coast (Figure 1). Sites were chosen in order to provide a diversity of habitats and local environmental conditions.

Collection of specimens

Specimens were collected at low tide, between April and October, from 2001 to 2003. *P. intermedia* and *P. vulgata* were collected at mid-shore, *P. rustica*, at high-shore, and *P. ulyssiponensis*, at low-shore levels. *P. intermedia* and *P. ulyssiponensis* occurred at all sites, and were collected from most of the sites. *P. rustica* was absent from AFI, AGU, FFZ and OEI, and was collected in most of the other sites. *P. vulgata* was absent from LUZ, MAR and SAL, and was collected from most of the other sites. A complete set of analyses (five metals, three samples for each metal) required at least 1.8 g of shell (see Assay of metals) and therefore only shells of a certain size were collected. Shell length of *P. intermedia*, *P. rustica*, *P. ulyssiponensis* and *P. vulgata* specimens was, respectively, (mean, minimum, maximum, in mm): 31, 26, 38; 30, 25, 35; 39, 32, 44; 36, 28, 44. Some shells of *P. intermedia* and *P. rustica* did not reach the minimum size for a complete analysis, and therefore only some metals were assayed in each shell, and/or only two samples were used for each metal. For each site, species and metal, the number of analyzed shells varied between six and sixteen.

Treatment of specimens

In the laboratory, the specimens were immersed for a few minutes in boiling water to separate the shell from the soft part. The radula was removed from the visceral mass by dissection, immersed in household bleach to remove mucilaginous substances, washed in distilled water, and measured to the nearest 1mm using a ruler. After air-drying, pluricuspid teeth were observed using a binocular microscope, at 80 x final magnification. The external and internal shell surfaces were examined and their characteristics were recorded. Shell length, width, widthante, height, apexante and apexpos (**Tab. 1** for the definition of these measures) were then determined to the nearest 0.01 mm using a digital calliper (Mitutoyo, model CD-15DC).

Identification of specimens

Identification of the specimens at species level was based on the morphology of the radula pluricuspid teeth and of the shell, by comparison with data reported in the literature (Evans, 1947, 1953; Fischer-Piette & Gaillard, 1959; Christiaens, 1973; Fretter & Graham, 1994).

Assay of metals

The shell of each specimen was broken into very small fragments using a plastic hammer, and these were treated with commercial bleach for 24 h at 45°C in a closed plastic tube. This resulted in the removal of all adhering organisms and the periostracum layer – samples were therefore composed of the crystalline calcium carbonate and its organic matrix. The shell fragments were then thoroughly washed with distilled water and dried. Calcium, sodium and strontium were assayed in nitric acid digests, and iron and potassium in hydrochloric acid digests. For the preparation of the nitric acid digests, 200-300 mg of shell were weighted to the nearest 0.1 mg, 4 ml HNO_3 68% was then added, the plastic tube was closed and placed in a boiling water-bath until complete digestion. After cooling, the digest was diluted with distilled deionized water up to 50 ml. Hydrochloric acid digests were prepared similarly using 400-500 mg shell and 2 ml HCl 37%. Typically each shell was divided into six samples, three for the assay of Ca, Na and Sr, and three for the determination of Fe and K. Only results based on at least two samples are reported in this work.

Calcium was determined by titration with EDTA with Eriochrome Blue Back R as indicator, adapted from APHA (1995). One ml of the nitric acid digest was diluted to 50 ml with distilled deionized water and placed in a baker. The diluted digest was neutralized with concentrated NaOH to pH 13. Two drops of indicator solution were added, and EDTA added dropwise by drop until colour change.

Iron was assayed spectrophotometrically by the phenanthroline reaction, adapted from APHA (1995). To a 5 ml sample, 0.1 ml of a hydroxylamine solution was added, and the volume was heated in a boiling-water bath for 30 min. After cooling, 1 ml of acetate buffer was added, followed by 0.4 ml of the phenanthroline solution. After 30 min at room temperature, the absorbance at 510 nm was determined in a Jasco model V-530 double-beam spectrophotometer.

Potassium and sodium were determined by flame photometry using a Jenway model PFP7 flame photometer.

Strontium was assayed by flame atomic spectrophotometry in a Philips model PU9200X atomic absorption spectrometer.

The methods were calibrated using standards prepared by diluting 1000 ppm standard metal solutions. Calcium, potassium and sodium solutions were prepared according to APHA (1995). Iron and strontium solutions were purchased from Panreac Quimica SA. All reactions were carried out in plastic tubes previously decontaminated with hot diluted nitric acid. The limit of detection for each method was determined as described in Miller & Miller (1988) and is indicated in **Tab. 2**.

The determination of metals other than calcium in these shell acid digests is technically difficult since in certain analytical techniques, the huge calcium concentration can interfere with the assay of the other metals. For these reasons, the accuracy of the procedures used in the present work was checked by analyzing a reference material. Bone ash was chosen because it provided a matrix similar to that of the analysed shell, with a very high calcium concentration as the analyzed shells. Results presented in Tab. 2 showed that the methods used in the present work were accurate and reliable.

