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Evaluation of the ^{137}Ba mass-marking technique and potential effects in the early life history stages of *Sepioteuthis lessoniana*

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Abstract. The use of mass-marking techniques of enriched stable isotopes has increased in studies of ecology, movement patterns and the dispersal of marine organisms. However, the efficacy of this technique and its potential effects on hatchling size and statolith chemistry of cephalopods are yet to be investigated. *Sepioteuthis lessoniana* egg capsules were collected from northern Taiwan and assigned randomly to ^{137}Ba-spiking experimental groups at 0.2, 0.5 and 1 ppm and three immersion durations (1, 3 and 7 days). Immersion duration >3 days produced significantly lower ^{138}Ba : ^{137}Ba ratios, with 100% marking success, indicating that it is a reliable marking technique. The ^{137}Ba mass marking had a positive effect on size at hatch and was likely to affect statolith trace element incorporation, including Cu, Zn and Pb. These findings highlight that it is necessary to consider the species-specific effects on hatchling size and physiological responses in when using stable isotopes mass-marking techniques.

Additional keywords: barium isotopes, hatchling size, statolith chemistry.

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Introduction

Cephalopods are crucial in commercial fisheries (Hunsicker *et al.* 2010; Jereb and Roper 2010) and are a vital component in marine food webs as a dietary source for numerous marine organisms (Clarke 1996; Klages 1996). The growth and population dynamics of cephalopods respond to environmental conditions, such as food abundance, temperature and water (Jackson and Moltschaniwskyj 2002; Forsythe 2004). Similar to most marine organisms, the degree of larval dispersal and distributional range of cephalopods determine connectivity of the population and are related to conservation of the ecosystem (Swearer *et al.* 1999; Cowen and Sponaugle 2009). Several methods for investigating dispersal patterns and migratory behaviours of marine animals in their early life history stages have been developed, but are not always applicable. For example, external tags may harm organisms and increase mortality rates (Sauer *et al.* 2000; Replinger and Wood 2007; Barry *et al.* 2011). In addition, the large tag size is impossible to use on squid paralarvae or juveniles (Nagasawa *et al.* 1993; Semmens *et al.* 2007). Although alternative biomarkers, like parasite communities and molecular markers, are not restricted by larval size, these methods do not provide detailed information on larval dispersal and movement patterns (Bower and Margolis 1991; Buresch *et al.* 2006). Traditional marking methods clearly have their limitations, and advanced marking techniques for early life history stages are required.

Recently, enriched stable isotope-marking techniques have been used, such as injecting enriched stable isotopes into mature females (Thorrold *et al.* 2006; Almany *et al.* 2007) or immersing offspring in water with enriched stable isotopes (Munro *et al.*

2008; Smith and Whitledge 2011; Woodcock *et al.* 2011*a*). Marked individuals with unique isotopic signatures in their biogenic carbonates are distinguishable from natural populations (Munro *et al.* 2008; Smith and Whitledge 2011). Stable isotopes of barium and strontium are commonly used in the marking experiments. Both elements have similarities in their ionic radius to Ca^{2+} and will likely be a substitute for Ca^{2+} in biogenic carbonates (Speer 1983). It is easier to perform this technique with barium for marine organisms because barium concentrations are low in natural seawater (varying in the range 0.007–15 ppm in seawater and fresh water; Bernat *et al.* 1972; Kresse *et al.* 2007). In addition, marking by feeding Ba-enriched dietary items has been suggested as a more effective method in marine systems (Woodcock and Walther 2014). The ^{137}Ba isotope is stable in lower abundance (11.23%) and is not the major barium isotope (71.1% for ^{138}Ba; Rosman and Taylor 1998). Because enrichment with ^{137}Ba in calcified structures is greater than environmental levels, the mark is easily detected and shows a difference from natural seawater signatures (Thorrold *et al.* 2006). Therefore, ^{137}Ba mass-marking techniques are likely suitable for tracking larval dispersal and movement patterns of cephalopods in the natural environment.

To date, only two studies have evaluated the enriched stable isotope mass-marking technique in cephalopod early life stages (Pecl *et al.* 2010; Payne *et al.* 2011). Maternal injection of stable isotopes in cephalopods may not mark offspring because there is difficulty in determining the oocyte maturity stage in cephalopod ovaries (Pecl *et al.* 2010). By contrast, Payne *et al.* (2011) marked *Sepia apama* hatchlings by immersing their spawning eggs in solutions spiked with different ^{137}Ba concentrations (0.3 and 1 ppb) and for different durations (2, 5 and 8 days). These authors demonstrated the potential of using stable isotope-marking techniques to assess the population dynamics of cephalopods in the field (Payne *et al.* 2011). However, the efficacy of marking techniques varies among species. Thus, which method produces high-quality marks with lower costs in target species must be assessed (Warren-Myers *et al.* 2018).

