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Negative temperature dependence of statolith Sr/Ca and its intraspecific variability in experimentally maintained spear squid *Heterololigo bleekeri*

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Research Article

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Abstract

The strontium-to-calcium ratio (Sr/Ca) in aragonitic shells and statoliths often depends on temperature, and has been investigated for its potential availability as a sclerochronological record of the temperatures experienced during the life of the animal. Only a few cephalopod species have been subjected to rearing experiments to validate the temperature dependence of statolith Sr/Ca despite the strong demand for estimating their historical ecology, particularly of migratory squid populations. In this context, we examined the relationship between temperature and statolith Sr/Ca in the spear squid, *Heterololigo bleekeri*, by maintaining wild-caught immature individuals at one of three constant temperatures: 12, 14 or 16°C. A portion of statolith precipitated during the experiment was optically identified by daily increment analysis, and then subjected to Sr/Ca measurement using an electron probe micro-analyser. Regression analysis (N = 29) demonstrated the dependence of statolith Sr/Ca on temperature and the relationship was estimated as an equation $Y = 9.93\ (\pm 0.29) - 0.11\ (\pm 0.02)\ X$. However, ~90% of the total residual variance was accounted for by the among-individual variation of statolith Sr/Ca within each temperature group. Consequently, the 95% confidence interval ranged over ± 7.85°C when the temperature was estimated by inserting a Sr/Ca value into this equation. The statolith Sr/Ca values are unlikely to provide reliable estimates for absolute temperatures, but it may allow reconstruction of a time-series of relative temperatures experienced by a particular individual.

Introduction

As a possible archive of environmental conditions experienced by an individual, elemental composition in biogenic carbonates precipitated by marine organisms has been intensively studied over the last two decades. Elemental analysis of molluscan shells or statoliths has been conducted for the purposes of palaeoclimatological reconstruction (e.g. Schöne & Gillikin, 2013 for bivalve shells), estimation of population connectivity (Zacherl, 2005 for gastropod statoliths and protoconchs) or migratory patterns (Ikeda *et al.*, 2003; Liu *et al.*, 2016; Jones *et al.*, 2018; all for cephalopod statoliths), and individual age determination (Durham *et al.*, 2017 for bivalves; Hollyman *et al.*, 2018, 2019 for gastropod statoliths; Arkhipkin *et al.*, 2018 for cephalopod statoliths). In particular, strontium (Sr) is the most useful element because it is incorporated into aragonitic structures at a relatively high concentration and often reflects environmental conditions in many species (reviewed by Doubleday *et al.*, 2014; Avigliano *et al.*, 2020). The strontium-to-calcium ratio (Sr/Ca) in aragonitic structures has been shown to record the temperatures experienced by an individual in some species (e.g. Schöne *et al.*, 2011; Shirai *et al.*, 2014 for *Arctica islandica*), although otolith Sr/Ca is often used for estimating the age at a transition between marine and freshwater habitats in diadromous fish species (e.g. Murase *et al.*, 2019). In molluscs, the relationship between temperature and shell Sr/Ca is usually reported to be negative (Schöne *et al.*, 2011; Füllenbach *et al.*, 2015), but it may be positive (Wanamaker *et al.*, 2008; Irie & Suzuki, 2020), and sometimes there may be no clear relationship (Vander Putten *et al.*, 2000), depending on species. Furthermore, skeletal Sr/Ca shows considerable variability among conspecific individuals reared at the same temperature, which potentially limits the applicability of a Sr/Ca-based thermometer (Irie & Suzuki, 2020 for gastropods).

