

**THE INFLUENCE OF TEMPERATURE AND SALINITY ON THE STATOLITH
OF THE OVAL SQUID *SEPIOTEUTHIS LESSONIANA* LESSON, 1830
DURING EARLY DEVELOPMENTAL STAGES**

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ABSTRACT: *Sepioteuthis lessoniana* Lesson, 1830 is an important fishery resource in Taiwan. Its distribution is concentrated around the northeastern and the southern coasts of Taiwan, and the Peng-Hu Archipelago. In this study, we use the known-age statoliths incubated in the different conditions to relate with those factors, *i.e.*, temperature, salinity, and photoperiod, which influenced the ring formations during the embryonic and larval stages. Although the growth increments can be counted in each specimen, the numbers of embryonic increments do not correspond with the time span from stage 24 to hatching. Squid larvae were maintained as long as possible. The trend which older squids have more increments on the statolith than younger squids is consistent, but the slope between the increments and survival days is less than one. This study indicates that the hypothesis of daily-increment for the whole life history requires further verification with larger sample size and wider size ranges of the tropical squids in future.

INTRODUCTION

Sepioteuthis lessoniana Lesson, 1830 is a loliginid squid with a wide distribution in the Indo-Pacific Ocean. This species is reported to range from northern Japan to northern Australia and from the Hawaiian Islands in the eastern Pacific to the Gulf of Aqaba in the upper Red Sea (Dunning, 1998). It is an important fishery resource in Taiwan. Its distribution is concentrated around the northeastern and the southern coasts of Taiwan, and the Peng-Hu Archipelago. The capture method is usually with jigging, producing a high quality product. Little information has been published on this species in this region. Taiwan Fisheries Research Institute had tried to rear *S. lessoniana* in 1989, but no further studies on aquaculture were continued (Yu and Tung, 1989). Diversity of cephalopods from the waters around Taiwan was published by Lu (1998). Other pieces of information were based on personal observations by scuba divers and fishermen, *i.e.* spawning

behaviour, spawning grounds, and fishery catch.

The ability to age individual organisms in recent years had been one of the most significant advances towards our understanding of the population dynamics of the squid. The way in which environmental conditions affected the statolith increment-deposition ratio remained virtually unknown. Attempts to validate the “one ring equals one day” hypothesis had been only partially successful. Some results of studies tended to support the hypothesis (Jackson, 1989; Jackson, 1990; Jackson and Moltschaniwskyj, 2001; Jackson *et al.*, 1993; Lipinski, *et al.*, 1998; Durholtz, *et al.*, 2002). In those studies, statoliths in live captive squid were marked by administering tetracycline and growth increments were later counted outside a marked increment or within a marked zone. There are some other evidences against the “one ring equals one day” hypothesis (Morris, 1991; Morris, 1993; Villanueva, 2000b). The region of the statolith corresponding to the

experimental treatments could not be identified from daily increment counts. Most of studies are concentrated on subadult and adult individuals; there are just a few studies that focus on the early life stages, *i.e.*, embryos, larvae or juveniles. Our knowledge on the formation of rings during the cephalopod's life history is still incomplete, and the "one ring equal one day" hypothesis might not be applicable to all life history stages.

Based on the study of *S. lessoniana*, the statolith could be observed clearly at stage 24 during the embryonic development (Segawa, 1987). The duration from statolith formed in the embryo to hatching took more than one week at 25°C (personal observation). Consequently, the duration of embryonic development was highly temperature dependent. In this study, we used the known-age statoliths incubated in the different conditions to relate with those factors, *i.e.*, temperature, salinity, and photoperiod, which influenced the ring formation during the embryonic and larval stages.

MATERIALS

1. Collection of egg-strings

Eggs of *Sepioteuthis lessoniana* can be collected by trawling from February to October, although the main season is from May to September. The main sampling site in this study was at 25°04' N, 121°54' E in the Chin-Sha Bay of northeastern Taiwan. The sampling area was covered with coral reef, which dispersed from the coast to 400 m seawards. Beyond the coral reef, an area covered with coral sands, about 200 m long and 60 m wide, was chosen to be the sampling site. Fresh bamboos were collected from the hill near the sampling area, and 10–12 bamboos were fastened together to be a cluster for attracting squid spawning. From April to September, more than twenty clusters of bamboos, 3–4 m long, 1–2 m wide, were set on the sea bed at a depth of 16 to 20 m. The mature individuals of *S. lessoniana* were quickly attracted to the artificial spawning site. During the main season, we undertook 1–4 scuba-divings a week to check the bamboo clusters. If the bamboo cluster was filled with egg-strings, we built a new one for squid to spawn. The egg-strings were

usually filled on all the clusters of bamboo in one or two weeks. Five maximum-minimum thermometers were set at the base of the clusters. The range of temperatures of maximum and minimum was recorded during every scuba diving interval. The salinity at the surface and bottom of the sampling site was measured during every dive.