Acronym	Description
SL	Shell length: greatest distance between anterior and posterior ends
SW	Shell width: greatest distance perpendicular to the anterior-posterior axis
SWA	Shell widthante: greatest distance perpendicular to the anterior-posterior axis passing through the apex
SH	Shell height: greatest vertical distance from apex of the shell to the plane of aperture
SAA	Shell apexante: greatest distance between apex and anterior end
SAP	Shell apexpos: greatest distance between apex and posterior end
SV	Shell volume = $(\pi/3) \times (SW/2) \times (SL/2) \times SH$
RL	Radula length

Tab. 1. Characters used in the canonical discriminant analysis.

Tab. 1. Caratteri utilizzati nell'analisi canonica discriminante.

Metal	Certified	Found			
	Mean	Mean	Coefficient of variation (%)	Number of samples	Limit of detection
Calcium	38.18%	38.9%	4.6	18	0.5%
Iron	660 µg × g^{-1}	610 µg × g^{-1}	5.1	13	5.2 µg × g^{-1}
Potassium	186 µg × g^{-1}	168 µg × g^{-1}	0.7	7	4.1 µg × g^{-1}
Sodium	0.6%	0.601%	10.7	7	0.15%
Strontium	249 µg × g^{-1}	263 µg × g^{-1}	3.1	11	100 µg × g^{-1}

Tab. 2. Analysis of Bone Ash reference material (Standard 1400, National Institute of Standards & Technology, 1992, Department of Commerce, USA), certified for calcium, iron, potassium and strontium, and with indicative values for sodium.

Tab. 2. Analisi del materiale di riferimento delle ceneri ossee (Standard 1400, National Institute of Standards & Technology, 1992, Department of Commerce, USA), certificata per il calcio, il ferro, il potassio e lo stronzio, e con valori indicativi per il sodio.

Environmental variables

To assess the correlation between the shell metal content and the seawater surface temperature, we searched for available data for this parameter along the Portuguese continental coast. The most complete set of data was obtained from the Portuguese Metereology Institute and comprehended monthly means for the period 1981-2000, but only at seven sites along the coast (Carvalho & Soares, 2001). Inspection of the data revealed that the seawater surface temperature increased smoothly along the west coast, from north to south, showing an appreciable rise on passing from the west to the south coast (**Fig. 1**). In light of this information, the following procedure was used. Shells were divided into groups, corresponding to areas with minor variations in the seawater temperature, and the respective seawater temperature taken from the nearest one or two stations. Group I, included shells from the sites AFI, AGU, FFZ, FZA and BAL, and corresponded to the mean seawater temperature of Leixões and Peniche. Group II, included shells from the sites SJE and OEI, and corresponded to the seawater temperature of Santa Marta. Group III, included shells from the sites CVI and TEL, and corresponded to the seawater temperature of Sines. Group IV, included shells from the sites MRT, SAL, LUZ and MAR, and corresponded to the seawater temperature of Praia da Rocha and Cabo de Santa Maria. At each group, the mean metal content was calculated by averaging values for all shells. Only *P. intermedia* and *P. ulyssiponensis* were considered since only these species occurred at most of the sites along the coast.

Statistics

The dependence of the shell metal content on age was assessed by plotting, for each site, species, and metal, the metal content *vs.* shell length of each specimen. The significance of the correlation was assessed by analysis of variance (ANOVA).
For each site, species and metal, *intra* specimens metal content variability was assessed by calculating the coefficient of variation of the metal content of the replicate samples for each shell. *Inter* specimens metal content variability was assessed by calculating the coefficient of variation of the metal content of the assayed shells. The values for each species and metal, were averaged for each site, and then for all sites.
Comparisons of two means were carried out by a Student t-test. Comparisons of three or more means were carried out by ANOVA.
The correlation between the concentrations of the different metals in the shells of each limpet species was assessed by plotting, for each species and all sites, the concentration of one of the metals *vs.* the concentration of another metal, for each specimen. The significance of the correlation was assessed by calculating the linear correlation coefficient.
Descriptive statistics, linear correlation coefficients, t-tests, and ANOVAs were carried out using Microsoft® Excel XP program.
Canonical discriminant analysis on shell (size, form and metal content) and radular variables was used to identify the variables that were responsible for the separation between the four studied species. Discriminant analyses were carried out using *a posteriori* probabilities and four different sets of variables. Analysis I used shell and radula sizes – SL, SW, SWA, SH, SAA, and RL. Analysis II used analysis I variables plus shell metals content variables. Analysis III used shell form and radula relative size variables – SH/SL, SAA/SAP, RL/SH and RL/$\sqrt[3]{}$(SV). Analysis IV used analysis III variables plus shell metal content variables. Discriminant analysis was carried out only at sites with a considerable number of shells with a complete set of metal analyses (AFI, BAL, MAR, OEI and SJE). Discriminant analyses were performed using XL-STAT® 5.1 (Addinsoft, Paris, France) software package.

