

Geographical Variations in the Chemical Compositions of Veined Squid *Loligo forbesi* Statoliths

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Chia-Hui Wang, Audrey J. Geffen, and Richard D.M. Nash (2012) Geographical variations in the chemical compositions of veined squid *Loligo forbesi* statoliths. *Zoological Studies* 51(6): 755-761. The chemical composition of statoliths of the veined squid *Loligo forbesi* Steenstrup (Cephalopoda: Loliginidae) was analyzed by inductively coupled plasma-mass spectrometry (ICP-MS) to determine geographical differences. Statolith samples from the North Sea and Irish Sea were collected, and samples of 2 consecutive years from the Irish Sea were also included in the analyses to test temporal stability of the statolith chemical composition. Seven trace elements were detected, including strontium, barium, lithium, lead, manganese, nickel, and cobalt, in decreasing order of abundance. The elemental compositions of the statoliths significantly differed among sampling areas, and no significant difference in chemical composition was found between the 2 consecutive yearly Irish Sea samples. <http://zoolstud.sinica.edu.tw/Journals/51.6/755.pdf>

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Cephalopod statoliths are composed of calcium carbonate crystallized as aragonite (Clarke and Fitch 1975, Dilly 1976, Radtke 1983) with a small percentage of organic material that was ascertained to be protein (Radtke 1983). Statoliths are analogous to fish otoliths with similar neurophysiological functions and grow by periodic incremental deposition (Radtke 1983). The material deposited in fish otoliths is metabolically inert after deposition and is not susceptible to resorption, which makes otolith microchemistry a useful tool for identifying environments the fish have experienced (Radtke and Shafer 1992, Wang et al. 2010) and for distinguishing among fish stocks (Campana 1999, Thresher 1999). As an analog to fish otoliths in function and chemical composition, and by assuming a close similarity between otolith and statolith formation, analyses of statolith chemical composition were applied to

studies of cephalopod life history in recent years, by modifying the methodology of otolith studies (Arkhipkin et al. 2004, Zumholz et al. 2007, Warner et al. 2009).

Early studies of statolith growth concentrated on the biomineralization process, and the elements controlling increment formation. High levels of magnesium (Mg) in the statocyst endolymph fluid were found to inhibit statolith calcification in *Alloteuthis subulata* (Morris 1991). The strontium (Sr) concentration was hypothesized to control statolith biomineralization in *Loligo vulgaris reynaudii* (Lipinski 1993), and it also resulted in better visualization of increments in the wing zone which contained higher Sr concentrations than the lateral dome (Lipinski 1993, Durholtz et al. 1997). Furthermore, Sr²⁺ ions are vital for the development of cephalopod statoliths (Hanlon et al. 1989). However, microprobe measurements (Bettencourt

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and Guerra 2000) of Sr/calcium (Ca) ratios in both the wing and lateral dome of *Sepia officinalis* and *L. vulgaris* statoliths along the frontal plane showed that despite a higher level of Sr in the wing than in the lateral dome of both species, the increments did not have better definition in that area due to different patterns of calcium crystallization (Radtke 1983, Lipinski 1993). Ca^{2+} and Mg^{2+} ions along with the protein content in the endolymph seemed to be more closely related with growth increment definition in these species (Bettencourt and Guerra 2000).

Statolith Sr/Ca ratios have been used in reconstructing thermal histories (Ikeda et al. 1998, Arkhipkin et al. 2004) and ontogenetic changes (Rodhouse et al. 1994) of cephalopods. In addition to Sr and Ca concentrations, other trace and micro-elements were reported from statoliths such as chromium (Cr), manganese (Mn), iron (Fe), copper (Cu), zinc (Zn), bromine (Br), and lead (Pb) (Durholtz et al. 1997, Lipinski et al. 1997). The concentrations reported are highly variable, but it is not clear whether this reflects species or geographic trends, since little is known about the relationships of environmental (i.e., salinity and temperature) and biological parameters (i.e., somatic growth rate, feeding, maturation, vertical migration, etc.) with statolith element incorporation (Ikeda et al. 2002a b). There are relatively few published studies applying statolith compositions to investigate population differences and geographic variations (Arkhipkin et al. 2004, Zumholz et al. 2007, Warner et al. 2009), which were successfully applied to many fish species. Therefore, we undertook a study of a widely distributed squid, with good background information on the genetic structure and abundances throughout its range.

