1 **Highlights**

- 2 • Elemental chronologies of Ba and Sr were constructed for statoliths of *Doryteuthis gahi* using
- 3 LA-ICP-MS.
- 4 • Temporally distinct spawning cohorts had unique elemental chronologies.
- 5 • Elemental chronologies displayed significant variation throughout ontogeny.
- 6 • Cohort-specific chronologies were most dissimilar during 80-160 d post-hatching.
- 7 • Sr/Ca and Ba/Ca ratios were both negatively correlated with near-bottom water temperature.

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22 **Using statolith elemental signatures to confirm ontogenetic migrations of the squid *Doryteuthis***
23 ***gahi* around the Falkland Islands (Southwest Atlantic)**

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36 **Abstract**

37 The Patagonian long-finned squid *Doryteuthis gahi* is an abundant commercial species within
38 Falkland Island waters. The population consists of two temporally distinct spawning cohorts, inferred
39 to have markedly different patterns of migration and timings of ontogenetic events. Ontogenetic
40 migrations of each cohort were confirmed by analysis of the chemical composition of statoliths
41 collected from both cohorts in two consecutive years. Trace element concentrations were quantified
42 using laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS), to determine
43 temporal and cohort-specific variation. Individual ablation craters, ablated in a transect from the
44 nucleus to the rostrum edge, were aged to produce high-resolution elemental chronologies.

Generalised additive mixed models (GAMM) indicated that cohort and life history stage had a significant effect on Sr/Ca and Ba/Ca ratios. Sr/Ca and Ba/Ca ratios were both negatively correlated with near-bottom water temperature, with Ba/Ca also potentially correlated to depth. Statolith elemental chronologies have useful applications as natural tags, discriminating between spawning cohorts.

Keywords

Doryteuthis gahi, Laser Ablation ICP-MS, Trace Elements, Statolith, Migrations, Cephalopods

1 Introduction

Cephalopods have become an increasingly important fisheries resource over the last few decades, as evidenced by the rapid increase in global landings (Arkhipkin et al. 2015; Doubleday et al. 2016; Hunsicker et al. 2010; Pierce and Portela, 2014). They have an unusual life history characterised by: short lifespan, complex population structure and for many species extensive ontogenetic migrations. Understanding the degree of migration, connectivity and structure of a population is fundamental to the design of effective conservation and management strategies (Gillanders, 2005). Traditional techniques such as tagging allow for insights into an individual's movement and behaviour throughout ontogeny (Gilly et al. 2006; Thorrold et al. 2002). However, these techniques are difficult to implement on small species such as coastal loliginid squid, which are too fragile for an external tag and are lacking a suitable attachment site that does not inhibit their behaviour (Arkhipkin, 2005). An alternative method that requires no prior handling is the geochemical analysis of calcified structures in marine organisms. This method has been shown to have applications in determining population structure (Arbuckle and Wormuth, 2014), migration patterns (Ikeda et al. 2003), assigning natal origin (Pecl et al. 2011) and as a proxy for environmental parameters (Beck et al. 1992). Analysis of elemental signatures has been applied to a wide range of taxa such as; scleractinian corals (Beck et al.

70 1992), teleost fish (Campana, 1999), gastropods (Zacherl et al. 2003), medusae (Mooney and
71 Kingsford, 2012), bivalves (Gillikin et al. 2008) and cephalopods (Arbuckle and Wormuth, 2014;
72 Warner et al. 2009).

73 Statoliths are paired calcareous concretions found within the statocysts, responsible for the detection
74 of linear and angular acceleration in cephalopods (Arkhipkin and Bizikov, 2000; Clarke, 1978).
75 Analogous to fish otoliths, these hard structures grow continually throughout life and are formed by
76 the deposition of calcium carbonate crystals, primarily in aragonite form, within a protein matrix
77 (Radtke, 1983). Throughout the accretion process, trace elements are incorporated into this matrix
78 (Arkhipkin, 2005; Bettencourt and Guerra, 2000). Uptake of these elements into the statolith
79 microstructure is considered to reflect the ambient environmental conditions at the time of
80 incorporation. For example, Sr/Ca ratios have been suggested to have a negative relationship with
81 temperature in many biogenic calcified structures in a wide range of taxa (Beck et al. 1992; Campana,
82 1999; Galante-Oliveira et al. 2015; Zacherl et al. 2003). Statoliths are appropriate for use as a natural
83 tag on account of their metabolic inertness after deposition and the incorporation of continuous
84 growth increments, which give a temporal scale for analysis (Wang et al. 2012).

85 The Patagonian long-finned squid *Doryteuthis gahi* (D'Orbigny, 1835) is a cold water loliginid most
86 abundant in Falkland Islands waters, where it is subject to an important and economically valuable
87 commercial fishery. It is a small species, typically attaining an adult size of 13-17 cm mantle length
88 (Arkhipkin et al. 2013). As well as being a key fisheries resource, *D. gahi* is involved in the transfer
89 of organic and inorganic material across various parts of the shelf ecosystem and has an important
90 role both as predator and prey (Arkhipkin, 2013). It is an important food source for marine mammals
91 (Arkhipkin, 2013), commercially important finfish (Laptikhovsky et al. 2010) and seabirds
92 (Piatkowski et al. 2001).