In cephalopods, the statolith is the only calcified structure allowing the Sr/Ca-based reconstruction of age-associated experienced temperatures, because daily increment periodicity in statolith growth enables the estimation of individual age by counting growth rings (reviewed by Jackson, 2004). However, there have been only a few studies published so far that have experimentally evaluated the relationship between temperature and statolith Sr/Ca in cephalopods and no consistent conclusions have been reached among the species examined. Zumholz

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et al. (2007*a*) found no relationship between water temperature and statolith Sr/Ca in *Sepia officinalis* by rearing hatchlings at one of three different temperature treatments for 60 days. Gillanders *et al.* (2013) reared hatchlings of the giant Australian cuttlefish, *Sepia apama*, at three temperatures combined with three seawater Sr/Ca concentrations, and found positive relationships between temperature and statolith Sr/Ca at the medium and high Sr concentration treatments. Yamaguchi *et al.* (2015) demonstrated experimentally that there is a negative relationship between statolith Sr/Ca and temperature in the swordtip squid, *Uroteuthis edulis*, which was the first experimental study using a loliginid squid. These differences imply that the physiological mechanism of Sr/Ca uptake differs among cephalopod species, such that the sclerochronological thermometer role for Sr/Ca established for one species cannot be assumed to apply to another species.

Here, we examined the relationship between water temperature and statolith Sr/Ca in the spear squid, *Heterololigo bleekeri* (Keferstein 1866), aiming to evaluate its potential as a sclerochronological thermometer. This species is distributed in temperate waters around the Japanese archipelago. It is among the most commercially important squids for coastal fisheries, and the annual landing of this species shows a deceasing trend from 20,000 tons in 1979 to 3900 tons in recent years (Arkhipkin *et al.*, 2015). The habitat of this species is generally limited to the continental shelf and the depth of habitat is deeper in the warmer southern region but shallower in the colder northern region (Natsukari & Tashiro, 1991). Mature individuals migrate close to shore in the winter spawning season, and are the target of the fishery (Iwata *et al.*, 2010). The migration pattern of this species through its life history is not well understood, but Toriyama *et al.* (1987) showed that juveniles distribute at 14–15°C, immature at 11–16°C, and mature individuals at 12–14°C in southern Japan, suggesting that ontogenic change of the optimal temperature seems to be little. However, the optimal temperature for spawning is 10–12°C in the northern region (Sato, 1990), suggesting that the optimal temperature for each life history stage is different among geographic regions. In addition, males of this species show dimorphism associated with alternative reproductive tactics (Iwata & Sakurai, 2007), but the environmental influences on the male phenotypes are still elusive. Analysis of statolith elemental composition in cultured individuals is a potential important step to a better understanding of the migratory ecology of *H. bleekeri* and the effects of environmental conditions experienced during the life history of wild individuals. Therefore, wild-caught individuals were maintained at one of three different temperatures in the laboratory to examine directly the relationship between water temperature and statolith Sr/Ca.

Materials and methods

Immature *H. bleekeri* were obtained from an inshore set net (fixed at a depth of ~30 m; temperature was roughly estimated as 16°C using FRA-ROMS II) in southern Hokkaido in October 2016, and transported using a 500 litre tank (Live fish tank 500; Suiko) filled with fresh seawater saturated with O_2 to the Graduate School of Fisheries Science, Hokkaido University. The ambient sea surface temperature at capture was 19.2°C when we hauled a net containing squid onto the boat. Rearing experiments were performed by holding the squid in one of three 1000 litre circular FRP tanks with closed filtering systems and natural seawater pumped from the Kumaishi coast of Hokkaido (salinity 34‰). The inner wall of each tank was painted with vertical black stripes to decrease the likelihood of injuries through squid colliding with the wall. A 12:12 h photoperiod was maintained using white fluorescent tubes during the day 06:00–18:00 h.

After five days of acclimation to the captive conditions, squid were reared at three constant temperatures of 12, 14 and 16°C. Temperature was gradually adjusted to that assigned to each tank over 24 h, controlled by electric coolers and heaters with thermostats, and monitored daily (Table 1). Squid were fed fillets of jack mackerel, *Trachurus japonicus*, once daily. There were 16, 12 and 21 individuals at 12, 14 and 16°C, respectively, of which 11, 10 and 18 individuals survived longer than 7 days. At the end of the experiments, all surviving individuals were euthanized under anaesthesia with 5% ethanol and stored in a freezer for later processing. All procedures performed in the present study followed the ethical standards of the Life Science Research Ethics and Safety Committee of the University of Tokyo and the animal experiments were approved by the committee.