2. Field specimens

New spawning egg-strings, which were marked with red ropes and covered by nets above, were allowed to develop in the field until near hatching (after stage 26). They were collected to observe the ring formation during embryonic development in the natural condition.

EXPERIMENTAL METHODS

Experiment I (The effects of different temperatures and salinities on the hatchling's statolith):

When embryonic development reached stage 15, egg-strings were collected from the field. Egg-strings were maintained in 1:1 of oxygen water ratio for transporting to the laboratory. The total time in transit was less than 10 hr and the packing of frozen chemical ice packs limited temperature change during transport to no more than 2°C from the field water temperature. Squid eggs were kept in a closed, recirculating seawater system consisting of nine 120 l cylindrical plastic rearing tanks and twelve 80 l glass rearing tanks, five submerged biofilter beds and one activated carbon filter. The entire system held a volume of approximately 3600 l. Water was completely circulated through a 40 W ultraviolet sterilizer twice daily. When embryos reached stage 24, they were transferred into different rearing conditions, *i.e.*, salinity of 20, 25, 30, 35 ppt. and temperature of 15, 20, 25, 30°C, a total of 16 combinations of the rearing conditions. During the course of the experiment, the embryos were exposed to the photoperiod of 12 hours light and 12 hours dark. When the larvae hatched, the hatchlings were preserved in the 70% neutral buffer EtOH until statoliths were taken.

Experiment II (The effects of two different photoperiods on the larval ring formations):

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After hatching, squid hatchlings were separated to incubate in both of the photoperiod regimes, 12 hr: 12 hr and 24 hr constant light conditions. The larvae were fed with adult brine shrimps enriched with PACK BOOST Enrichment Diets (Golden Prawn Enterprise Co, Kaohsiung, Taiwan) and the live larvae of *Tilapia* sp. three times a day (7:00, 14:00, 22:00). The nutrient in the enriched brine shrimps was too low for larval squid after 8 hours of feeding. These residual brine shrimps were removed before further feeding. Squid larvae were maintained as long as possible. When they died, they were kept in the 70% neutral buffer EtOH until statoliths were removed.

Statolith analysis:

For every specimen, mantle length of the fixed specimens was measured and statoliths were extracted with fine forceps. Statoliths were washed with a supersonic cleaner for one minute, and mounted whole in Crystal Bond thermoplastic cement. Owing to the small size of the statoliths, it was not necessary to grind or polish them. Statoliths were viewed using Zeiss Axiolab microscope and photographed with the Nikon coolpix 990 digital camera. The statolith of the larvae was small and transparent, the image program (Adobe Photoshop 6.0) can be used to modify the contrast between the rings to make the increments easy to count. Total statolith length (TSL) was measured and the rings were counted with the program Matrox Inspector 2.0. The data was analyzed with the statistics program SAS 8.1. Linear equations were fitted to the relationships between TSL and ML for different incubated conditions. Student's two-tailed t-test was used to determine the significance of the difference between slopes and intercepts of the calculated equations. The Duncan multiple-range test was used to describe in words the relationship among the numbers of the rings in the hatchling statoliths.

RESULTS

The field specimens:

A total of 71 scuba-dives were made in 2002, more than 4,500 minutes dive-time. Movement of

the water current in the sampling site was southward after the flood tide, and northward after the ebb tide. Bottom temperature at the sampling site usually changed quickly according to the temperature of the current. Temperatures ranged from 20.5 to 29.5 °C during the main spawning season, from May to September. During these months, the temperature usually fluctuated in 3–5 degree range in a week, but sometimes the temperature range differed by 7 degrees in one day in July and August (Fig. 1). Three typhoons passed through the sampling site on 3 July, 10 July, and 2 September 2002. When the typhoon passed, sea temperature had a narrow range of 27–29 °C for a week. The salinity of the sampling site at that time was 35 ppt. and did not fluctuate with the tide or rain.