Compared with fluorescent dyes (e.g. alizarin complexone), techniques for mass marking with enriched stable isotopes are usually seen as non-toxic to experimental offspring (Williamson *et al.* 2009; Woodcock *et al.* 2011*a*; Warren-Myers *et al.* 2018). Yet, there is evidence to suggest that enriched stable isotope mass-marking techniques may affect hatchling size (Williamson *et al.* 2009; Starrs *et al.* 2014*a*, 2014*b*). The size at hatch is crucially related to swimming and foraging abilities, which subsequently affect survival rate and reproduction (Sogard 1997). The effect of marking method on the hatchling size of cephalopod species is unclear. Moreover, the process of enriched stable isotope marking may alter physiological regulation, which may cause erroneous interpretations of cephalopod behaviours (de Vries *et al.* 2005). Such effects need to be carefully evaluated and considered in enriched stable isotope mass-marking experiments.

The present study used the method of immersing eggs of *Sepioteuthis lessoniana* with the enriched isotope of ^{137}Ba and aimed to evaluate the optimum spiked concentration and immersion duration for successful marking of their statoliths. In addition, we examined hatchling size (mantle length (ML) and bodyweight) and growth condition factor (Fulton's condition factor *K*) after marking, in addition to analysing elemental concentrations (^{24}Mg, ^{25}Mn, ^{63}Cu, ^{64}Zn, ^{88}Sr and ^{208}Pb) in hatchling statoliths. Changes to statolith chemistry may be induced by physiological regulation and varying accretion rates of calcium carbonate (Hamer and Jenkins 2007; Sturrock *et al.* 2015). This study looked at the potential effects of the enriched stable isotope mass-marking technique on cephalopod size at hatch and statolith chemistry.

Material and methods

Bigfin reef squid *S. lessoniana* are widely distributed in the neritic waters of the Indo-Pacific Ocean (Okutani 2015). They spawn almost throughout the entire year, with the embryo development period being ~23–26 days when the temperature is ~25°C (Segawa 1987). *S. lessoniana* egg capsules were collected by SCUBA diving from artificial bamboo reefs (depth ~20–25 m) at Wanghaixiang Bay in northern Taiwan in August 2015. The egg capsules were put in an opaque plastic bucket with natural seawater and immediately transported (<2 h) to the aquaculture station of the National Museum of Marine Science and Technology (Keelung, Taiwan). Before the experiments, all the egg capsules were suspended on nylon threads in a 200-L tank for initial acclimation. Natural seawater was collected from Wanghaixiang Bay and pumped through a filter bed to supply the rearing system. During the experiment, the seawater temperature was maintained at a mean (±s.d.) temperature of 25 ± 1°C, salinity was maintained at 34.1–34.7 PSU and experiments were conducted under a 12-h light–dark cycle.

In all, 150 eggs with visible embryos at 23–25 developmental stages, which were classified according to Segawa (1987), were randomly selected for each group and reared in a 10-L tank. There were nine experimental groups in total: three ^{137}Ba spike concentrations (0.2, 0.5 and 1 ppm) and three immersion durations (1, 3 and 7 days). These groups were compared against a control group with no spiking. Different ^{137}Ba concentrations were prepared by dissolving ^{137}Ba-enriched $BaCO_3$ (≥91% ^{137}Ba and 8% ^{138}Ba; Trace Sciences International, Richmond Hill, ON, Canada) in ultrapure water. For groups immersed for >1 day, half the rearing seawater was replaced daily and extra ^{137}Ba spike was added to maintain the concentration of the ^{137}Ba spike. After immersion, eggs were returned to the natural seawater until they hatched. The ML (mm) and bodyweight (mg) of the hatchlings were measured. Individuals were then killed by exposure to a high concentration of ethyl alcohol and their statoliths extracted. The experimental procedures followed the Guidelines for the Care and Welfare of Cephalopods in Research – A Consensus Based on an Initiative by CephRes, FELASA and the Boyd Group (Fiorito *et al.* 2015). The growth condition factor of hatchlings was estimated based on Fulton's condition factor *K*, calculated as follows (Ricker 1975):

$$K = (\text{bodyweight} \div \text{ML}^3) \times 100$$

Statoliths were extracted under a stereomicroscope (SteREO Discovery, V12; Carl Zeiss Microscopy GmbH, Jena, Germany), cleaned ultrasonically with 70% hydrogen peroxide to remove adhering tissue, rinsed three times in ultrapure water, placed into acid-washed Eppendorf microcentrifuge tubes and