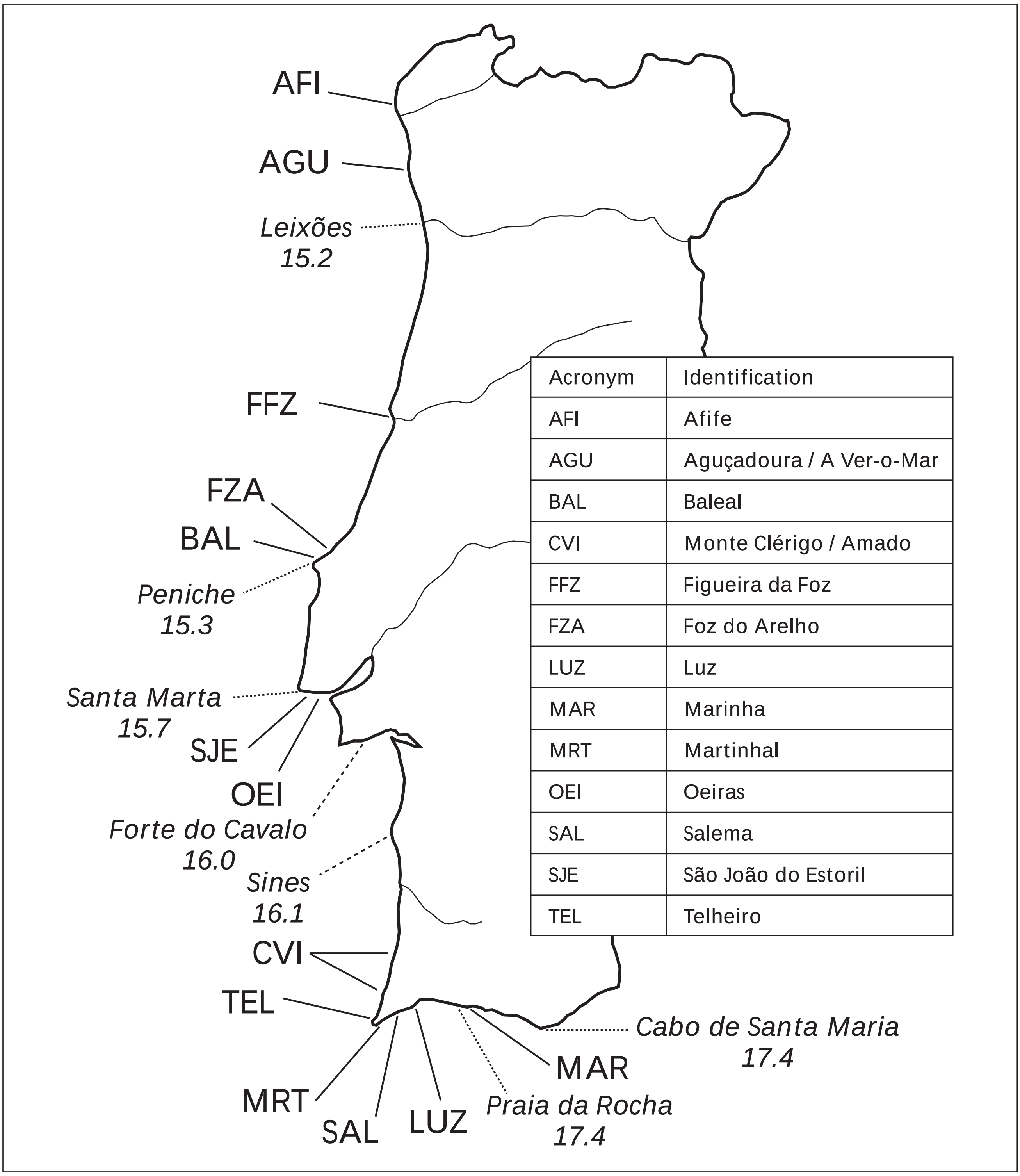
Results

Radula and shell characteristics of the limpet species

The differential characteristic of the four *Patella* species are shown in **Tab. 3**.

Was the shell metal content correlated with the age of the specimens?

The concentration of all five metals in the studied limpet shells was, for the great majority of the cases, not significantly correlated with the age of the specimen. Of the 161 tests carried out, only in 15 was the correlation significant at 0.05 level (nine with $0.02 < P \leq 0.05$, three



Acronym	Identification
AFI	Afife
AGU	Aguçadoura / A Ver-o-Mar
BAL	Baleal
CVI	Monte Clérigo / Amado
FFZ	Figueira da Foz
FZA	Foz do Arelho
LUZ	Luz
MAR	Marinha
MRT	Martinhal
OEI	Oeiras
SAL	Salema
SJE	São João do Estoril
TEL	Telheiro