Among the 39 individuals maintained in tanks longer than 7 days, only 29 individuals showed readable growth rings at the rostrum (i.e. the longitudinal edge of a statolith) and thus were subjected to the following analysis (12°C, N = 9; 14°C, N = 9; 16°C, N = 11; Table 1). After thawing specimens, mantle length was measured and both the left and right statoliths were removed from each individual. Each statolith was temporarily glued onto a glass slide with thermoplastic adhesive (Crystalbond 509-1; Aremco) and the convex side was ground with SiC Foil (grit #1000, #2000, #4000 [=FEPA P1200, P2400, P4000, respectively]; Struers) on a polishing wheel (RotoPol-35; Struers) to approach the core. After detaching the statolith from the glass slide, the flattened surface was embedded in epoxy resin (EpoFix; Struers) and then mounted on another glass slide in an inverted manner. The concave surface was ground with SiC Foil (grit #1000, #2000, #4000 [=FEPA P1200, P2400, P4000, respectively]; Struers) to expose the core, and then buffed with an active oxide polishing suspension (OP-S suspension, 0.25 µm; Struers) on a polishing wheel fitted with a semi-automatic specimen mover (MD-Chem and RotoPol-35/PdM-Force-20; Struers). Daily rings were counted using a system consisting of a light microscope with 50–1000 × magnification, a couple-charged device (CCD) camera, and a computer-controlled image analyser (Jiseki 8, Ratoc System Engineering Company; Figure 1A). The aim of this procedure was to identify the region precipitated during the experimental period and to estimate mean statolith growth rates from daily increments of the statolith diameter (see Jackson, 2004 for the daily periodicity of growth rings). Individual age at the end of the experiment was also indicated in Table 1.

The glass slide with a statolith attached was cleaned in an ultrasonic bath, rinsed for 1 min with 99% ethanol, for 1 min with deionized water, and then dried by placing in a vacuum desiccator (at 0.1 atm) at room temperature for 24 h. The upper surface was coated with platinum-palladium (60 s) in an ion-beam sputter coater (Hitachi E-1030) to enhance electrical conductivity. Statolith elemental composition was obtained using a JEOL JXA-8230 electron probe microanalyser (EPMA) at the Atmosphere Ocean Research Institute, University of Tokyo. The wavelength-dispersive spectrometry (WDS) method was used to measure calcium (Ca) and strontium (Sr) concentrations in the rostrum region formed during the experimental period. Exposure times were set at 10 s for peak measurements and 5 s for background measurements with an accelerating voltage of 15 kV and a beam current of 15 nA. Strontium titanate ($SrTiO_3$) and wollastonite ($CaSiO_3$) were used as standards for the ZAF correction procedure.

In the WDS analysis, electron beams measuring 3 µm in diameter were focused on a line with 3 µm intervals. Ten points were beamed along the longest growth axis of a statolith if the rostrum portion formed during the experimental period had enough length; otherwise, 10 points were divided into multiple lines drawn in parallel (Figure 1B). After polishing the statolith surface

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Table 1. Growth rate and statolith Sr/Ca ratio for *Heterololigo bleekeri* reared under constant temperature