oven dried overnight. The statoliths were then transferred to 1.5-mL acid-washed high-density polyethylene vials and weighed on a microbalance to the nearest 10 μg. Individual pairs of statoliths were dissolved in 0.5 mL of 0.3 M ultrapure nitric acid. Solutions were analysed using inductively coupled plasma–mass spectrometry (ICP-MS; ELEMENT XR ICP-MS; Thermo Scientific, Bremen, Germany) at the Institute of Earth Science, Academia Sinica, Taipei, Taiwan. Nine isotopes (^{25}Mg, ^{43}Ca, ^{55}Mn, ^{88}Sr, ^{137}Ba, ^{138}Ba and ^{208}Pb) were analysed in a low-resolution mode and two isotopes (^{63}Cu and ^{64}Zn) were evaluated in a medium-resolution mode. Element concentration is shown as a ratio relative to the concentration of calcium (mean element (Me) : Ca ratio). The carbonate (otolith)-certified reference material FEBS-1 (National Research Council, Ottawa, ON, Canada) was used to determine the Me : Ca ratio of samples and analysed every fifth sample to instrument drift. In regard to the matrix effect, statolith solutions in various calcium concentrations (0.5, 1, 5, 25 and 50 ppm) were tested and the Me : Ca ratios of every sample were normalised at the same level of matrix concentration. The relative standard deviations of the Me : Ca ratio measurements of FEBS-1 were lower than 4% for most elements expect Mn : Ca (Mg : Ca 3.37%; Mn : Ca 5.16%; Sr : Ca 1.79%; Ba : Ca 3.44%; Pb : Ca 2.46%; Cu : Ca 1.85%; Zn : Ca 3.06%), and the percentage accuracy of the Me : Ca ratios was better for Mg : Ca, Sr : Ca, Ba : Ca and Pb : Ca (1.04, 0.36; 0.60 and 1.21% respectively) than for Mn : Ca, Cu : Ca and Zn : Ca (7.20, 7.91 and 6.76% respectively). However, Mn concentrations detected were close to the background level and were excluded from further analyses.

All statistical analyses were performed using SPSS (ver. 20, IBM Corp., Armonk, NY, USA), as described below. A Shapiro–Wilk was used to assess the normality of the data, and Ba stable isotope ratios were found to be non-normally distributed. Therefore, a non-parametric Scheirer Ray Hare extension of the Kruskal–Wallis test was used to examine the effects of spiked concentration and immersion duration on ^{138}Ba : ^{137}Ba ratios. In addition, the effect of ^{137}Ba spikes on the size and condition of marked hatchlings were analysed by two-way analysis of variance (ANOVA). If significant differences were detected, Tukey's post hoc test was used to evaluate the difference between groups. For statolith chemistry, a forward stepwise canonical discriminant analysis was used to evaluate variations in element composition (Mg : Ca, Sr : Ca, Zn : Ca, Cu : Ca and Pb : Ca) among the control and all treatment groups, and cross-validation was further conducted to assess the percentage of successful classifications. In addition, Spearman's ρ test was used to assess correlations between barium stable isotopes (^{137}Ba : Ca and ^{138}Ba : Ca) and other trace elements.

Results

Barium isotope ratios and mark success

The ^{137}Ba spike was successfully marked in statoliths because ^{138}Ba : ^{137}Ba values decreased with increasing spike concentration or immersion duration. The mean (±s.d.) ^{138}Ba : ^{137}Ba ratio in statoliths in the control group was 6.28 ± 0.17, which decreased to 3.50 ± 0.22 after 7 days of immersion in 1-ppm ^{137}Ba-spiked solution (Fig. 1). Significant interactions were found between immersion duration and the concentration of the ^{137}Ba spike on

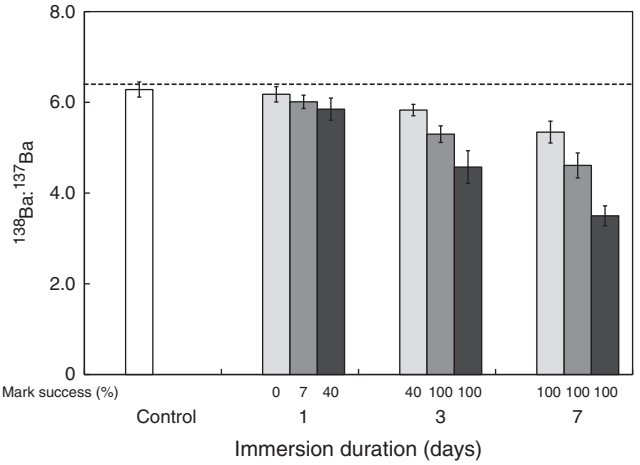

Fig. 1. Mean (±s.d.) Ba isotope ratios in statoliths of hatchlings immersed in water with different concentrations of ^{137}Ba spike, namely 0.2 ppm (light grey bars), 0.5 ppm (dark grey bars) and 1 ppm (black bars), for 1, 3 and 7 days. The percentage of mark success for each group is shown below the columns. The dashed line indicates the natural ^{138}Ba/^{137}Ba ratio.