The veined squid *L. forbesi* Steenstrup (Cephalopoda: Loliginidae) is widely distributed in the eastern Atlantic Ocean along the European coasts, and around the island groups of Madeira, the Azores, and the Canaries. It is believed to be a migratory species, and groups of squid might experience different water masses after hatching. Using probe techniques (wavelength dispersive spectrometry (WDS) and laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS)), it is possible to analyze discrete areas of the statolith, such as the statolith core or edge for explaining different routes of migration experienced by an individual squid. However, dissolving the entire statolith for a solution-based ICP-MS enabled us to detect more trace elements and quantify low concentrations with greater

accuracy precision, and sensitivity, thus offering an opportunity to detect differences between groups of squid from different areas. This study identified the elements that occur in the statoliths of different groups of *L. forbesi* and analyzed their absolute concentrations as a means of distinguishing among squid populations.

MATERIALS AND METHODS

Sample collection

Veined squid *Loligo forbesi* were collected from the North Sea and Irish Sea. Samples of 2 consecutive years from the Irish Sea (Nov. 1999 and Oct. 2000) were also included in this analysis to test the temporal stability of the statolith chemical composition. After collections, the dorsal mantle length (DML) and body weight (BW) of each squid were measured (Table 1). Sex and maturity stage were determined according to Boyle and Ngoile (1993), and all specimens used in present study were mature individuals at stage IV or V (Boyle and Ngoile 1993). Statoliths were removed, rinsed in distilled water, air-dried, and then stored in microcentrifuge tubes.

Sample preparation

All equipment was acid-washed with 10% nitric acid and rinsed in distilled water before use, and contact with metallic materials was avoided. Prior to the elemental analysis, all statoliths were decontaminated by immersion for 10 s in 1% diluted nitric acid to remove surface contamination, rinsed in distilled water, and stored in acid-washed microcentrifuge tubes. Then, each statolith was weighed on a microbalance (CAHN29 Automatic Electrobalance®, (Cahn Instruments, Inc., Cerritos, CA, USA) to 0.001 mg (Table 1).

Chemical analysis

Statolith samples were dissolved in 100 μl of 69% nitric acid. Then, 2 separate dilutions were prepared for different analyses. Samples were diluted 1000 times to measure the concentrations of Ca with atomic absorption spectrophotometry (AAS, Perkin-Elmer 5000). Further dilutions were prepared to measure the other elements with a solution-based ICP-MS (VG Elemental Plasma Quad 3, Thermo Scientific, UK). A nitric acid (1%) spike with an internal standard of 10 ppb indium

(indium (In) atomic absorption standard solution®, Aldrich, St. Louis, MO, USA) were used for all dilution procedures, and also analyzed as blanks solutions for monitoring the intensity drift. The results showed that only ⁷Li (lithium), ⁵⁵Mn, ⁵⁹Co (cobalt), ⁶⁰Ni, ⁸⁸Sr, ¹³⁷Ba (barium), and ²⁰⁸Pb were reliably detected from these statoliths, with an optimum dilution factor of 5000.

Statistical analysis

The homogeneity of the variance of the original data was tested using Cochran's C test. All element concentrations required log(x+1) transformation to improve the homogeneity of the variance. However, variances of the Li and Ni data still exhibited non-homogeneous variance, even after transformation.

An analysis of covariance (ANCOVA) was used to test the effect of statolith weight for each element. While elemental concentrations were presented in terms of parts per million (ppm) in statolith weight, fish otolith studies noted that trends in elemental incorporation with individual length and/or otolith weight might confound any stock-specific differences in the elemental composition. Statolith weight is related to squid DML, but the size range slightly differed in the different groups (Fig. 1). Using statolith weight as the covariate, all trace element concentrations, apart from Ni, were significantly negatively correlated with statolith weight (ANCOVA, *p* < 0.05). Statolith weight was therefore used to correct the elemental concentrations for each individual, using the equation:

$$AC = C - r \times SW;$$

where AC is the corrected element concentration, C is the original element concentration, r is the common slope (Table 2), and SW is the statolith weight.