Temp. (°C)	Sex	Mantle length (mm)	Age (days)	No. days in a tank	Statolith increment (μm)	Growth rate (μm day^{-1})	Sr/Ca (mmol mol^{-1})
12°C							
11.9	Male	150	200	34	84.7	2.49	8.64
11.9	Male	172	226	34	85.4	2.51	8.66
11.9	Male	138	185	15	30.9	2.06	8.55
11.9	Male	138	191	12	23.7	1.97	8.91
11.9	Male	126	212	34	72.1	2.12	8.35
11.9	Male	150	202	12	27.3	2.28	9.01
11.9	Female	120	185	8	19.2	2.40	8.37
11.9	Male	106	206	7	16.6	2.37	8.57
11.9	Male	134	200	8	16.2	2.03	8.72
14°C							
13.9	Male	170	221	14	31.5	2.25	8.47
13.9	Male	148	219	14	32.1	2.29	8.11
13.9	Female	172	221	15	34.1	2.27	8.26
13.9	Male	182	211	14	26.2	1.87	8.42
13.8	Male	176	239	13	29.6	2.27	8.35
13.8	Female	156	220	12	24.6	2.05	8.41
13.8	Male	184	192	13	30.3	2.33	8.27
13.8	Male	196	220	13	19.5	1.50	8.64
13.8	Male	154	230	13	27.7	2.13	8.62
16°C							
16.2	Male	148	217	34	86.9	2.56	7.94
16.2	Male	156	233	34	79.2	2.33	7.97
16.2	Male	132	190	34	94.0	2.76	8.08
16.2	Male	152	209	34	95.9	2.82	7.89
16.1	Male	130	209	34	76.9	2.26	8.22
16.1	Male	148	200	11	24.6	2.24	8.22
16.1	Male	130	204	11	26.8	2.44	8.38
16.1	Male	127	190	11	28.5	2.59	8.30
16.1	Male	126	216	12	23.9	1.99	8.15
16.1	Male	150	206	11	23.0	2.09	8.46
16.2	Male	142	191	11	29.2	2.65	8.38

with the OP-S suspension for several seconds, the 10-points measurement was conducted again. By repeating this process, a total of 40 points of Sr/Ca values was collected from each statolith. A mean Sr/Ca value was calculated from the 40 measurements, and treated as a statistically independent unit in the statistical analysis. Averaging multiple measurements seems necessary for obtaining biologically meaningful information from the WDS approach, because the Sr/Ca value from a single measurement is highly dispersive ($\sigma = 0.39$ mmol mol^{-1}). Figure 2 illustrates how the estimated standard error of the mean Sr/Ca depends on the number of replicate observations, which should be inversely proportional to $\sqrt{n}$ in theory. According to this relationship, we estimated the measurement error of the mean statolith Sr/Ca value obtained from 40 replicate observations to be 0.06 mmol mol^{-1}. Our estimation is based on a premise that the spatial distribution of Sr is homogeneous across the rostrum region we measured, because it is unlikely that a single measurement is precise enough to detect a possible finer-scale spatial variation in Sr/Ca and because the heterogeneity would be successfully averaged out by replicate observations if any.

An ordinary linear regression model was applied to the mean Sr/Ca values to obtain least-squares estimates of the slope and y-intercept against rearing temperature (N = 29; Table 1). Data from both sexes were pooled because the sex ratio of experimental individuals was considerably biased. An *F*-test was conducted to check the null hypothesis that the regression slope is equal to 0. The ordinary linear regression model assumes that the observation Y_i from the i-th individual (i.e. individual Sr/Ca value) is described by the relationship

$$Y_i = \beta_0 + \beta_1 X_i + \epsilon_i \text{ and } \epsilon_i \sim N(0, \sigma_E), \quad (1)$$

where β_0 and β_1 are parameters to be estimated and X_i is the individual rearing temperature. ϵ_i denotes an individual Sr/Ca