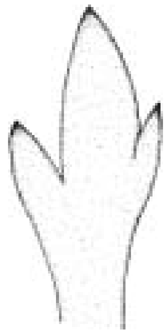
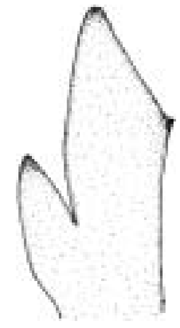
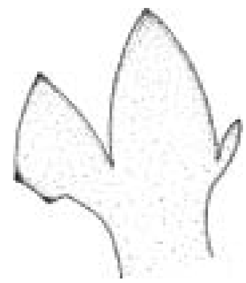
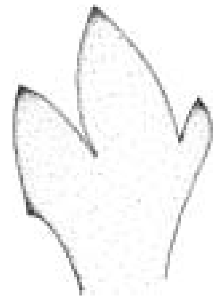
Fig. 1. Localization of the sampling sites in the Portuguese continental coast, and (italics) annual mean seawater temperature for the period 1981-2000, at the seven stations of the Portuguese Meteorological Institute (data from Carvalho & Soares, 2001).

Fig. 1. Localizzazione dei siti campionati sulla costa continentale portoghese, e (corsivo) temperatura annuale media dell'acqua di mare nel periodo 1981-2000 nelle sette stazioni dell'Istituto Meteorologico Portoghese (dati da Carvalho & Soares, 2001).

with $0.01 < P \leq 0.02$, and three with $P \leq 0.01$). These included examples in all species and metals.

Intra and inter specimens metal content variability

The studied *Patella* species exhibited similar *intra* and *inter* specimens variability in metal content (**Tab. 4**). However, the assayed metals exhibited different variabilities in their concentration in the limpet shells. The concentration of calcium was very constant. The mean coefficient of variation, both *intra* and *inter* specimens, was at maximum 2%. On the contrary, the concentration of iron varied substantially both in the same shell, and between shells. This variability was not due to the method used to assay this metal, since the mean coefficient of variation of duplicate analyses of 1058 samples was only 10.4%. The concentration of potassium, sodium and strontium displayed reduced variability, both in the same shell and between different shells of the

Species / Characteristics	*Patella intermedia*	*Patella rustica*	*Patella ulyssiponensis*	*Patella vulgata*
Radula's pluricuspid teeth Cusp 1: right Cusp 2: centre Cusp 3: left	 Three unequal teeth. Cusp 2 much taller and broader than cusps 1 and 3.	 Cusp 1 vestigial. Cusp 2 much taller than cusp 3, and with a rounded extremity.	 Three unequal teeth. Cusp 1 very small. Base of cusp 3 wider than cusp 2. Cusp 3 with a protuberance on its outer side – a vestigial fourth cusp.	 Three unequal teeth. Cusp 1 small. Cusps 2 and 3 subequal. Cusp 3 with a pointed protuberance on its outer side border.
Shell's characteristics				
Margin	Rimose, with pointed extensions connected to the rays.	Entire or slightly indented.	Finely crenulate.	Entire or slightly indented.
External surface	Few and prominent ribs.	Brown dots on the ribs.	Crowded by numerous, closely spaced, and well-marked ribs, of unequal size.	Smooth at the apex, with flat and spaced ribs below.
Internal surface	Alternating dark and light rays, in the lower part. Head scar yellow-orange, but creamy in a few specimens.	Alternating dark and light brown rays, in the lower part. Head scar dark, brown to grey.	Homogenous, porcellanous white. Head scar white, creamy or pale orange.	Often with a green or blue iridescence. Silvery head scar.

Tab. 3. Characteristics of the radula and shell of the studied *Patella* species.

Tab. 3. Caratteri radulari e conchiliari delle specie di *Patella* studiate.

same site. The coefficient of variation was generally lower then 10%.

Was the concentration of assayed metals constant along the coast?

The calcium content of the shells was very constant at all sites and species. The minimum was 37.0% and the maximum 42.3%. A comparison of the means simultaneously for all sites and species, indicated that the differences between the means were not significant (P > 0.50). On the contrary, the concentration of most the other metals showed, for most of the species, significant differences between sites. From 16 combinations of metals