Corrected concentrations of each element were tested using univariate and multivariate analyses of variance (ANOVAs) to see whether differences existed between areas. Homogeneous group tests were followed the Tukey's multiple-comparison test at a significance level of $\alpha = 0.05$ when significant differences were detected from the univariate ANOVA. Significant results of multivariate ANOVA (MANOVA) were followed by a canonical discriminant analysis to test for the source of differences. All analyses were carried out using STATISTICA (vers. 5.5, StatSoft, Tulsa, OK, USA).

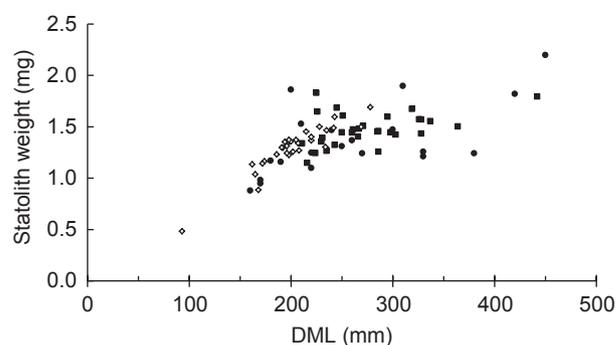


Fig. 1. Relationship between dorsal mantle length (DML) and statolith weight of all samples from different areas. North Sea (●), Irish Sea 1999 (■) and Irish Sea 2000 (◇).

Table 1. Summary of squid samples *Loligo forbesi* for statolith chemical analyses. Shown are the area and date of sampling, sample size (*n*), sample number of each sex (female (F): male (M)), and overall mean value (\pm standard deviation) of dorsal mantle length (DML), body weight (BW), and statolith weight (SW)

Area	Date	<i>n</i> (F:M)	DML (mm)	BW (g)	SW (mg)
North Sea	16 Nov. 2000	5 (0:5)	222 \pm 65	300 \pm 202	1.27 \pm 0.38
	14 Dec. 2000	5 (1:4)	252 \pm 116	491 \pm 627	1.31 \pm 0.52
	18 Jan. 2001	6 (1:5)	258 \pm 69	417 \pm 265	1.47 \pm 0.23
	15 Feb. 2001	4 (0:4)	335 \pm 66	675 \pm 407	1.41 \pm 0.28
Total		20 (2:18)	263 \pm 85	458 \pm 392	1.37 \pm 0.35
Irish Sea	22 Nov. 1999	30 (16:14)	277 \pm 51	552 \pm 259	1.48 \pm 0.16
	30 Oct. 2000	30 (12:18)	203 \pm 35	259 \pm 113	1.30 \pm 0.22
Total		60 (28:32)	240 \pm 57	406 \pm 247	1.39 \pm 0.21

RESULTS

ICP-MS results indicated the Sr comprised about 0.3% of the total statolith weight. Dividing it by the calcium concentration, the average Sr/Ca ratio ranged 0.0062–0.0126 with an average of 0.0085 ± 0.0013 for statolith samples from all areas combined. Samples from the Irish Sea had higher Sr/Ca ratios at 0.0086 ± 0.0012 (mean \pm S.D.) than those from the North Sea (0.0081 ± 0.0015). Site differences were significant (ANOVA, $F_{(2, 98)} = 19.233$, $p < 0.001$). Looking at a smaller scale of temporal variation, statoliths collected from the North Sea showed significant differences between months (Fig. 2A, ANOVA, $F_{(3, 19)} = 6.174$, $p < 0.01$). The Nov. 2000 samples had the highest average value of the Sr/Ca ratio, and significantly differed from those in Jan. and Feb. 2001. However, the Sr/Ca ratio of statoliths from the Irish Sea did not differ between the years 1999 and 2000 (Fig. 2B, *t*-test, *d.f.* = 58, $p = 0.546$).

The concentration of each element was examined by a univariate analysis, and the results showed highly significant differences among the 3 groups (North Sea, Irish Sea 1999, and Irish Sea 2000) in all 7 elements (ANOVA, $p < 0.05$, Fig. 3). Homogeneous groups were examined using Tukey's honest significant difference (HSD) method. Concentrations of Mn, Co, Ni, and Ba were the same between 1999 and 2000 samples from the Irish Sea, while Sr and Pb were slightly higher and Li was slightly lower in 1999. Statoliths from the Irish Sea contained higher Sr, Ba, and Pb and lower Mn, Co, and Ni than those from the North Sea (Fig. 3).