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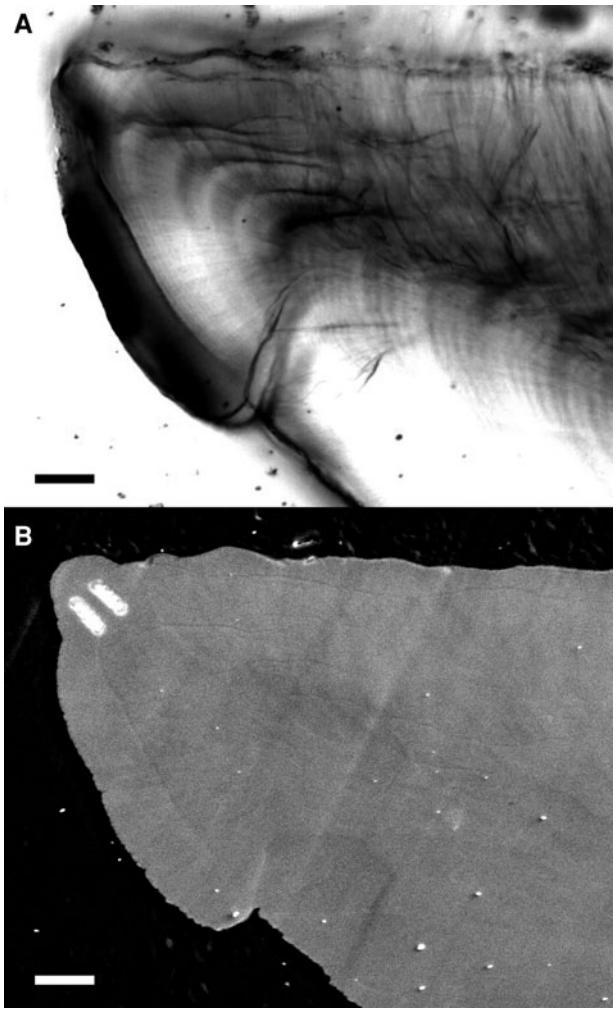


Fig. 1. The rostrum of a statolith extracted from an immature *Heterololigo bleekeri* (7th individual at 14°C in Table 1). (A) Light photomicrograph of ground surface (scale bar: 20 μm). (B) The corresponding SEM image with 10 beamed spots (scale bar: 20 μm).

deviation from the corresponding expected value, $\hat{\beta}_0 + \hat{\beta}_1 X_i$, and is assumed to follow a normal distribution with zero mean and σ_E standard deviation, $N(0, \sigma_E)$. However, in reality the linear relationship between temperature and Sr/Ca is variable among individuals within a species (e.g. Hayashi *et al.*, 2013). This can be formulated by a random intercept model

$$Y_i = \alpha_i + \beta_1 X_i + \epsilon_i \text{ with } \alpha_i \sim N(\mu_\alpha, \sigma_\alpha) \text{ and } \epsilon_i \sim N(0, \sigma_e), \quad (2)$$

if one most optimistically assumes that the slope β_1 is common across all individuals. Standard deviations for the intercept (σ_α) and pure error (σ_e) cannot be separately estimated using our data, but in a previous study it was implied that the former is much greater than the latter (i.e. $\sigma_\alpha >> \sigma_e$; see Irie & Suzuki, 2020). Based on this argument, the 95% pointwise confidence interval at $X = X_0$ was estimated using

$$\hat{Y}_0 \pm t(n-2, 0.975)\left\{1 + \frac{1}{n} + \frac{(X_0 - \bar{X})^2}{\sum (X_i - \bar{X})^2}\right\}^{1/2} s, \quad (3)$$

where $\hat{Y}_0 = \hat{\beta}_0 + \hat{\beta}_1 X_0$, and s can be replaced with the estimated residual standard deviation, $\hat{\sigma}_E$ (see eq. [3.1.6] in Draper & Smith, 1998). $t(n-2, 0.975)$ signifies T^* satisfying $P(T > T^*) = 0.975$ when a random variable T follows the t-distribution with $n-2$ degrees of freedom on which s^2 is based. The aim was to calculate the 95% confidence interval for the temperature predicted by applying a Sr/Ca value measured for a new statolith specimen to equation (1). All statistical analyses were conducted in the JMP Pro statistical package (version 13 for Windows; SAS Institute) and Mathematica (version 11.0.1 for Windows; Wolfram Research).