Species		Calcium			Iron			Potassium			Sodium			Strontium		
		N	CV (%) *Intra*	CV (%) *Inter*	N	CV (%) *Intra*	CV (%) *Inter*	N	CV (%) *Intra*	CV (%) *Inter*	N	CV (%) *Intra*	CV (%) *Inter*	N	CV (%) *Intra*	CV (%) *Inter*
Patella intermedia	Mean	10	1.7	1.8	10	24.1	33.6	11	4.9	13.2	10	4.7	8.7	9	6.1	6.3
	Max	15	2.5	2.5	15	28.9	59.1	16	7.0	21.2	16	5.7	13.9	14	8.9	10.1
	Min	7	0.8	1.0	6	18.3	18.1	6	2.0	5.3	7	3.5	5.9	6	4.2	3.3
Patella rustica	Mean	9	2.1	1.7	9	19.6	32.4	10	4.9	5.1	9	5.0	5.7	9	4.8	5.8
	Max	11	2.8	2.2	11	23.6	51.0	10	5.7	7.2	11	6.8	7.7	11	7.3	8.1
	Min	8	1.1	1.2	8	15.6	18.8	9	4.4	3.7	8	3.3	4.3	8	3.5	4.4
Patella ulyssiponensis	Mean	9	2.0	1.6	10	22.9	34.0	10	5.2	10.4	9	5.8	7.7	9	4.3	5.5
	Max	12	3.2	4.0	15	33.6	54.5	16	6.4	17.6	12	7.6	10.2	12	6.8	10.9
	Min	6	1.1	0.6	8	16.9	10.8	7	3.9	5.4	6	3.6	5.0	6	2.2	2.6
Patella vulgata	Mean	10	1.6	1.3	11	26.3	41.4	12	5.2	10.7	11	4.8	8.9	10	5.9	7.4
	Max	14	2.3	1.8	14	35.0	76.1	17	6.2	16.9	14	7.2	16.2	14	7.9	16.3
	Min	8	0.9	0.8	7	20.4	23.0	9	4.0	6.2	9	3.7	4.1	9	2.3	4.6

Tab. 4. The variability of the shell metals content. *Intra* and *inter* specimens coefficient of variation for each *Patella* species.

Tab. 4. La variabilità del contenuto di metalli nella conchiglia. Coefficiente di variazione *intra* ed *inter* individuale per ciascuna specie di *Patella*.

and species (four species, four metals), in 12 there was a significant difference (P < 0.05) in the metal content between sites. Only in four situations (Iron: *P. rustica* and *vulgata*; Potassium: *P. intermedia*; Strontium: *P. vulgata*) there was no significant difference (P > 0.05) in the shell metal content between sites, and from these only one had P > 0.10 (**Figs 2-5**).

Was the concentration of the assayed metals different between species?

Although there was a considerable variation in the concentration of iron, potassium and sodium in the shells along the coast, these metals displayed a general trend in the studied limpet species, for most of the studied sites.
P. ulyssiponensis had the highest iron shell content at northern sites (AFI, AGU, BAL, OEI and SJE), and *P. rustica* at southern sites (CVI and MAR). *P. intermedia* or *P. vulgata* usually had the lowest iron levels (**Fig. 2**). An inverse situation was found for potassium and sodium (**Figs 3-4**). The concentration of strontium, however, showed more variability along the coast (**Fig. 5**).
From 28 combinations of sites and metals (AFI, AGU, BAL, SJE, OEI, CVI, MAR, four metals), in 19 there was a significant difference (P ≤ 0.05) in the shell metal content between species, and in additional two this difference was significant at P = 0.10. At all sites, there was at least one metal differing between the species. The minimum was observed at AFI – only the concentration of potassium was different between species. The maximum was observed at SJE – there was a significant difference in the concentration of iron, potassium, sodium and strontium between species.
The two species with closer shell metal content were *P. intermedia* and *P. vulgata*. From 17 combinations of sites and metals, in 11 there was no significant difference (P > 0.05) in the shell metal content between these two species.

Were the concentrations of the different metals correlated in each limpet species?

Several very significant (P < 0.02) linear correlations were found in the concentration of the studied metals (**Tab. 5**). The highest number of correlations was found in *P. intermedia*, and the lowest in *P. rustica*. From the ten possible pairs, only two combinations were not significant (P > 0.05) in any species. Iron and sodium were significantly correlated with all other metals in at least one limpet species.

Was the concentration of iron, potassium, sodium and strontium in the limpet shells correlated with the seawater temperature?

It was studied, for *P. intermedia* and *P. ulyssiponensis*, and metals showing significant differences between sites, if there was a significant correlation between the shell metal content and seawater temperature. In no case was the correlation significant (P > 0.20).