93 These squid undergo horizontal ontogenetic migrations from shallow inshore spawning and nursery
94 grounds to feeding aggregations on the shelf edge and continental slope, where they are targeted by
95 the fishery (Arkhipkin et al. 2004a; Hatfield et al. 1990). The fishery operates within two seasons

96 corresponding to two temporally distinct cohorts (Patterson, 1988), the autumn spawning cohort
97 (ASC) and spring spawning cohort (SSC), both of which have an annual life cycle. The duration of
98 embryogenesis, extent of offshore migration and time of spawning differ between cohorts (Arkhipkin
99 et al. 2013).

100 An initial study which determined the elemental composition of *D. gahi* statoliths using solution-
101 based inductively coupled plasma mass spectrometry (ICP-MS) found significantly different
102 elemental signatures between cohorts and geographic regions (Arkhipkin et al. 2004a). As the study
103 dissolved the entire statolith and quantified its elemental composition, it was unable to determine how
104 the elemental signal changed throughout ontogeny. Alternative analytical techniques such as ion-
105 probe or laser ablation analysis are able to target specific regions of the statolith microstructure to
106 gather stage-specific information on life history and migration patterns.

107 A good understanding of migration patterns is of particular importance in short-lived species such as
108 *D. gahi*, which show a strong inter-annual variability and are more sensitive to factors such as
109 increased fishing pressure and environmental change (Doubleday et al. 2016). Therefore, the aims of
110 this study were to generate temporally resolved elemental chronologies for each individual by
111 quantifying trace elements in statoliths, to compare the elemental chronologies generated for each
112 spawning cohort and to explore possible relationships between environmental factors and the
113 element/Ca ratio at the corresponding time of incorporation.

114

115 **2 Materials and methods**

116 *2.1 Statolith Collection and Preparation*

117 Squid were collected and frozen by scientific observers on board commercial trawling vessels within
118 the Falkland Islands Interim Conservation and Management Zone (FICZ). Data collection took place
119 during both fishing seasons (Season 1: March-May, Season 2: July-October) of both 2014 and 2015
120 within the designated fishing zone, the ‘Loligo box’ and to the west of the Falkland Islands (Fig. 1).

121 Mature individuals of both sexes were selected to ensure that each individual had a long elemental
122 chronology representing as many life history stages as possible (Table 1).

123

124 **Figure 1: Sample locations within the Falkland Interim Management and Conservation Zone (FICZ). The Loligo box**
125 **denotes the fishing area. Monthly oceanographic data were collected along the CTD transect (dotted line). The inset**
126 **map indicates the position of the Falkland Islands in relation to mainland South America.**
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130 Squid samples were processed in the Falklands Island Fisheries Department laboratory. Samples
131 were measured (dorsal mantle length ± 1 mm), weighed (total weight ± 1 g) and visually assessed for
132 sex and maturity stage according to Lipinski (1979). Statoliths were dissected from the cephalic
133 cartilage and stored in 96% ethanol. One statolith per specimen was mounted for elemental and age

analysis concave side up, then ground and polished on one side to expose the nucleus (Arkhipkin and Shcherbich, 2012).

Table 1: Summary of samples collected. N = number of samples collected.

Collection Year	Cohort	Mean Collection Depth (m) $\pm$ SE	Sex	N	Mantle Length (cm)	Age range (days)	Mean age (days)
2014	ASC	185 ± 2.3	M	23	9.0-34.5	181-322	241.8
			F	11	9.0-18.0	185-274	223.7
	SSC	230 ± 1.2	M	49	7.0-38.0	152-312	252.7
			F	11	8.5-16.5	173-237	205.2
2015	ASC	178 ± 1.8	M	34	10.0-33.0	169-289	226.9
			F	21	10.5-20.0	165-258	218.7
	SSC	230 ± 3.1	M	24	10.0-31.5	171-288	227.6
			F	12	10.0-21.5	163-248	206.1

2.2 Trace Element Analysis

Statoliths were analysed using laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) at the British Geological Survey, Nottingham. Ground statoliths (N = 271) were remounted using Crystalbond 555 in batches of 12, with contaminants removed from the ground surfaces using a solution of 1% HNO₃/ 0.5% HCL prior to analysis. Elemental concentrations were obtained using a 193 nm Class 4 Nd: YAG solid state excimer lamp-pumped laser ablation system (New Wave Research, USA) coupled with an *in situ* Agilent 7500c ICP-MS. Values for limit of detection (LOD) were calculated as 3 standard deviations of the background signal. The following trace elements were quantified at each ablation spot (with mean values for LOD in ppm included in brackets); ²³Na (22.807), ⁸⁸Sr (1.373), ²⁴Mg (0.581), ¹¹B (2.381), ⁷Li (0.183), ¹³⁸Ba (0.018), ²⁷Al (1.298), ⁵⁵Mn (1.207), ⁵⁶Fe (1.663), ⁶⁶Zn (0.269), ⁶³Cu (0.833), ¹¹⁴Cd (0.167) and ²⁰⁸Pb (0.022), with ⁴²Ca used as an internal standard to account for variation in ablation yield. Elements Pb, Cu, Zn, Fe, Al, Cd and B were consistently below the theoretical LOD and were subsequently removed from further analysis.