The rearing setup of our experiment is not statistically desirable because multiple individuals are kept in the same tanks, which potentially causes pseudoreplication in the obtained data. However, we were not able to prepare more than three tanks of the size large enough to maintain spear squid, as space in the laboratory was limited. This type of pseudoreplication can be theoretically avoided by sequentially conducting solitary rearing (i.e. keeping each individual in an independent tank), but this causes another type of pseudoreplication by ignoring a time-related factor unless repeated measurements from the same individuals are performed according to the within-subject design. The repeated measures design was not feasible in this study because spear squid are not likely to survive until the second measurement as well as because an unacceptable length of time is required to accomplish the entire experiment. We discussed a potential impact on statolith elemental composition by culturing multiple individuals in the same tank (see Discussion).

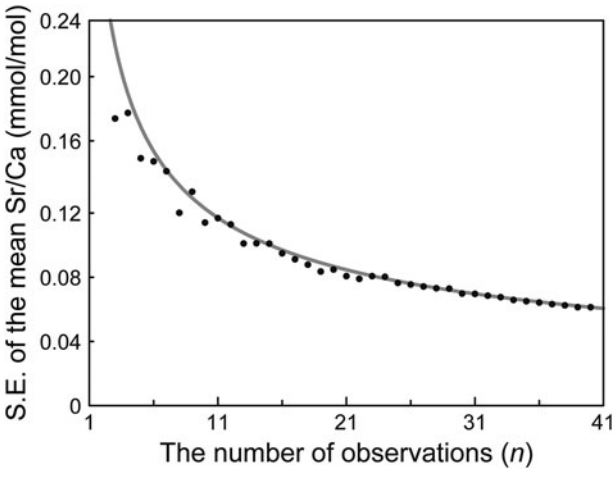


Fig. 2. The standard error of the mean statolith Sr/Ca as a function of the number of replicate observations (grey solid line). Closed dots indicate the average SE values directly calculated from the measurements.

Results

As a result of the regression analysis on the data collected from the part of statolith deposited during the experimental period, a negative linear relationship was found between temperature and Sr/Ca ($R^2 = 0.52$; Figure 3). Slope and y-intercept (and their standard errors) were estimated to be −0.11 (±0.02) and 9.93 (±0.29), respectively. The impact of temperature on Sr/Ca was statistically significant (df = 1, 27; $F = 29.38$; $P < 0.0001$). The estimated residual standard deviation, $\hat{\sigma}_E$, was 0.19 mmol mol^{-1}, which was much greater than the estimated EPMA measurement error, 0.06 mmol mol^{-1}; or, equivalently, 90% of the residual variance ($\hat{\sigma}_E^2$) was accounted for by the among-individual variance of statolith Sr/Ca at the same temperature. The 95% pointwise confidence interval estimated according to equation (3) was as broad as [10.07°C, 17.92°C] when the temperature experienced was calculated by applying a hypothetical Sr/Ca value, 8.4 mmol mol^{-1}, to equation (1). There were also negative correlations between

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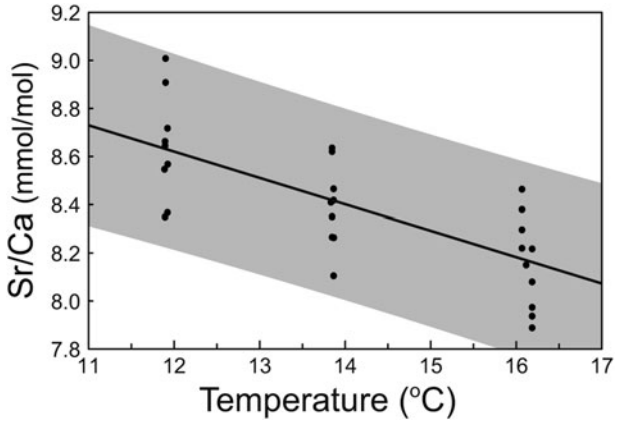


Fig. 3. Relationship between rearing temperature and statolith Sr/Ca of immature *Heterololigo bleeleri* reared under constant temperature, indicated as individual observations (closed dots) and regression (solid line), with the 95% pointwise confidence interval displayed as a grey band.