The MANOVA indicated a significant difference in elemental compositions (elemental fingerprints) of statoliths from the 3 samples (MANOVA, Wilks' lambda: 0.193, $F_{(14, 142)} = 12.945$, $p < 0.001$). In order to test which variables (elemental concentrations) could separate squid groups, a discriminant analysis was carried out, using

$\log(x+1)$ -transformed data. The 1st 2 canonical variables explained 83% of the variance. The elements that contributed most to the 1st canonical variable were Sr and Li, while Ni contributed to the 2nd canonical variable. Figure 4 shows the canonical variables of the 1st and 2nd roots of each individual sample. The sample from the North Sea and the sample in 1999 from the Irish Sea showed a clear separation, while the sample from the Irish Sea in 2000 could not be clearly separated from the others. Using the discriminant functions described above, an average of 79% of

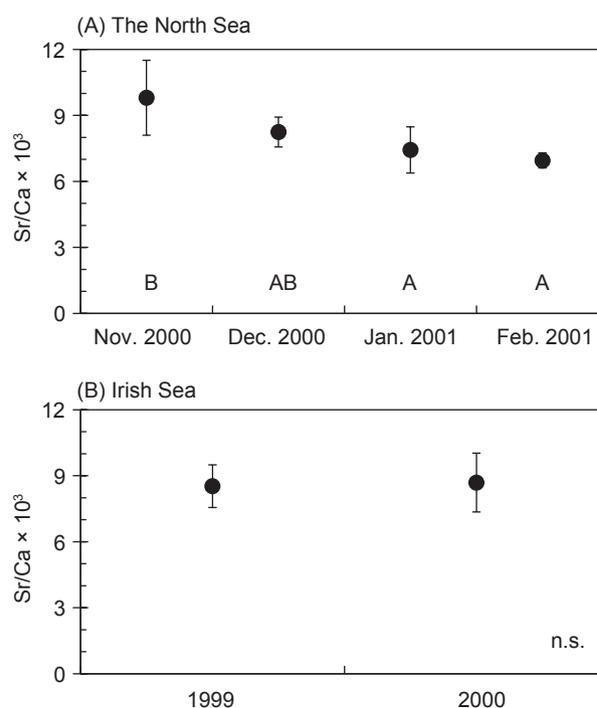


Fig. 2. Sr/Ca ratios in statoliths collected from different (A) months (North Sea samples) and (B) years (Irish Sea samples). Different capital letters (A, B, C) indicate a significant difference after Tukey's multiple-comparison test, and the same letter indicates that mean Sr/Ca ratios were homogeneous between groups. n.s., indicates a non-significant difference between groups.

Table 2. Classification matrix from the discriminant function analysis of statolith elemental compositions

Actual group	Percent correct	Predicted		
		North Sea	Irish Sea 1999	Irish Sea 2000
North Sea	80%	16	0	4
Irish Sea 1999	90%	0	27	3
Irish Sea 2000	67%	9	1	20
Total	79%	25	28	27

the samples could be categorized into the correct groups (Table 2).

DISCUSSION

In the present study, statolith elemental compositions were sufficiently distinct to

separate different sample groups of *L. forbesi*. Statolith Sr/Ca ratios, in particular, showed significant geographic differences. Sr/Ca ratios are considered to be useful proxies of water temperature in coral (Shen et al. 1996) and molluscs (Zacherl 2005), whereas the relationship between Sr/Ca and temperature in fish otoliths is more complex (Kalish 1990, Elsdon and Gillanders