^{138}Ba : ^{137}Ba ratios in hatchling statoliths (Scheirer–Ray–Hare extension of the Kruskal–Wallis test, d.f. = 9, SS = 166601.9, $H = 34.577$, $P < 0.001$), so separate Dunn's tests were used to compare the mean ^{138}Ba : ^{137}Ba ratios within groups. Overall, 7 days of immersion produced significantly lower mean ^{138}Ba : ^{137}Ba ratios than 1 day immersion for the same spiked concentration ($Z > 4.057$, $P < 0.001$), and the mean ^{138}Ba : ^{137}Ba ratios of the 1-ppm treatment were significantly lower than those of the 0.2-ppm treatment for the same immersion duration ($Z > 3.510$, $P < 0.01$). Longer immersion durations (3 and 7 days) with higher spiked concentrations (0.5 and 1 ppm) produced significantly lower ^{138}Ba : ^{137}Ba ratios than seen in the control group ($Z > 4.564$, $P < 0.001$). An additional significant difference was detected between 3- and 1-day immersions in the 0.2-ppm ^{137}Ba-spiked group ($Z > 3.510$, $P = 0.015$).

Following the criteria of Payne *et al.* (2011), the critical value of successfully marked squid was set at 5.78, which was the mean ratio of the control group minus 3 s.d. for ^{138}Ba : ^{137}Ba. A successfully marked statolith was defined as a ^{138}Ba : ^{137}Ba ratio in the hatchling statolith that was lower than this value. Higher spiked concentrations and longer immersion duration both increased the success rate of statolith marking (Fig. 1). For example, no mark was found after 1 day of immersion with a 0.2-ppm ^{137}Ba spike, but the success rate increased to 40% after 3 days of immersion with the same concentration. In total, 100% of squid were successfully marked after 3 days of immersion with the 0.5- and 1-ppm concentrations and after 7 days of immersion with all concentrations.

Hatchling size and growth condition factor

All eggs hatched 1–5 days after marking. The mean ML of the hatchlings in each group ranged from 5.54 to 5.99 mm, the mean bodyweight ranged from 24.4 to 31.3 mg and mean Fulton's condition factor K ranged from 12.98 to 16.43 (Fig. 2). No interaction between spike concentration and immersion duration was found for ML ($F = 0.795$, $P = 0.622$), bodyweight