Equally incremented ablations (35 μ m $\varnothing$, spacing 70 μ m) were made along the axis of growth from the nucleus to the marginal edge of the rostrum (Fig. 2). The number of ablation sites ranged from 12 to 20 per statolith depending on statolith total length, which has been shown to have a linear

155 relationship with mantle length (Hatfield, 1991). Ablation parameters were as follows; a pulse rate of
156 10 Hz with an average irradiance of 0.67 GW/cm², an average fluence of 3.33 GW/cm² and a dwell
157 time on each ablation spot of 30 s. Ablated material was transferred from the laser ablation cell to the
158 ICP-MS in a flow of helium (0.8 l min⁻¹) which was then combined with a stream of argon carrier gas
159 (0.9 l min⁻¹).

160 Due to a lack of appropriate matrix matched calibration standards, the glass reference materials NIST-
161 610 and NIST-612 (National Institute of Standards and Technology, USA) were used for external
162 calibration (Limbeck et al, 2015). The concentration of Na in the NIST-610 glass is approximately
163 two orders of magnitude higher than that of statoliths, which may give rise to inaccuracies, and is
164 therefore not recommended for calibration (O'Neil and Gillikin, 2014). Following Limbeck et al.
165 (2015), To to minimise this the risk of inaccuracies, cross-calibration factors and the detector voltages
166 are were regularly updated. NIST-610 was ablated at the start and end of each slide, and after every
167 fourth sample to calibrate elemental concentrations and assess changes in instrumental sensitivity.
168 NIST-612 was measured at the start of each slide and treated as an unknown sample to assess
169 measurement accuracy (for further details see Limbeck et al., 2015). Periods of gas blank collected
170 for 40 s prior to each ablation were defined as background, the average of which was subtracted from
171 subsequent samples. Raw counts (cps) of each element were processed using Igor Pro 6. 34
172 (WaveMetrics) with the extension package Iolite v. 2.5.

173 2.3 Age Estimation

174 Ablated statoliths were flipped and ground on the other side, overturned again to better visualise
175 ablation craters, embedded in mounting medium (Canada BalsamTM) and covered with a cover glass
176 for observation. Statoliths were read under the transmitted light of an Olympus BX51 compound
177 microscope at x400 magnification, with a phase-contrast Nomarski effect used to improve readability
178 (Arkhipkin and Shcherbich, 2012).

179 Statoliths were first read in the lateral dome from the natal ring to the first prominent check. This
180 check was followed into the rostrum and read in the rostrum to the tip. As the one ring=one day

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Is this why Limbeck et al did what they did? If you are using Limbeck et al as justification, please make this clearer

hypothesis has been validated in other loliginid species (e.g. *Loligo plei* [Jackson and Forsythe, 2002], *Loligo vulgaris reynaudii* [Lipinski et al., 1998], *Sepioteuthis lessoniana* [Jackson et al., 1993]), total increment number was assumed to represent post-embryonic age in days. To minimise counting errors, total number of growth increments for each specimen was taken as the mean of at least two counts. If the difference between these counts exceeded 10% a third count was made. If the difference between the second and third readings still exceeded 10% the statolith was rejected from further age analysis. A sub-sample of 10 statoliths was counted by a second reader to further minimise error (First reader J. Jones, second reader A. Arkhipkin). A total of 272 statoliths were prepared, of which 185 (68%) were successfully ablated and read. Statoliths were discarded throughout the process either because 1.) Ablation craters were too shallow to be visualised under the microscope for ageing 2.) The statolith was over ground or 3.) Initial data exploration suggested contamination (indicated by the combined presence of Zn, Cu and Fe), which is most likely a result of material settling in cracks in the surface during grinding. This has been indicated to be a common issue during elemental analysis of statoliths (Arbuckle and Wormuth, 2014). Statoliths were ablated prior to age analysis as thin ageing sections are prone to breakage during slide transfer and because each individual ablation spot is individually aged after LA-ICP-MS. Age analysis prior to LA-ICP-MS with a large sample size would add a significant amount of time to the process.

Hatch date was determined by back-calculation of total number of increments (=age in days) from the date of capture. Based on hatch date, each squid was assigned to a spawning cohort; those with hatch dates in July – October were assigned to the ASC and those with hatch dates from November – May were assigned to the SSC (Arkhipkin et al., 2004a). Each individual ablation spot was aged to produce time-delimited elemental signatures. Visual inspection indicated that each ablation spot represents approximately one week during an individual's life. The central growth ring of each ablation spot was considered to be the ablation spot age (days post hatching - Fig. 2).

Figure 2: Image of ground statolith. Region delimited by the white line contains 7 growth increments. Lines between growth increments are optical effects. Circles indicate regions where material has been ablated. Inset image shows a statolith with 12 ablation spots from the central nucleus to the rostrum edge.

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210 2.4 Oceanographic Data Collection

211 Monthly oceanographic data have been routinely collected by the Falkland Islands Fisheries
 212 Department on board the Falkland Islands fisheries patrol vessel (Fig. 1). These data were collected
 213 along a transect located in the southern part of the ‘Loligo box’ using a SeaBird Electronics SBE 25
 214 CTD (Sea-Bird Electronics Inc. Bellevue, WA, USA). Transects were carried out approximately mid-
 215 month subject to vessel availability and weather conditions. The CTD was lowered to the seabed and
 216 retrieved at a rate of 1 ms⁻¹. Temperature (°C) was measured directly and salinity (PSU) was derived
 217 from conductivity (S m⁻¹) using Seasoftware v. 4.326 software (Sea-Bird Electronics Inc.). Transect
 218 stations (with depths of 55 m, 60 m, 100 m, 200 m and 300 m) covered the reported species depth
 219 range (Arkhipkin et al. 2013). Near-bottom temperature and salinity profiles were interpolated for
 220 each transect using data collected from May 2013 to December 2015.