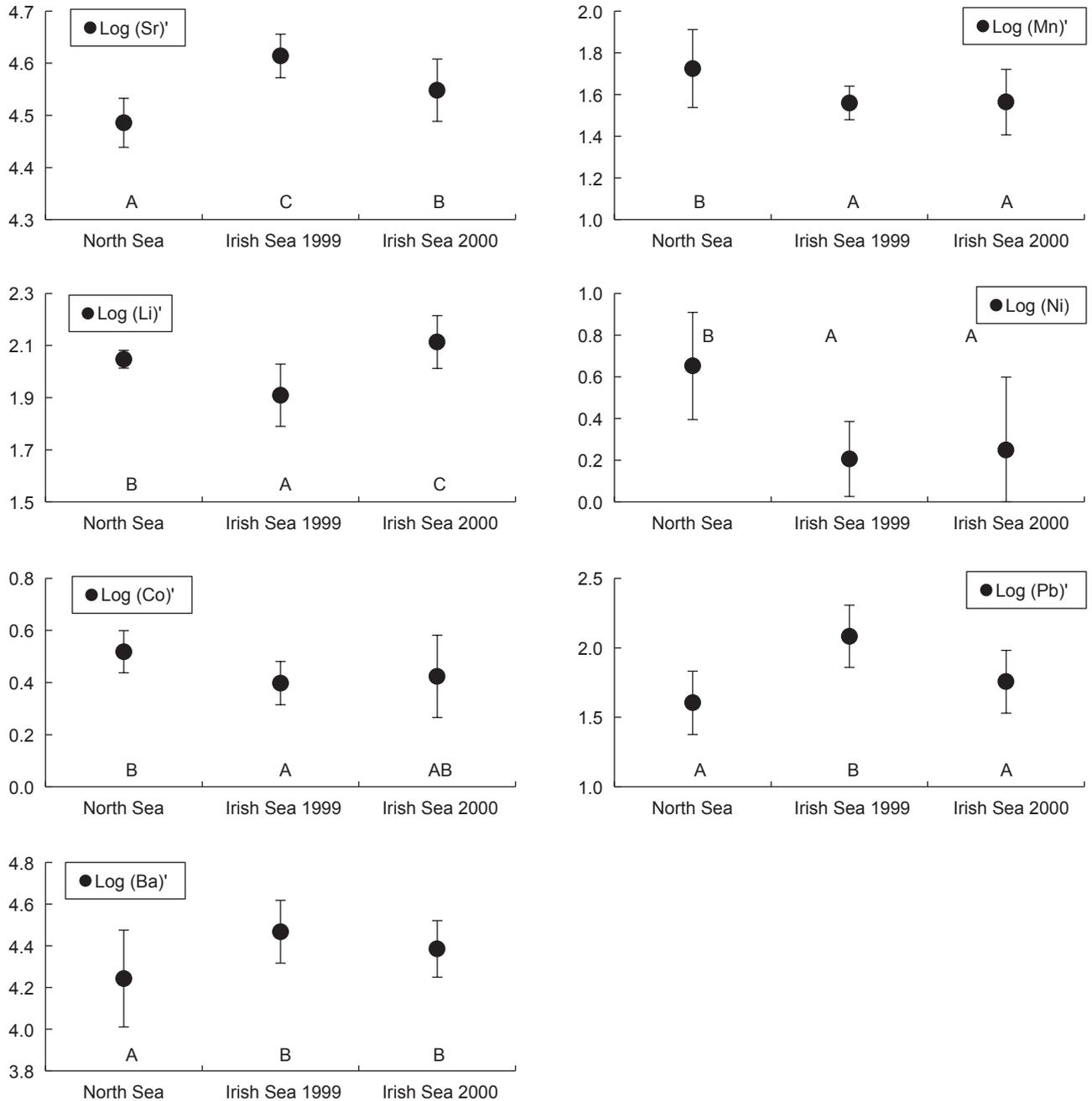


Fig. 3. Mean concentration of each element that significantly differed among areas. Vertical bars indicate the standard deviation. Values of Sr, Li, Mn, Co, Ba, and Pb were detrended. Different capital letters (A, B, C) indicate a significant difference after Tukey's multiple-comparison test, and the same letter indicates that the mean elemental ratios were homogeneous between groups.

2002, Bath-Martin et al. 2004). Concentrations of Sr in statoliths of Japanese common squid *Todarodes pacificus* were negatively correlated to the water temperature (Ikeda et al. 1998), and the same pattern was also found in *L. gahi* statoliths (Arkhipkin et al. 2004). According to the Sr/Ca thermometer hypothesis, a low Sr/Ca ratio is consistent with living in relatively warm water (Ikeda et al. 1998, Arkhipkin et al. 2004). The present study analyzed chemical concentrations of the entire statolith, and the results were consistent with the inverse relationship between the temperature and Sr/Ca ratio. Effects of other oceanographic factors, such as salinity, should be further investigated when applying the Sr/Ca ratio in statoliths as an environmental recorder.