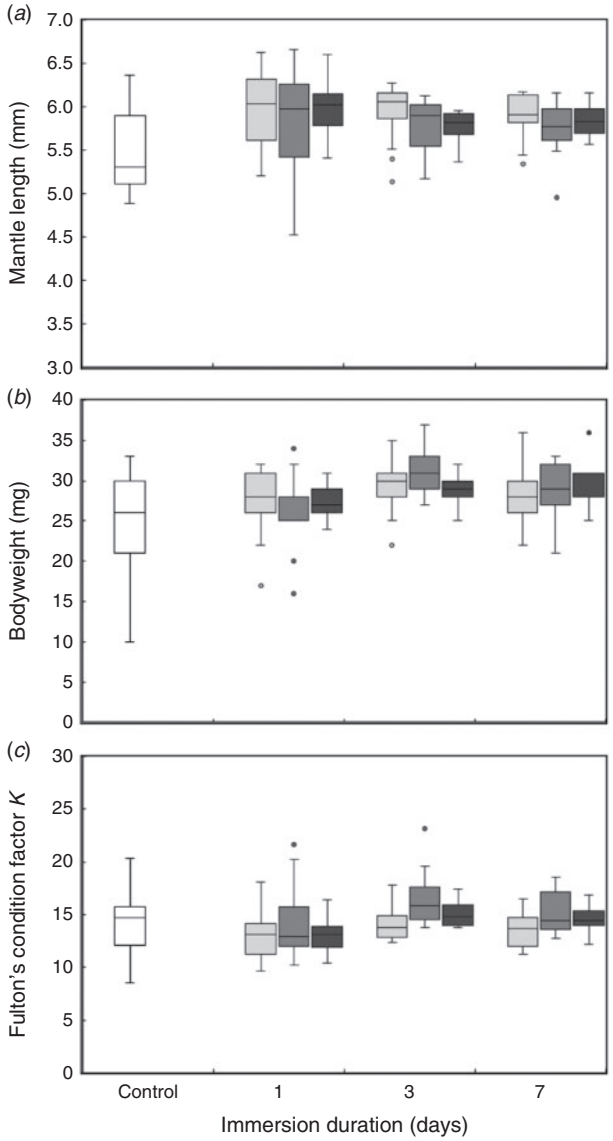

Fig. 2. Mantle length, bodyweight and Fulton's condition factor *K* of hatchlings immersed in water with different concentrations of ^{137}Ba spike, namely 0.2 ppm (light grey bars), 0.5 ppm (dark grey bars) and 1 ppm (black bars), for 1, 3 and 7 days and the control group. The boxes show the interquartile range, with the median value indicated by the horizontal line; whiskers show the range. Circles indicate outliers in each experimental group.

($F = 1.162$, $P = 0.321$) or Fulton's condition factor *K* ($F = 0.821$, $P = 0.597$) of hatchlings. The spiked concentration of ^{137}Ba significantly affected ML ($F = 5.789$, $P = 0.001$) and bodyweight ($F = 6.687$, $P < 0.001$) of hatchlings, but not Fulton's condition factor *K* ($F = 2.530$, $P = 0.058$). Hatchlings exposed to spike concentrations of 0.2 and 1 ppm were significantly longer than those in the control group (Tukey's honest significant difference (HSD), $P = 0.001$ and 0.008 respectively; Fig. 2*a*). The bodyweight of hatchlings in the control group was significantly lower than that of hatchlings in all spiked groups ($P < 0.01$, Fig. 2*b*). Conversely, the ML ($F = 5.190$, $P = 0.002$), bodyweight ($F = 8.222$, $P < 0.001$) and Fulton's condition factor *K* ($F = 3.214$, $P = 0.024$) of hatchlings differed significantly among immersion duration treatments. Individuals in most immersion duration groups had a larger size in terms of ML and bodyweight than those in the control group, except for bodyweight observed after 1 day immersion ($P = 0.063$). In addition, there was a significant difference in Fulton's condition factor *K* between 1 and 3 days of immersion ($P = 0.013$; Fig. 2*c*).

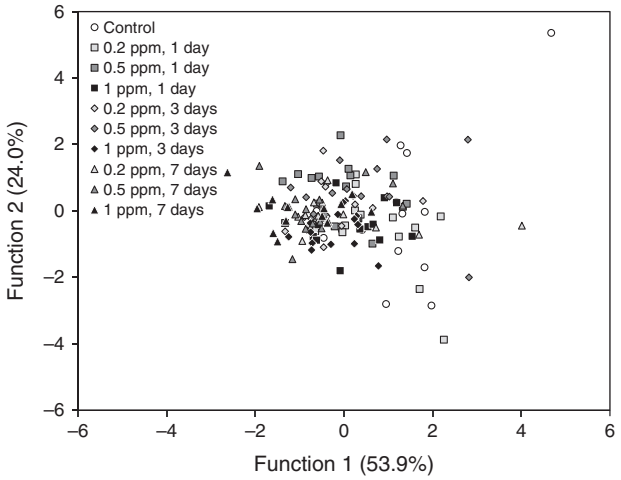

Fig. 3. Forward stepwise canonical discriminant analysis using Mg, Sr, Zn, Cu and Pb in the statoliths of hatchlings among control and all experimental groups immersed in water containing different concentrations of ^{137}Ba spike (0.2, 0.5 and 1 ppm) for 1, 3 and 7 days.

Table 1. Structure matrix coefficients for Discriminant Function (DF) 1 and DF2 for each mean element : Ca ratio used in canonical discriminant analysis for hatchling statoliths among the control and experimental groups

Main elements contributing are in bold

Element	DF1	DF2
Cu : Ca	**0.689**	0.254
Zn : Ca	0.506	**0.484**
Pb : Ca	0.336	−0.339
Mg : Ca	0.094	0.042
Sr : Ca	0.082	0.350

Element discrimination and correlation

According to canonical discriminant analysis, hatchling statolith element composition did not show a clear pattern of discrimination between the control and all experimental groups (Fig. 3). The variations explained by Functions 1 and 2 were 53.9 and 24.0% respectively. Cu primarily contributed to Function 1 and Zn contributed to Function 2 (Table 1). The cross-validated classification success for all hatchlings was 24.7%, and ranged from 0% (7 days of immersion with 0.2 ppm of ^{137}Ba) to 53.3% (7 days of immersion with 1 ppm of ^{137}Ba) (Appendix 1).