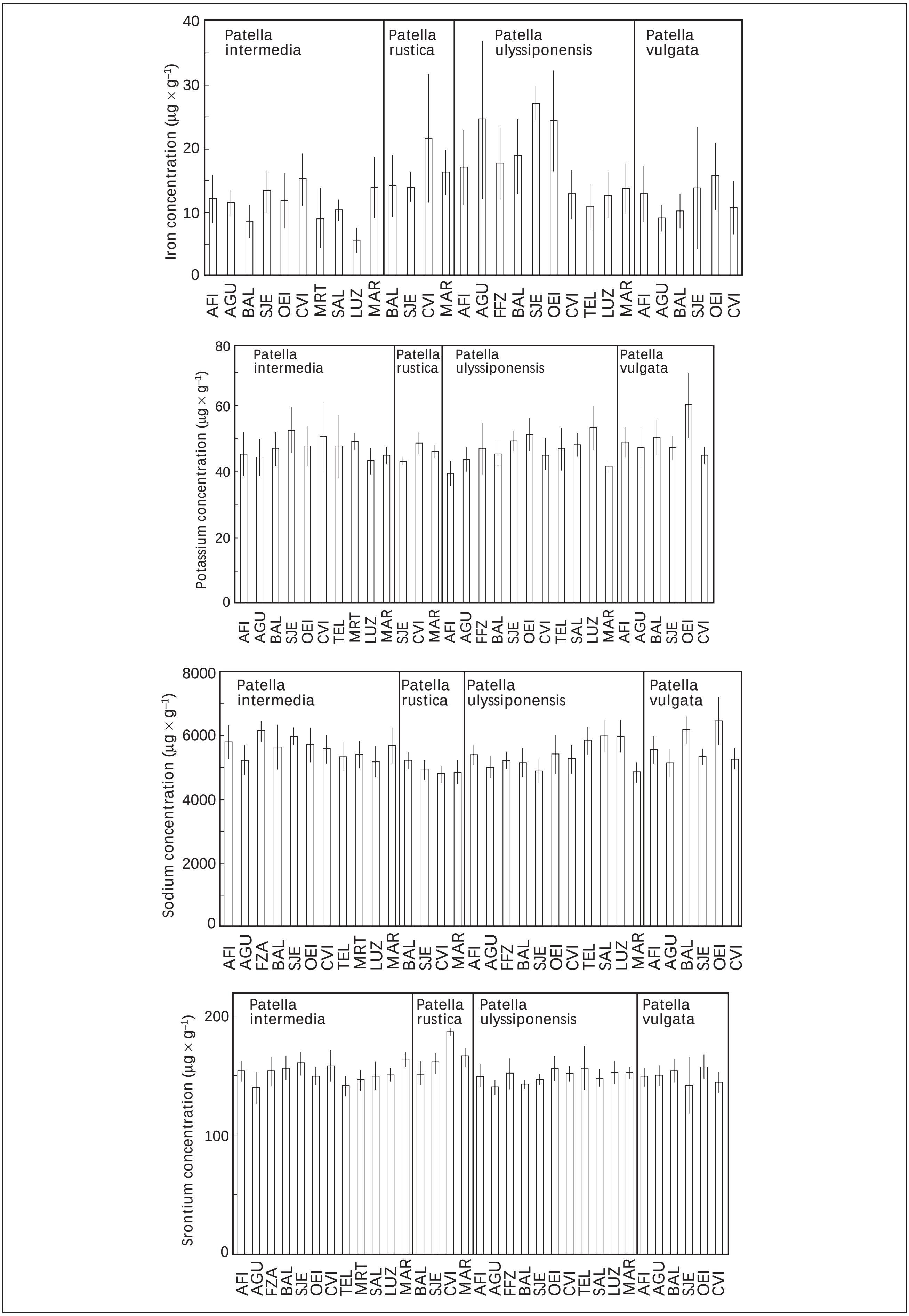
The importance of the shell metals content in the discrimination between limpet species

Analysis I and III, using variables describing shell size or form and radula size, resulted in an acceptable or good discrimination between species, with most of the specimens *a posteriori* correctly identified to species (**Tab. 6**). Shell and radula size variables (Analysis I) performed better than shell form and radula size variables (Analysis III). The addition of the metal content variables to shell size or form and radula size variables (Analyses II and IV) improved appreciably the identification of the specimens. The best results were obtained using both shell size and metals and radula size variables (Analysis II). All but one specimen were *a posteriori* correctly identified to species.
Almost all *P. rustica* and *P. ulyssiponensis* specimens were correctly identified with any set of variables. Lower values were obtained for *P. intermedia* and *P. vulgata* specimens.
Using variables describing shell size or form and radula size (Analysis I and III), radula absolute or relative size variables (RL, RL/$\sqrt[3]{}$(SV, RL/SH) were almost always the most discriminating variables (**Tab. 7**). Using the metal content variables in combination with shell size or form and radula size variables (Analysis II and IV), iron and potassium had the highest loadings among the assayed metals.

Metal	Calcium	Iron	Potassium	Sodium	Strontium
Calcium	—	—	—	—	—
Iron	*P. intermedia* (neg) P < 0.02 *P. ulyssiponensis* (neg) P < 0.001	—	—	—	—
Potassium	None	*P. vulgata* P < 0.001	—	—	—
Sodium	*P. ulyssiponensis* P < 0.005	*P. ulyssiponensis* (neg) P < 0.001	*P. intermedia* P < 0.001 *P. ulyssiponensis* P < 0.001 *P. vulgata* P < 0.001	—	—
Strontium	*P. intermedia* (neg) P < 0.001	*P. intermedia* P < 0.01 *P. rustica* P < 0.005	none	*P. intermedia* P < 0.005 *P. ulyssiponensis* P < 0.001 *P. vulgata* P < 0.02	—

Tab. 5. Significant linear correlations between the concentrations of metals in the shells of a given *Patella* species.

Tab. 5. Correlazioni lineari significative tra le concentrazioni di metalli nelle conchiglie di una data specie di *Patella*.



Figs. 2-5. Mean metal shell content at each sampling site for the studied limpet species. Sampling sites are identifyed in Figure 1, and are ordered from north to south, at each species. Bar, Standard Deviation.