Temporal variations in Sr/Ca was not observed in statoliths collected in 2 consecutively years from the Irish Sea, but significantly varied between 4 consecutive monthly samples from the North Sea. Since the sample size from the North Sea in each month was relatively small, the significant difference might reflect the large variation between individuals. Considering the highly migratory habits of squid, no stock differences in the *L. forbesi* population on the European shelf was hypothesized. However, by comparing all samples collected in winter 2000 (including 5 groups, Irish Sea Oct. 2000 and North Sea Nov. 2000, Dec. 2000, Jan. 2001, and Feb. 2001), the average value of Sr/Ca ratio of

statoliths from the Irish Sea Oct. 2000 did not differ from those of the North Sea Nov. and Dec. 2000 statoliths, but was significantly higher than those of statoliths from squid collected in Jan. and Feb. 2001. This might indicate the existence of 2 different stocks or different spawning groups in the *L. forbesi* population around the Irish Sea. Interestingly, when samples of the Irish Sea 2000 (collected in Oct. 2000) were added to this analysis, Sr/Ca ratios showed a significant difference between samples collected in the same season (winter 2000) (ANOVA, $F_{(4,49)} = 4.17$, $p = 0.006$). The average Sr/Ca ratio of the Irish Sea 2000 sample was between those of the Nov. and Dec. 2000 North Sea samples. All 2000 samples could be grouped together, and significantly differed from the Jan. and Feb. 2001 samples (Tukey's HSD, $\alpha = 0.05$). Weaker geographic differences could represent the squid's highly migratory behavior, while small-scale temporal variations might indicate the existence of different squid populations or cohorts.

In addition to Sr and Ca concentrations, the composition of other elements present in statoliths was first reported by Lipinski et al. (1997). Particle Induced X-ray Emission (PIXE) results showed that elements such as Cr, Mn, Fe, Cu, Zn, Br, and Pb were detectable in some statoliths of chokka squid *L. vulgaris reynaudii* (Durholtz et al. 1997, Lipinski et al. 1997). Concentrations were highly variable with inconsistent trends possibly due to the methods of analyses and conditions of sample preparation, precluding any biologically relevant explanation. The same technique was applied to statoliths of the Japanese common squid, *T. pacificus*, collected from 3 geographical regions (Ikeda et al. 1998). Concentrations of Fe and Cu in *T. pacificus* statoliths were not related to the integrated water temperature (which was used to express the wide range of temperature due to daily vertical movements) or water region, but was more physiologically or ontogenetically related (e.g., production). Cr, Mn, and Zn were also detected but not further analyzed due to the small set of reliable data.

In the present study, statolith elemental compositions were used to distinguish different sampling groups of *L. forbesi*. Using the concentrations of 8 elements, about 79% of the individuals were correctly assigned to their group of origin. The results support the use of statolith elemental fingerprints to serve as natural tags for studying geographic patterns of squid populations.

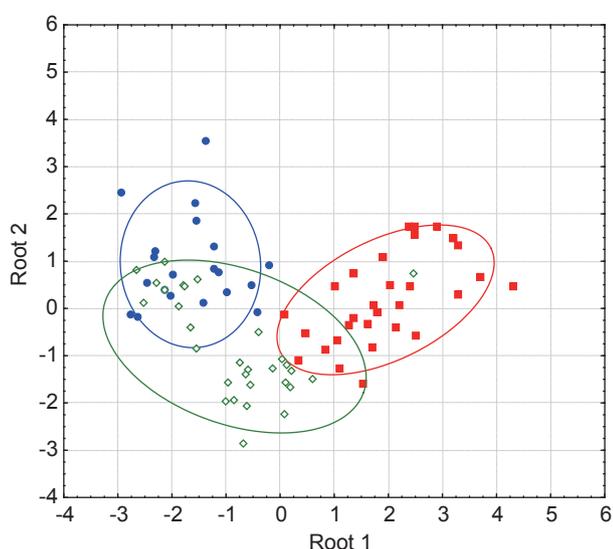


Fig. 4. Plot of the 1st 2 canonical variates from the discriminant analysis comparing the elemental composition of statoliths from the 3 groups of North Sea (●), Irish Sea 1999 (■) and Irish Sea 2000 (◇).

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