Although statoliths were enriched with ^{137}Ba, their elemental : Ca ratios (Cu : Ca, Zn : Ca and Pb : Ca) positively correlated to ^{138}Ba but not to ^{137}Ba (Table 2; Fig. 4). The

Table 2. Summary of Spearman's ρ test between Ba stable isotopes and trace elements in the statoliths of hatchlings
Significant correlations ($P < 0.05$) are in bold

	Mg : Ca (mmol mol^{-1})	Sr : Ca (mmol mol^{-1})	Zn : Ca (μmol mol^{-1})	Cu : Ca (μmol mol^{-1})	Pb : Ca (μmol mol^{-1})
^{137}Ba : Ca (μmol mol^{-1})					
r_s	0.503	−0.122	0.055	−0.067	−0.030
P-value	0.138	0.738	0.881	0.855	0.934
^{138}Ba : Ca (μmol mol^{-1})					
r_s	0.588	0.012	**0.794**	**0.794**	**0.794**
P-value	0.074	0.973	**0.006**	**0.006**	**0.006**

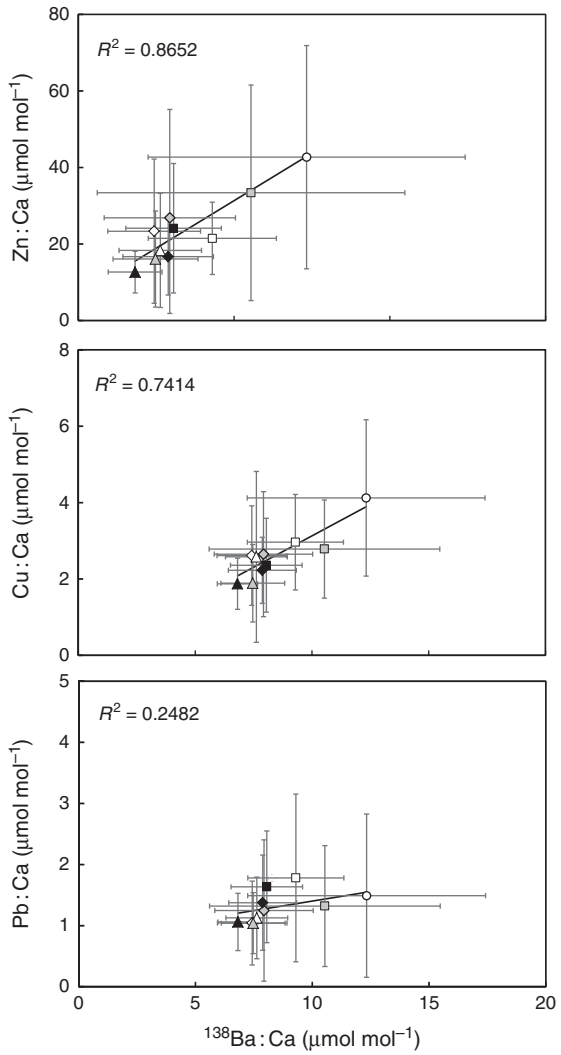

Fig. 4. Linear regressions between mean element : Ca ratios and ^{138}Ba : Ca in the statoliths of hatchlings. Symbols indicate different treatment durations (circle, control; square, 1 day; diamond, 3 days; triangle, 7 days) and difference ^{137}Ba spike concentrations (white, 0.2 ppm; grey, 0.5 ppm; black, 1 ppm). Error bars indicate the s.d.

regressions of ^{138}Ba : Ca with Cu : Ca, Zn : Ca and Pb : Ca were significant ($P < 0.01$), with determination coefficients (R^2) of 0.865, 0.741 and 0.248 respectively.

Discussion

Because of its crucial role in marine ecosystems and being a highly attractive fishery target, effective ecological monitoring and resource management of *S. lessoniana* are needed. In particular, larval dispersal patterns and demographic population connectivity have significant effects on marine organism resources (Cowen *et al.* 2000; Thorrold *et al.* 2001; Jones *et al.* 2005; Cowen and Sponaugle 2009). There are many factors influencing the success rate of mass marking (e.g. spike concentration or developmental stage; Payne *et al.* 2011; Woodcock and Walther 2014). Consistent achievement of 100% mark success is a vital goal for any mass-marking technique (Warren-Myers *et al.* 2018). For fish larvae or eggs, concentrations of ≥0.1 ppm of ^{137}Ba have been used to achieve 100% mark success by immersion (Woodcock *et al.* 2011*a*, 2011*b*; de Braux *et al.* 2014, Warren-Myers *et al.* 2015). However, the eggs of many cephalopod species (e.g. myopsid squid and sepioidea cuttlefish) are coated with encapsulation substances (i.e. a capsule) that are effective barriers against metal uptake into the embryo (Rosa *et al.* 2015). Therefore, the present study examined higher ^{137}Ba spike concentrations to mark large numbers of *S. lessoniana* hatchlings and found that 100% mark success was achieved steadily after 3 days of immersion with concentrations >0.5 ppm of the enriched barium stable isotope.