Figg. 2-5. Contenuto conchiliare medio di metalli in ciascun sito di campionamento per le specie di patella studiate. I siti di campionamento sono identificati nella Figura 1, e sono ordinati da nord a sud per ciascuna specie. Trattino, Deviazione Standard.

Site	Species	Number of specimens	Analysis I	Analysis II	Analysis III	Analysis IV
			Shell and radula sizes	Shell and radula sizes and Metals	Shell form and radula relative size	Shell form and radula relative size and Metals
AFI	*P. intermedia*	6	6	6	5	6
	P. ulyssiponensis	10	10	10	10	10
	P. vulgata	13	12	13	12	13
BAL	*P. intermedia*	6	5	6	3	4
	P. rustica	4	4	4	4	4
	P. ulyssiponensis	8	8	8	8	8
	P. vulgata	7	6	6	4	7
MAR	*P. intermedia*	5	5	5	5	5
	P. rustica	6	6	6	6	6
	P. ulyssiponensis	5	5	5	5	5
OEI	*P. intermedia*	8	6	8	4	6
	P. ulyssiponensis	6	6	6	6	6
	P. vulgata	7	5	7	6	7
SJE	*P. intermedia*	9	9	9	9	9
	P. rustica	9	9	9	9	9
	P. ulyssiponensis	9	9	9	8	9
	P. vulgata	9	8	9	7	8

Tab. 6. Number of specimens *a posteriori* correctly identified to species, based on canonical discriminant analysis using different sets of variables.

Tab. 6. Numero di esemplari identificati correttamente *a posteriori* come specie, in base all'analisi canonica discriminante utilizzando differenti insiemi di variabili.

Discussion

Results presented in this work on the shell metal content agreed with data reported in the literature for other characteristics, namely shell size, form and colour, that the limpet shell can vary appreciably between different sites (Fretter & Graham, 1994). Except for calcium, the concentration of most of the studied metals showed, for most of the species, significant differences along the coast. Therefore, it could be concluded that the iron, potassium, sodium and strontium shell content are not reliable taxonomic characters in *P. intermedia*, *P. rustica*, *P. ulyssiponensis* and *P. vulgata* species. However, at each site, the concentration of these metals in shells was relatively constant within each species, and, for most sites and metals, significant different between the studied limpet species. It appears therefore that although *P. intermedia*, *P. rustica*, *P. ulyssiponensis* and *P. vulgata* shells can vary appreciably between different locations, at each site, each species maintains its individuality, and differences with respect to the other limpet species.

In the discrimination between *P. intermedia*, *P. rustica*, *P.*

Site	Analysis I	Analysis II	Analysis III	Analysis IV
	Shell and radula sizes	Shell and radula sizes and Metals	Shell form and radula relative size	Shell form and radula relative size and Metals
AFI	RL SH SWA	RL K Fe Na	RL/$\sqrt[3]{(SV)}$ RL/SH SH/SL	RL/$\sqrt[3]{(SV)}$ RL/SH K Fe
BAL	RL SL SAA	Fe RL SAA SL	RL/$\sqrt[3]{(SV)}$ RL/SH SH/SL	Fe RL/SH RL/$\sqrt[3]{(SV)}$ Sr
MAR	RL SH SAA	RL K SH Sr	RL/$\sqrt[3]{(SV)}$ SH/SL RL/SH	RL/$\sqrt[3]{(SV)}$ RL/SH SH/SL K
OEI	RL SAA SL	Fe SL RL SAA	RL/$\sqrt[3]{(SV)}$ RL/SH SH/SL	Fe RL/$\sqrt[3]{(SV)}$ RL/SH Na
SJE	RL SL SAA	RL Fe SL SAA	RL/$\sqrt[3]{(SV)}$ RL/SL SH/SL	RL/$\sqrt[3]{(SV)}$ RL/SH SH/SL K

Tab. 7. Variables with the highest loadings in the discrimination between species.

Tab. 7. Variabili con il carico più elevato nella discriminazione tra le specie.

ulyssiponensis and *P. vulgata*, radula absolute or relative size variables were more important than shell size, form and metal content variables. Results presented in this work therefore confirmed data reported in the literature (Hernández-Dorta, 1992; Cabral, 2003) on the importance of the radula in the characterization and delimitation of these limpet species.

Results presented in this work on the shell metal content agreed with data reported in the literature for other shell and soft part characteristics, that *P. intermedia* and *P. vulgata* are close to each other, and distant from *P. rustica* and *P. ulyssiponensis*. At each site, the lowest shell iron and the highest shell potassium or sodium were exhibited by either *P. intermedia* or *P. vulgata*, whilst *P. rustica* and *P. ulyssiponensis* showed opposite behaviour. At most sites, there was no significant difference in the concentration of most metals between *P. intermedia* and *P. vulgata* specimens. In the discriminant analysis almost all *P. rustica* and *P. ulyssiponensis* specimens were correctly identified to species, whilst some *P. intermedia* specimens were identified as *P. vulgata*, and vice-versa. Similarly, radula relative size ranges reported in the literature for *P. intermedia* and *P. vulgata* partially overlap, whilst those for *P. ulyssiponensis* are much lower, and those for *P. rustica* are much higher (Fisher-Piette, 1934, 1935, 1941, 1948, Evans, 1947, 1953, 1958; Brian & Owen, 1952; Fisher-Piette & Gaillard, 1959; Christiaens, 1973; Ibañez, 1982; Feliu & Ibañez, 1984; Fretter & Graham, 1994; Cabral, 2003).