221 2.5 Statistical Analysis

Each element was standardised to calcium to produce element/Ca ratios and further analysed in R V.3.3.0 (R Core Team, 2016). For the purposes of this study, particular focus was placed on Sr/Ca and Ba/Ca ratios, as these are often cited to have a relationship with environmental parameters and were found in high concentration within the statolith microstructure.

Data exploration was undertaken following procedures described in Zuur et al. (2010). Extreme outliers observed in Cleveland dotplots were removed prior to analysis. For visual presentation, element/Ca ratios for individual ablation spots were pooled by the number of weeks post-hatching, separately for both assigned cohorts. Mean concentrations for each week were plotted with a 3-point moving average applied to improve visualisation. For statistical modelling, raw post-hatching ages (in days) were used.

The data set consisted of multiple time observations for each statolith. We therefore applied two generalised additive mixed models (GAMM), one using Sr/Ca ratios and the other using Ba/Ca ratios as the response variable, with the random effect *Statolith.no* (a unique identifier for each individual) nested within cohort as a way to model dependency (Zuur and Ieno, 2016). Statistical modelling was performed in the R package “mgcv” (Wood, 2006).

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A Gaussian GAMM (identity link) was used for Sr/Ca (Equation 1) and Ba/Ca (Equation 2) to determine whether element/Ca ratios could be linked with cohort assignment and ontogenetic time. Fixed covariates available were sex (categorical with two levels), year (of collection; categorical with two levels) and cohort (categorical with two levels). The addition of an interaction term between year and cohort was investigated. A The categorical variable of *cohortyear* (with 4 levels: ASC-2014, SSC-2014, ASC-2015 and SSC-2015) was also investigated and compared to model performance with cohort and year included as separate factors. The variable day (within the year, a categorical variable ranging from 1-365) had a smoother fitted using cyclic cubic regression splines, penalised cubic regression splines whose ends meet up. The remaining variable, age (number of days post-hatching) is continuous and a smoother was fitted using thin plate regression splines (Wood, 2003). To incorporate the dependency among observations of the same statolith, we treated *Statolith.no* as a

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248 random variable. The random effect of location was also explored, using station as a proxy to capture
 249 the spatial component (each station was a unique trawl identifier, with each trawl having a different
 250 spatial location).

251 Equation 1:

$$g(E(Sr_{ij})) = Statolith.no_i + f_{ij}(Age \times Cohort) + Cohort \times Year + \varepsilon_i$$

$$\varepsilon_i \sim N(0, \sigma^2)$$

252 Equation 2:

$$g(E(Ba_{ij})) = Statolith.no_i + f_{ij}(Age \times Cohort) + Cohort + \varepsilon_i$$

$$\varepsilon_i \sim N(0, \sigma^2)$$

253 Where element_{ij} is the j^{th} observation in Statolith.no i , and ε_i is the random intercept, which is assumed
 254 to be normally distributed with mean 0 and variance σ^2 .

255 Examination of residuals suggested a transformation was necessary to avoid violating the assumptions
 256 of normality and to reduce heterogeneity. A box-cox transformation was applied (Box and Cox,
 257 1964) which improved the residual fit, defined as:

$$y_i^\lambda = \begin{cases} \frac{y_i^\lambda - 1}{\lambda_i}, & \lambda \neq 0 \\ \ln y, & \lambda = 0 \end{cases}$$

258 Optimal GAMs were selected via backwards selection on the basis of the lowest value of Akaike
 259 Information Criterion (AIC), providing that there were no serious patterns in residuals and all
 260 remaining explanatory variables had a significant effect. The mixed model random components were
 261 then added to the optimised GAM.

262

263 3 Results and discussion

264 3.1 Temporally resolved elemental ratios

265 Growth ring analysis indicated an age range of 152 – 322 d and a mean age of 233 d (Table 1).
 266 Elemental concentrations of *D. gahi* statoliths, expressed as element/Ca ratios are displayed in Table 2
 267 for elements which normally have concentrations above the LOD. The mean values for each
 268 element:Ca ratio were generally similar to those found in the previous solution-based ICP-MS study
 269 on *D. gahi* (Arkhipkin et al. 2004a), which reported an approximate Sr/Ca ratio of 8 mmol.mol⁻¹ (this
 270 study $\mu = 9.46$ mmol.mol⁻¹) and Ba/Ca ratios ranging from 3 to 8 μ mol.mol⁻¹ (this study 4-16
 271 μ mol.mol⁻¹). Mn/Ca ratios were also similar, ranging from 0.02-9.9 μ mol.mol⁻¹ (1-3 μ mol.mol⁻¹ in
 272 the previous study). However, the ranges of values found in the present study are much broader for
 273 each element. In particular, Mg (30 – 535 μ mol.mol⁻¹) had a much broader range than the previous
 274 solution based study (70 – 170 μ mol.mol⁻¹). This is likely to be due to methodical differences. If
 275 element concentrations vary widely across an individual statolith, this would not be apparent in a
 276 study based on measurements for whole statoliths. It is also possible that measurement error is higher
 277 for the ablation method, however sampling procedures ensured that measurement error (due to
 278 instrumental drift and high sensitivity detecting contaminants) were minimized. The present study is
 279 consistent with the previous study (Arkhipkin et al. 2004a) in that elemental signatures of *D. gahi*
 280 varied significantly geographically and between spawning cohorts.