This study revealed that 7 days of immersion with lower spike concentrations could also achieve 100% mark success, indicating that immersion duration is a critical factor for marking *S. lessoniana* statolith through egg immersion. This may be because the perivitelline fluid, which is in the capsule and encasing the embryo, is conducive to ambient seawater influx and swells gradually during the late development stage (Cronin and Seymour 2000). Extension of immersion during egg swelling results in the uptake of the spiked water, decreasing the ^{138}Ba : ^{137}Ba ratio within eggs. A similar effect of immersion duration on Ba stable isotope ratios in otoliths of fish species has been reported (Munro *et al.* 2008; de Braux *et al.* 2014). Yet, this is inconsistent with the results reported for *S. apama* by Payne *et al.* (2011), who found a significant interaction between the concentration of enriched ^{137}Ba and immersion duration, but no significant differences among immersion durations for the lower concentration tested (0.3 ppb). Species and physiological differences may explain these different results. For example, egg swelling time varies according to embryo development period, thus the longer embryo development of *S. apama* (3–5 months; Hall and Fowler 2003) would dilute the contribution of immersion time to the ^{138}Ba : ^{137}Ba ratios in *S. apama* statoliths.

Moreover, the low enriched ^{137}Ba concentration may need a longer time of immersion, and the effect of immersion duration would become significant. In the study of Payne *et al.* (2011), extension of immersion duration from 2 to 8 days did decrease ^{138}Ba : ^{137}Ba ratios for the higher-concentration (1 ppb) treatment group. Therefore, determining the appropriate concentration and corresponding time of immersion before using this technique on a species of interest is important, because life history characteristics (e.g. developmental stage) and habitats (e.g. seawater or fresh water) may affect the effectiveness and the costs for mass marking.

The ML of hatchlings in this study was consistent with that reported by Lee *et al.* (1994), who continuously cultured *S. lessoniana* through three successive generations and whose hatchlings averaged 5.3 mm ML, ranging from 3.5 to 6.4 mm ML. The bodyweight of hatchlings in past studies varies, from a range of 4.3–12.0 mg (mean 8.2 mg; Lee *et al.* 1994) to 50 mg (Segawa 1987); the bodyweight of hatchlings in the present study fell between values published in the literature. In the present study, ^{137}Ba mass marking slightly increased the ML and bodyweight of marked hatchlings in some of the experimental groups. Larger hatchling size may benefit from an increased attack speed (Sugimoto and Ikeda 2013) and a reduction in the distance required to capture prey accurately (Chen *et al.* 1996). In addition, hatchling size is linked to vulnerability to predators (Blaxter 1986; Sogard 1997), so that larger size hatchlings would have a greater survival rate in the early life history stages. Moreover, the growth condition (K) is related to embryo development and environmental variables, and individuals in a better condition (K) have higher survivorship and greater growth rate (Bolger and Connolly 1989). However, the K values of hatchlings in the present study only differed significantly between two immersion duration groups, indicating that ^{137}Ba mass marking did not affect hatchling growth condition. Previous experimental results of the effects of transgenerational marking (i.e. injection method) on the condition of larval fish were species specific. Positive (Starrs *et al.* 2014*a*, 2014*b*), negative (Williamson *et al.* 2009) and no significant effects (Zitek *et al.* 2013; Warren-Myers *et al.* 2015) on size at hatch, yolk sac area, oil globule area and eyeball diameter were found among species. The findings of the present study provide additional information on cephalopod species marked using the immersion method. As noted by Starrs *et al.* (2014*b*), the effects of such mass marking with stable isotopes on hatchling morphology require additional research, as does the roles of barium during the developmental of *S. lessoniana* embryos.