For most of the studied limpet species and assayed metals, there was a significant difference in the metal content between sites and between species. What was responsible for this variation?

Data reported in the literature indicates that the metal composition of a marine mollusc shell can be influenced by (a) the metal composition, salinity and temperature of the seawater; (b) the proportion between the calcite and aragonite content; (c) specific characteristics of the species such as the mode of shell construction; and/or (d) the animals' physiology, in particular the activity of the mantle (Turkian & Armstrong, 1960; Pilkey & Goodell, 1963; Dodd, 1965; Harriss, 1965; Hallam & Price, 1968; Carriker, Swann, Prezant & Counts, 1991; Foster & Chacko, 1995; Vander Putten, Dehairs, Keppens & Baeyens, 2000).

Differences in the iron, potassium, sodium and strontium concentration of the seawater at the sampling sites used in the present work were not to be expected since the concentrations of these metals in the ocean are very constant (Odum, 1951; Pilkey & Goodell, 1963). The limpet shell is mainly formed by crossed-foliated and crossed-lamellar layers (MacClintock, 1967). In *Patella*, both are calcitic in nature, although crossed-lamellar layers are usually made of aragonite (Dauphin & Denis, 2000). Therefore, from the mentioned factors, only the seawater temperature and animals' physiology appeared as plausible to explain the variation in the shell metal content along the coast.

Several works have been published on the effect of the seawater temperature on the metal content of the marine mollusc shell. Two basic methodologies have been used. One approach uses shells of sites with different water temperatures, and the other compares different layers of the same shell, corresponding to different growing seasons and temperatures. Opposite conclusions have been reported using both methods. Some studies found significant linear correlation between the shell metal content and the seawater temperature (Pilkey & Goodell, 1963; Dodd, 1965; Lerman, 1965; Hallam & Price, 1968, whilst others reported opposite conclusion (Rucker & Valentine, 1961).

In the present work, no significant correlation was found between the concentration of calcium, iron, potassium, sodium and strontium in the shells of *P. intermedia* and *P. ulyssiponensis*, and seawater temperature along the Portuguese continental coast. It appears therefore that the accumulation of these metals in the shells of these limpets was not a simple function of the seawater temperature and therefore can not be used as a high resolution seawater temperature proxy in the Portuguese continental coast. However, although we sampled specimens from almost the far north to the far south rocky shores of the Portuguese coast, the difference between the highest and the lowest mean seawater temperature was lower than 3°C. This range of temperatures is indeed much lower than those reported in the literature for those molluscs showing a significant correlation between shell metal content and water temperature. There is therefore the possibility of the existence of a correlation in a wider range of temperatures.

Shell building in molluscs is mediated by an organic framework synthetized by the mantle, into which the appropriate ions are actively introduced, and thus induce crystal formation and growth (Lowenstam, 1981; Falini, Albeck, Weiner & Addadi, 1996; Sudo, Fujikawa, Nagakura, Ohkubo, Sakaguchi, Tanaka & Nakashima, 1997). Calcium is by far the most abundant metal in the mollusc shell, but minor quantities of other alkaline-earth (Mg, Sr) and alkaline (Na, K) metals are always present. However, the processes that determine, and the factors that influence the discrimination between these different cations in the incorporation in the mollusc shell are only partly understood. In *Mytilus edulis*, Rosenberg & Hughes (1991) concluded that, metabolic gradients within the mantle determined shell chemistry. In limpets, metabolic rates are influenced by environmental parameters, namely the position of the animal in the shore and consequent duration of immersion and exposure to air (Davies, 1969; Fretter & Graham, 1994). In the present work, sampling sites displayed a diversity of habitats and local conditions. Therefore, the observed variability in the limpet shell metal content along the coast might be related to differences in local environmental conditions.

Conclusions

The concentrations of iron, potassium, sodium and strontium in *P. intermedia*, *P. rustica*, *P. ulyssiponensis* and *P. vulgata* shells was generally not constant along the

Portuguese continental coast, and therefore are not reliable taxonomic characters for these species.
However, on a local scale, radula size together with the shell metal content could discriminate satisfactorily the studied limpet species.
The concentration of calcium, iron, potassium, sodium and strontium in *P. intermedia* and *P. ulyssiponensis* shells was not a simple function of the seawater surface temperature, and therefore can not be used as a high resolution seawater temperature proxy in the Portuguese continental coast.

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