281 **Table 2: Summary of element:/Ca ratios for *D. gahi* statoliths.**

Element	Minimum	Maximum	Mean $\pm$ SD
Na:/Ca (mmol.mol ⁻¹)	16.18	28.39	20.48 $\pm$ 1.47
Sr:/Ca (mmol.mol ⁻¹)	5.84	14.57	9.46 $\pm$ 1.24
Mg:/Ca (μ mol.mol ⁻¹)	33.89	535.38	140.43 $\pm$ 76.23 (139.74 $\pm$ 40.28) *
Li:/Ca (μ mol.mol ⁻¹)	0.58	280.47	33.13 $\pm$ 34.10
Ba:/Ca (μ mol.mol ⁻¹)	4.52	16.54	7.73 $\pm$ 1.48
Mn:/Ca (μ mol.mol ⁻¹)	0.02	9.89	2.61 $\pm$ 1.26

282 * Additional mean $\pm$ SD value where four Mg/Ca values $> 400 \mu\text{mol.mol}^{-1}$ removed that are likely to be anomalies

283 Typically, laser ablation studies using statoliths as sample material involve fewer than 40 individuals.
284 This is the first study to conduct this type of analysis on a much larger scale, allowing for cohort-
285 specific elemental chronologies to be generated, as observed in Fig. 4B and 4D. For both Sr and Ba,
286 element/Ca ratios exhibited considerable ontogenetic variation and the elemental signatures for each
287 cohort are noticeably different. Cohort-specific elemental signatures affirmed the results of Arkhipkin
288 et al. (2004a), with the inclusion of separate smoothers (for the Age effect) for the ASC and SSC
289 significantly improving the model fit for both response variables, as shown by analysis of deviance
290 (Sr model: $F = 6.65$, $p < 0.05$, Ba model: $F = 11.01$, $P < 0.05$). The chemical composition of statoliths
291 from the ASC and SSC are most dissimilar during 80-160 days post-hatching (12-23 weeks) where a
292 clear gap is seen between the 95% confidence intervals of the two cohorts (Fig. 3).

293 **Figure 3: Fitted curves based on model predictions for the concentration of A – Ba/Ca and B- Sr/Ca (on box-cox**
294 **transformed scale) ratios in relation to post hatching age in days, with individual smoothing curves constructed for**
295 **each cohort and 95% confidence intervals included, represented by dotted lines.**
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 299 Modelling results are found in(Table 3) and confirmed that time during ontogeny (squid age), and
 300 cohort assignment were significantly related to element/:Ca concentration for both Sr ($R^2 = 0.37$) and
 301 Ba ($R^2 = 0.28$). Error increases in the modelConfidence limits around fitted values are wider towards
 302 the latter stages of ontogeny, with fewer available data that were only available from the oldest (and
 303 largest) individuals. Although year itself was not significant, the interaction between year and cohort
 304 was significant for strontium ($F = 2.01$, $p = 0.04$). Contrary to the solution-based study (Arkhipkin et
 305 al. 2004a), inclusion of a spatial component in the model did not improve fit, implying that no
 306 significant difference could be found in elemental signatures from different geographic locations.

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Table 3 Estimated regression parameters for two-way nested generalized additive mixed models (GAMM) for the response variables Sr/Ca and Ba/Ca, as presented in eqn (1) and eqn (2). F_t-values and P- values are included, as well as expected degrees of freedom (edf) for smooth terms. Significance, *** = p<0.0001, ** = p<0.01, * = p<0.05.

Response variable	Explanatory variable	edf	F- value	P-Value
Sr/Ca	Cohort	-	-3.07	< 0.05**
	Year	-	-0.63	0.52
	Cohort:Year	-	2.01	< 0.05*
	Days:Cohort_ASC	7.65	178.6	< 0.05***
	Days:Cohort_SSC	7.35	156.5	< 0.05***
Ba/Ca	Sex	-	-2.99	< 0.05**
	Days:Cohort_ASC	6.69	121.4	< 0.05***
	Days:Cohort_SSC	6.20	101.8	< 0.05***

3.2 Sr/Ca ratios

Ontogenetic variation in mean Sr/Ca ratio for both cohorts (ASC and SSC) and consecutive years (2014 and 2015) is shown in Fig. 4B. In all four groups there is a sharp increase in Sr concentration during the first 5 weeks of life, with each individual's Sr/Ca ratio increasing by approximately 1.5 mmol.mol⁻¹ during this early stage of ontogeny. After the first 5 weeks of post-hatching life, ontogenetic patterns for each cohort diverge; The general trends for the ASC cohort is an increase in Sr/Ca concentration with a corresponding decrease during February - March. The SSC cohort is characterised by a progressive increase in Sr/Ca ratio over time. This is also observed in the fitted smoothing curves from the GAMM model in Fig. 3B. The smoothing curve applied to the SSC had lower expected degrees of freedom than the ASC indicating a less complex trend, closer to linear (Table 3).