We found different element compositions of statoliths in hatchlings that were related to size at hatch, and significant correlations were found between Me : Ca ratios (Cu : Ca, Zn : Ca and Pb : Ca) and ^{138}Ba : Ca. The effects on element composition of statoliths are not often mentioned in the literature when marking cephalopod offspring with enriched stable isotopes. Element uptake in cephalopod statoliths is presumably similar to the observations in fish otoliths (Gillanders *et al.* 2013) and is primarily associated with environmental changes, such as water chemistry composition (Arkhipkin *et al.* 2004) and ambient temperature (Ikeda *et al.* 2002; Zumholz *et al.* 2007). However, in this study the rearing seawater was maintained at consistent conditions and egg capsules in the same cluster were used to eliminate any possible affects from the maternal yolk (e.g. Lloyd *et al.* 2008). The difference in growth rate between control and experimental groups was a potential explanation for variations in element incorporation. Growth rate has been confirmed to be negatively correlated with the elemental partition coefficient in otoliths of teleost species (Walther *et al.* 2010). A faster growth rate could result in more calcium-binding proteins, altering relative ion concentrations in the calcifying fluid (Kalish 1989). Therefore, trace elements such as Cu and Zn have a greater likelihood of being associated with organic matrix protein (Miller *et al.* 2006). In addition, a fast growth rate usually occurs with higher calcium carbonate accretion rate (Ikeda *et al.* 1999), which raises the pH of the calcifying fluid and reduces trace element concentrations in the endolymph, resulting in a negative relationship between the Me : Ca ratio and accretion rate (Sinclair 2005; Sinclair and Risk 2006; Hamer and Jenkins 2007). The lower hatchling size in the control group could simultaneously lead to elevated patterns of Me : Ca in statoliths. Although the effects of growth rate on the element composition of cephalopod statoliths have not been adequately clarified, the physiological processes do significantly affect the microchemistry of biogenic carbonates. We emphasise that the mechanisms of trace element incorporation into statoliths should be carefully considered to avoid confounding environmental signatures with artificial marking.

Understanding the population connectivity and migration of cephalopods is critical in developing approaches for the resource management and conservation of marine ecosystems. Stable isotope mass-marking techniques can be successfully used in fishes. This study demonstrated unique signatures in *S. lessoniana* statoliths with 100% marking success after 3 days of immersion in ^{137}Ba and provides a method to unravel the questions regarding dispersal mechanisms and movement patterns in cephalopods. However, we also found potential effects of stable isotope mass marking on offspring size at hatch that are consistent with those reported by an increasing number of studies. The effects on embryo development and growth may induce variations in element composition in statoliths, probably reflecting physiological processes and statolith accretion, which affect statolith chemistry and may subsequently affect the accuracy of interpreting an individual's environmental history. The present study defined the successful marking conditions for use with stable isotope-marking techniques on *S. lessoniana* and their potential effects on cephalopod statoliths. We highlight that the effects of this technique need to be taken into consideration in field applications. Additional research investigating the relationships among multiple elements and physiological responses to enriched stable isotope incorporation will advance our knowledge for the application of these techniques to wild cephalopods.

Conflicts of interest

Chia-Hui Wang declares that she is a guest editor of the Otoliths Symposium special issue for *Marine and Freshwater Research* but took no part in the review and acceptance of this manuscript. The authors declare that they have no further conflicts of interest.

Declaration of funding

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Appendix 1 Cross-validated classification success for the statoliths of hatchlings in the control and experimental groups based on the discriminant function analysis scores

Correct classifications are in bold. Hatchlings in the experimental groups were immersed in water containing different concentrations of ^{137}Ba spike (0.2, 0.5 and 1 ppm) for 1, 3 and 7 days

Treatment	Control	0.2 ppm, 1 day	0.5 ppm, 1 day	1 ppm, 1 day	0.2 ppm, 3 days	0.5 ppm, 3 days	1 ppm, 3 days	0.2 ppm, 7 days	0.5 ppm, 7 days	1 ppm, 7 days
Control	**26.7**	26.7	0.0	0.0	6.7	13.3	13.3	6.7	6.7	0.0
0.2 ppm, 1 day	20.0	**20.0**	0.0	13.3	0.0	20.0	6.7	13.3	6.7	0.0
0.5 ppm, 1 day	0.0	0.0	**13.3**	20.0	6.7	26.7	6.7	6.7	6.7	13.3
1 ppm, 1 day	6.7	20.0	13.3	**26.7**	0.0	0.0	26.7	0.0	0.0	6.7
0.2 ppm, 3 days	13.3	6.7	13.3	0.0	**6.7**	13.3	13.3	13.3	13.3	6.7
0.5 ppm, 3 days	13.3	6.7	6.7	0.0	6.7	**20.0**	0.0	13.3	26.7	6.7
1 ppm, 3 days	0.0	13.3	0.0	6.7	6.7	6.7	**40.0**	6.7	6.7	13.3
0.2 ppm, 7 days	20.0	0.0	6.7	0.0	0.0	13.3	6.7	**0.0**	20.0	33.3
0.5 ppm, 7 days	6.7	0.0	6.7	0.0	0.0	13.3	13.3	0.0	**40.0**	20.0
1 ppm, 7 days	0.0	6.7	0.0	0.0	0.0	6.7	0.0	13.3	20.0	**53.3**