mean statolith growth rates and statolith Sr/Ca within treatments, but correlation coefficients (r) did not significantly deviate from 0 in the 12°C and 16°C treatments: $r = -0.19$ ($P > 0.05$), −0.67 ($P = 0.049$) and −0.36 ($P > 0.05$) at 12°C, 14°C and 16°C, respectively (Figure 4). No significant correlation was found between mantle length and statolith Sr/Ca at 12°C ($r = 0.46$, $P > 0.05$), 14°C ($r = 0.31$, $P > 0.05$) nor 16°C ($r = -0.36$, $P > 0.05$). Furthermore, statolith Sr/Ca showed no significant correlations with age at the end of the experiment irrespective of temperatures ($r = 0.02$ at 12°C, $r = 0.29$ at 14°C, $r = -0.52$ at 16°C; all $P > 0.05$).

Discussion

In the present study, it was demonstrated experimentally that, in *H. bleekeri*, there is a negative linear relationship between water temperature and statolith Sr/Ca. Similar negative relationships have been reported for *U. edulis* (Yamaguchi *et al.*, 2015) and *S. apama* (pre-hatched individuals; Gillanders *et al.*, 2013) reared in constant temperatures. Circumstantial evidence of a negative correlation between temperature and statolith Sr/Ca was obtained for the Patagonian longfin squid *Loligo gahi* collected from different localities across a thermal gradient (Arkhipkin *et al.*, 2004), as well as for the Japanese common squid *Todarodes pacificus* (Ikeda *et al.*, 1998) and the boreoatlantic armhook squid *Gonatus fabricii* (Zumholz *et al.*, 2007*b*). Negative temperature dependence of

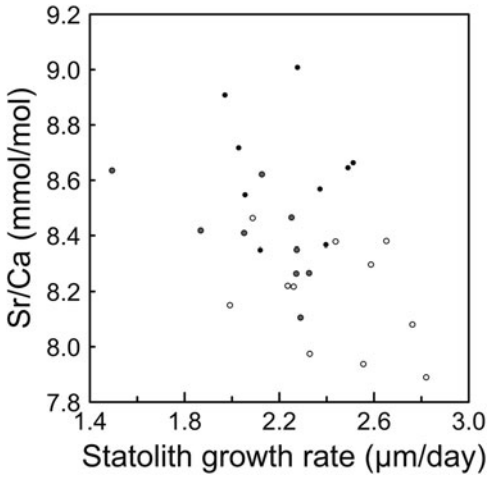


Fig. 4. Relationship between statolith growth rate and statolith Sr/Ca at 12°C (closed dots), 14°C (open dots filled with grey), and 16°C (open dots).

otolith Sr/Ca is typically seen in teleost fishes (reviewed by e.g. Hüssy *et al.*, 2020), which suggests the presence of some causal mechanism common across ectothermic calcifiers.

The analysis indicated that statolith Sr/Ca varies considerably among individuals within a given temperature, which significantly impairs its availability as a sclerochronological thermometer. It is unlikely that the variability arose from some environmental heterogeneity during the rearing experiment, because all individuals were kept in the natural seawater pumped from the same place. It is considered instead that this phenomenon results from some quantitative difference in the physiological basis of statolith precipitation, possibly ascribable to genetic variation among individuals. Similar variabilities are found in the skeletal Sr/Ca of a scleractinian coral (*Porites australiensis*; Hayashi *et al.*, 2013) and the shell Sr/Ca of an intertidal gastropod (*Monetaria annulus*; Irie & Suzuki, 2020) reared in a common temperature regime. The linear relationship between temperature and statolith Sr/Ca possibly varies in a parallel manner; alternatively, both slope and y-intercept may differ among individuals. The approach used here cannot distinguish these two possibilities, but data from Hayashi *et al.* (2013) support the latter.