Figure 4: A –Seasonal changes in near-bottom temperatures at various depths on the Falkland Islands shelf overlaid with a scheme of the ontogenetic migrations of the ASC (grey) and SSC (black), adapted from Arkhipkin et al. 2004b. Circles indicate periods of egg development. B –Sr/Ca elemental signatures for the ASC (grey) and SSC (black) in 2014 (dashed line) and 2015 (line). C –Seasonal changes in near bottom salinity. D – Ba/Ca elemental signatures for the ASC (grey) and SSC (black) in 2014 (dashed line) and 2015 (line).

333 It is evident from the cohort-specific ontogenetic patterns that Sr/Ca ratios show high potential to
334 discriminate between population components. An investigation into how elemental patterns (obtained
335 from 5-6 ablation spots per statolith) vary between geographically separate groups of the Humboldt
336 squid *Dosidicus gigas* similarly found that Sr/Ca ratios were significantly different between
337 population sub-groups (Arbuckle and Wormuth, 2014). Geographical variation in Sr/Ca ratios were
338 also found when comparing two groups of the ommastrephid squid *Todarodes pacificus* in the Sea of
339 Japan (Ikeda et al., 2003). Nevertheless, to date the only squid found to have cohort-specific
340 differences in the Sr/Ca elemental signature has been *D. gahi*. The only other study comparing
341 spawning cohorts, **who which analysed** the multivariate elemental signatures of the ommastrephid
342 squid *D. gigas*, found no significant differences between three cohorts (Liu et al., 2015). Unlike
343 loliginids, ommastrephid squid undergo extensive diel-vertical migrations that are likely to mask the
344 patterns arising from horizontal migration (Arkhipkin et al. 2004a).

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345 The dynamics of incorporation of trace elements into calcified structures of organisms may be
346 regulated by many different factors, both intrinsic and extrinsic in nature, for example; physiology,
347 ontogenetic stage, reproduction, diet, stress level, temperature, salinity and water chemistry (Radtke
348 and Shafer, 1992). In reality, the chemical composition of calcified tissues is most likely driven by a
349 multiplicity of factors at any particular moment of a squid's life. However, the primary factors driving
350 the deposition are likely to be different for each trace element.

351 Many experimental studies have shown a strong association between Sr/Ca ratios and temperature.
352 An inverse relationship was first found in scleractinian corals (Beck et al. 1992; Smith et al. 1979) and
353 was later found in a wide range of marine taxa. In the herring *Clupea harengus*, laboratory
354 experiments indicated that the greatest temperature effect occurred at lower temperatures, with
355 temperatures less than 5 °C physiologically impairing their ability to discriminate against the
356 incorporation of strontium into the otolith (Townsend et al. 1992). As *D. gahi* is the coldest water
357 loliginid, inhabiting near-bottom waters with minimum temperatures of ~ 4.9 °C, the temperature
358 effect on strontium uptake may be pronounced for this species. In cephalopods, attempts to establish
359 strontium as a temperature proxy have so far been unsuccessful. Those studyingSome studies of

360 ommastrephid squid, such as the Humboldt squid *D. gigas*, often fail have failed to establish a
 361 significant relationship between Sr/Ca and temperature (Ikeda et al. 2002; Liu et al. 2011, 2015),
 362 although some others have found negative relationships (Liu et al. 2013, 2016). As stated above, the
 363 extensive diel-vertical migrations undertaken by oceanic ommastrephids likely mask horizontal
 364 temperature effects (Arkhipkin et al. 2004a). For loliginid squid, some circumstantial evidence for a
 365 negative correlation between Sr/Ca and temperature has been found in *D. gahi*. Those living in
 366 warmer Peruvian waters at ~11-12 °C had significantly lower Sr/Ca ratios than those living in the
 367 South Atlantic at ~4.9-9 °C (Arkhipkin et al. 2004a). Comparisons of Sr/Ca concentrations between
 368 loliginid species has indicated that temperate *Loligo bleekeri* from Japanese waters have significantly
 369 higher concentrations of these elements in their statoliths than those of tropical *Loligo divauelei*,
 370 *Loligo chinensis* and *Loligo edulis* from the Andaman Sea and Gulf of Thailand (Ikeda et al. 1997).
 371 In cuttlefish (*Sepia officinalis*) reared under laboratory conditions found there was no relationship
 372 between Sr/Ca and temperature when comparing three different temperature treatments.
 373 Experimental studies on squid are required to ascertain the exact relationship between temperature
 374 and strontium uptake into squid statoliths. However, squid are notoriously challenging to rear under
 375 laboratory conditions so this may be difficult to resolve.
 376 Water chemistry and diet are also thought to be drivers of Sr incorporation in calcified tissues. The
 377 dietary effect on Sr incorporation was found to be up to 10% in experimental studies on statoliths of
 378 the cephalopod *S. officinalis* (Zumholz et al. 2006). For the damselfish *Acanthochromis*
 379 *polyacanthus*, effects of ontogenetic stage and diet interacted to drive the incorporation of Sr (Walther
 380 et al. 2010). In terms of water chemistry, dramatic drops in Sr/Ca values have been associated with
 381 ontogenetic movements from seawater, relatively rich in Sr to low-Sr freshwater environments
 382 (Radtke et al. 1988).
 383 *D. gahi* inhabits near-bottom layers of the Patagonian shelf and undergoes extensive inshore-offshore
 384 ontogenetic migrations from shallow nursery grounds to deep offshore feeding grounds on the shelf
 385 edge (Arkhipkin et al. 2013). This species therefore experiences significant variation in a multitude of
 386 environmental factors during migration which may affect elemental uptake into the statolith

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microstructure. Although squid of both cohorts have an annual life cycle and occur on the same feeding grounds (Agnew et al. 1998; Patterson, 1988), differences in the timing of ontogenetic events such as spawning and hatching, and inter-annual fluctuations in temperature and salinity mean that they are subject to very different environmental conditions throughout their lifetime (Arkhipkin et al. 2013). Therefore, when comparing elemental profiles to environmental events, profiles for each cohort should be considered separately.

A schematic diagram of the life-cycle of each cohort is presented in Fig. 4A. Post-hatching juveniles and paralarvae of the ASC remain inshore in shallow nursery grounds, undertaking gradual offshore migration to feeding grounds at 100-150 m depth as shallow nursery waters start to warm and squid start to mature (Arkhipkin et al. 2004b). In autumn (March-May), the ASC move inshore to spawn with peak spawning occurring in May to June (Hatfield and Des Clers, 1998). Squid of the SSC have a markedly different life history. From December-February paralarvae and juveniles stay inshore, gradually moving offshore during autumn and usually finishing their offshore migrations in May-June. The equality in temperatures from shallow sites to depths of 200 m in late autumn (April-May) allows this cohort to penetrate to deeper than the ASC. With winter cooling in shallow sites and the formation of a warm layer at 150-250 m, squid are restricted to this warm water layer and remain on feeding grounds (with no inshore migration) until the end of October, when they rapidly return to shallow depths to spawn (Arkhipkin et al. 2004b, 2013).

For both cohorts, ontogenetic patterns in mean Sr/Ca concentration over time are consistent with a negative relationship with temperature, when taking into account this lifecycle. For the ASC, the general pattern across the statolith microstructure is an increase in Sr/Ca ratio with a corresponding decrease during late summer (Fig. 4B). The increase in concentration occurs synonymously with the timing of the offshore migration to cooler deeper waters, as would be expected if a negative relationship with temperature existed. At depths where squid are feeding, i.e. at ~150 m, delayed warming occurs in March. Assuming a negative relationship with temperature, reduction in Sr/Ca from February- March corresponds to an increase in near bottom water temperatures on their feeding

grounds during this period. Further reductions in the Sr/Ca ratio correspond to a further increase in temperature as the squid undergo inshore migrations to shallower warmer waters in March - May.

The Sr/Ca elemental profile for the SSC is also consistent with a negative relationship with temperature (Fig. 4B). After the initial peak, Sr/Ca concentration progressively increases over time, consistent with the gradual migration of this cohort down the shelf into deeper and cooler waters. As samples were all collected prior to October (when this cohort undergoes its inshore migration), there is no return migration (if there were a negative relationship, a decrease in Sr/Ca ratio as squid move inshore to warmer waters) evident in the elemental profile. It is therefore likely that the elemental incorporation of strontium into statoliths is linked to the physiochemical properties of the ambient environment.

However, a negative correlation between Sr/Ca and temperature is not consistent with the sharp increase in concentration observed in the first five weeks of life when the squid do not undergo extensive migrations and are therefore unlikely to be exposed to such high temperature variability (Fig. 4B). As the majority of biological sampling of *D. gahi* takes place on fishing grounds, early ontogenetic phases prior to the size at which individuals recruit into the fishery (~6 months of age) have not been studied in such detail (Hatfield and Rodhouse, 1994). Factors driving this phenomenon during the first 5 weeks of life are therefore unclear. This pattern may be a result of the interactive effects of several factors, as during this period of ontogeny, squid are undergoing rapid development and significant morphological changes that are likely to affect elemental incorporation. In addition, changes in salinity and temperature are pronounced in the shallow waters where squid hatch (15-30 m) and where their nursery grounds are found (up to 70 m depth) compared to their offshore feeding grounds (> 100 m [Arkhipkin et al. 2013]). A combination of intrinsic and extrinsic factors is likely to be contributing to the observed patterns during this transitional period.

3.3 Ba/Ca ratios

Ontogenetic variation in mean Ba/Ca concentration for both cohorts (ASC and SSC) and consecutive years (2014 and 2015) is shown in Fig. 4D. An increase in Ba/Ca concentration, though not as

substantial as for the Sr/Ca ratio, is evident in the first 5 weeks of life for both cohorts and both years. Following this common pattern, Ba/Ca ratio gradually increases for the ASC followed by a corresponding decrease, similar to the pattern observed in the Sr/Ca elemental pattern. Comparison of GAMM smoothers for Ba and Sr indicates that the trends observed in the ASC for both elemental profiles are similar (Fig. 3). Ba/Ca ratio increases for the SSC throughout ontogeny, with a plateau in the elemental signature during March-April.