The physiological mechanism responsible for the negative relationship between temperature and statolith Sr/Ca remains to be elucidated. Since the molluscan statolith is precipitated from the endolymph in the statocyst (Morris, 1991; Bettencourt & Guerra, 2000), it is to be expected that statolith Sr/Ca will be affected by the Sr/Ca value in the endolymph. Transmembrane active ion transporters (i.e. Ca^{++}-ATPase) have been considered to selectively increase the Ca concentration of calcification fluid in bivalves (Carré *et al.*, 2006), corals (Sinclair & Risk, 2006; Allison *et al.*, 2011), and foraminiferans (Bentov *et al.*, 2009) to facilitate $CaCO_3$ precipitation. Ca^{++}-ATPase may also modify the elemental composition of the endolymph from ambient seawater in cephalopods. In this scheme, lower statolith Sr/Ca values at higher temperatures suggest that the activity of Ca^{++}-ATPase increases with increasing temperature in *H. bleekeri* within the range 12–16°C. This conjecture is qualitatively compatible with the result that the correlation coefficients between mean statolith growth rate and statolith Sr/Ca were consistently negative within treatments.

Our experiment was subject to a number of technical restrictions because Teuthid squids including *H. bleekeri* are very difficult to keep alive in laboratory for experiments, as notoriously recognized among cephalopod researchers (Jackson, 2004). For example, they require large-volume tanks for keeping alive, resulting in pseudoreplication as individual cages or tanks cannot be used. Another difficulty arises in setting up temperature regimes in experiments. In the present study, we maintained squid at 12–16°C, but this probably covers only the upper part of their thermal window (Arkhipkin *et al.*, 2015). As the experimental squid were caught at 19°C in coastal water, we did not expose squid to conditions colder than 12°C, considering that they might not be able to tolerate the physiological stress by the acute thermal acclimation. Consequently, the negative relationship between temperature and statolith Sr/Ca was demonstrated only between 12–16°C in this study and the relationship at lower temperatures remains an open question.

Findings from the present study may lack a generality across the sexes, because the sex ratios of experimental individuals were severely biased to males (Table 1). The male-biased sexual segregation did not result from sex-dependent mortality rates during the experiment, but arose at the collection with an inshore set net. Deviations from the 1:1 sex ratio are frequently observed at local population levels in squids (Arkhipkin & Middleton, 2002). Due to the extreme scarcity of females, we gave up on statistically examining whether statolith Sr/Ca values show a sexual

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difference. If any, however, it seems unlikely to detect a statistically significant difference in Sr/Ca between the sexes, even when the sex ratio was 1:1. This is because statolith Sr/Ca considerably varies among individuals (Figure 3) and the sample size is not large (N = 29). In fact, the detection of a sexual difference in otolith Sr/Ca is statistically marginal even in environmentally controlled rearing experiments on fishes (Mohan *et al.*, 2014; Sturrock *et al.*, 2015). Irie & Suzuki (2020) demonstrated that there is no sexual difference in shell Sr/Ca by conducting rearing experiments of an intertidal gastropod.

In summary, it was demonstrated that *H. bleekeri* statolith Sr/Ca shows a negative dependence on water temperature. Among-individual Sr/Ca variability is considerable at a constant temperature, such that it is impractical to obtain a reliable pointwise estimate of absolute temperature from a single statolith Sr/Ca measurement. Nevertheless, statolith Sr/Ca can be informative in ecological studies if a Sr/Ca time-series is measured perpendicularly across growth rings, because it can provide the ontogenetic history of the relative temperatures experienced. Future studies are expected to improve both accuracy and precision of sclerochronological thermometers based on skeletal Sr/Ca by achieving a better understanding of the physiological and crystallographic backgrounds behind the incorporation of elements into aragonite crystals.

Data. The data that support the findings of this study are available from the corresponding author, upon reasonable request.

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Author contributions. SH performed statolith measurements and writing. TI led data analysis and writing. JY, MN, YS, and YI collected squids, and conducted rearing experiments. TK revised the manuscript.

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Conflict of interest. The authors declare none.

Ethical standards. All procedures performed in the present study followed the ethical standards of the Life Science Research Ethics and Safety Committee of the University of Tokyo and the animal experiments were approved by the committee (approval number A16-15).

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