Ontogenetic variation in the concentration of Ba/Ca was substantially different for each cohort, with a significant effect of time during ontogeny and cohort assignment found during GAMM model fitting (Fig. 4C). Thus, as with strontium, it is likely that the Ba/Ca ratios may be useful as a natural tag to distinguish between cohorts. Similar results were reported in Arbuckle and Wormuth (2014), who found Ba/Ca patterns across the statolith microstructure to be highly variable among different subgroups (based on geographic location).

Barium has a nutrient-type distribution in the ocean, with concentration increasing with depth, or indicating upwelling events (Lea et al. 1989). Concentration of Ba in the surrounding seawater has been shown experimentally to be an important factor contributing to the uptake of Ba in fish otoliths (Walther and Thorrold, 2006) and has been inferred to be an important factor for uptake of Ba in cephalopod statoliths (Arkhipkin et al. 2004a), with Ba uptake increasing with depth. As well as water chemistry, temperature has been proposed to be an important factor affecting uptake of barium into biogenic aragonites. Laboratory experiments under different temperature and salinity conditions indicated no relationship with salinity and a negative relationship between temperature and Ba/Ca ratios for statoliths of the cuttlefish *S. officinalis* (Zumholz et al. 2007a). Although no diet effects have been detected in cephalopods thus far (Zumholz, 2006), interactive effects of food quantity, temperature and life history stage on Ba/Ca uptake have been shown experimentally for the damselfish *A. polyacanthus* (Walther et al. 2010).

Given that the ontogenetic patterns for Ba/Ca are similar to those of Sr/Ca for both cohorts, it is likely that Ba/Ca ratios (Fig. 4D) also conform to a negative relationship with temperature (Fig. 4A). The

timing of the offshore ontogenetic migration of the ASC into colder deeper waters is concomitant with
 an increase in Ba/Ca concentration. The timing of the subsequent decrease in elemental concentration
 corresponds to the timing of the ASC's inshore migration to their warmer shallower spawning habitat.
 The SSC, as with the Sr/Ca elemental signature, shows a gradual increase in Ba/Ca concentration
 corresponding to their offshore migration. Unlike the ASC, there is a plateau during March-April
 synonymous with the period of time when squid have arrived on their shared feeding grounds,
 followed by further increase in Ba/Ca concentration during late autumn when equality of temperatures
 allows for penetration into cooler water (Arkhipkin et al. 2004b). The plateau in the elemental
 signature during the period when squid are found at a constant depth suggests that depth
 (representative of changes in water chemistry) may also play a role in Ba uptake. Laboratory studies
 in which ambient water concentration and temperature could be experimentally manipulated, would
 be required to definitively confirm whether either of these factors have an effect on barium uptake in
D. gahi statoliths.

3.4 Inter-annual variation of element/Ca ratios

The interaction between year of collection and cohort had a significant effect on Sr/Ca ratio in the
 GAMM model. However, year was removed from the GAMM model for barium as its inclusion of
 year (or its interaction with cohort assignment) did not improve model fit. Visually, the elemental
 pattern for each cohort (and both elements) was consistent between years showing the same peaks and
 troughs. However, elemental concentrations (of both elements) were higher for the SSC in June-
 August 2015 than during the same period in 2014. As an example, SSC Sr/Ca ratios for the last week
 of July were $9.82 \text{ mmol.mol}^{-1}$ in 2014 and $10.66 \text{ mmol.mol}^{-1}$ in 2015. Comparison of near-bottom
 water temperatures from 2014 to 2015 ($T_{2015}-T_{2014}$) indicate that temperature values in 2015 are more
 extreme than the previous year (Fig. 5), with warmer temperatures during the first half of the year and
 comparably colder temperatures in late 2015. The higher Sr/Ca concentrations found in the SSC in
 2015 are likely due to individuals experiencing colder temperatures than 2014.

Figure 5: Near-bottom temperature differences between 2014 and 2015, defined as $T_{2015} - T_{2014}$.

The significant interaction between year and cohort for the strontium model suggest that there are cohort-specific inter-annual differences in the elemental signature. However, this was not the case for barium. Although inter-annual differences in elemental signatures were not significant for barium in the present study, given that only two years have been sampled it would be prudent to sample additional years, to determine whether a pattern exists on a multi-annual basis. If the magnitude of the elemental signal does vary with environmental anomalies, there may be potential predictive applications based on how the chemical signal changes inter-annually with environmental change and how this affects recruitment success in subsequent years. As this species has an annual lifecycle, there is no reservoir of older individuals to buffer the population against poor spawning success. Finer scale predictions of recruitment would prove invaluable for the real-time stock assessment of this fishery (Arkhipkin et al. 2008; Beddington et al. 1990; Winter and Arkhipkin, 2015).

4 Conclusions

The following conclusions can be made from this study:

- (1) Each cohort has its own unique elemental pattern for both strontium and barium
- (2) Analysis of cohort-specific elemental signatures in relation to environmental factors indicates a negative relationship between Sr/Ca and Ba/Ca with temperature
- (3) Life history stage and cohort assignment, have significant effects on Sr/Ca and Ba/Ca concentrations

These results demonstrate that elemental concentrations in *D. gahi* statoliths have applications as a natural tag, discriminating between components of a population and reconstructing ontogenetic changes. To maximise the potential of this laser ablation method, laboratory based research is required to manipulate environmental factors and assess their effect on elemental incorporation.

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