By field observation in the past three years (unpublished data), the individuals which spawned from April to the middle of June were larger, with a mantle length of 35–40 cm in females, and 50–60 cm in males. After June, sizes of the adults became significantly smaller, the females about 15–20 cm, and the males about 20–35 cm. Three main types of egg strings with different numbers of eggs were found, *i.e.*, 4–6, 7–9, and 10–12 eggs per string, although different numbers of eggs, from 0 to 12 eggs, in the egg-strings can be found. The type with 10–12 eggs was only found from July to September, less than 1 percent in number among all egg strings. The boundary of time between the two types of egg strings, 7–9 eggs and 4–6 eggs types, was found in July, the former was found before July and the later after July.

The marked egg-strings, 4–6 eggs, developed for 20–24 days to reach stage 28, the size of the external yolk sac was approximately the same as mantle length, in July and August in the field. Statoliths of 71 specimens were mounted for the ring counting. Mantle length of the hatching is 4.6 ± 1.33 mm and the total statolith length is 291.80 ± 85.73 μ m. The relationship between ML and TSL is shown with a linear equation in Table 1. Ring count in the left statolith are the same as in the right one ($P = 0.3817$, $df = 68$). The rings were counted in the left statolith. If the left statolith was unavailable, the right one was used for ring counting. The embryonic ring number in these specimens were

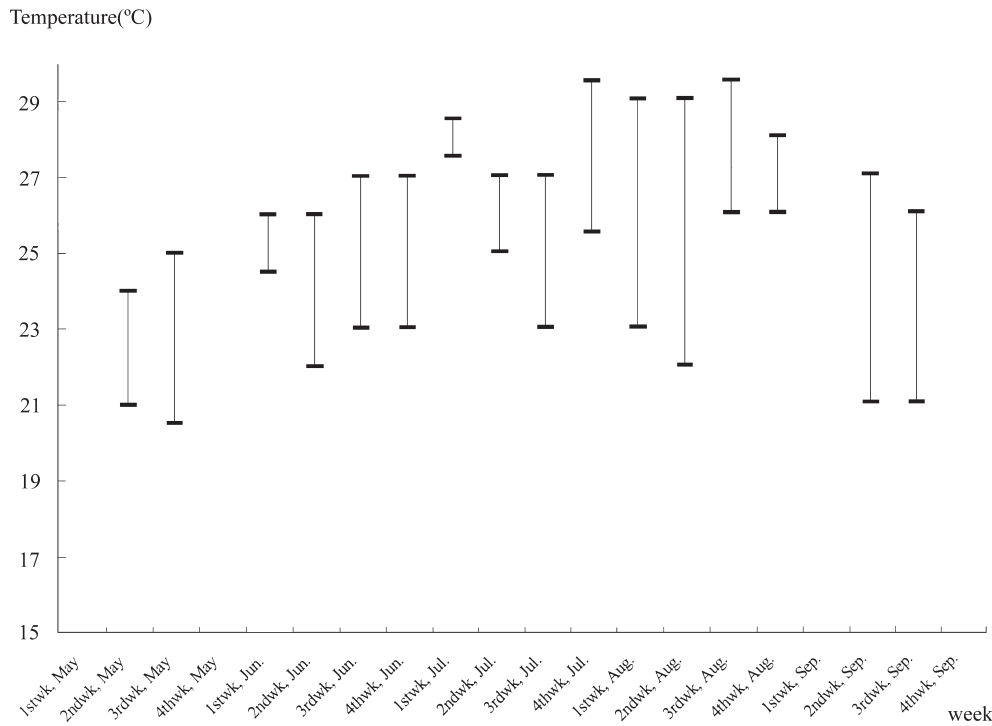


Figure 1. The temperature range during the spawning season in 2002.

from 0 to 13 at hatching, most of them concentrating from 0 to 5 (Fig. 2).

Experiment I (The effects of different temperatures and salinities on the hatchling's statolith):

All embryos died in 15 °C in all four salinities, and all embryos died in 20 and 25 ppt. in all four temperatures. Embryos developed normally in the following conditions, *i.e.*, 20 °C 30 ppt., 20 °C 35 ppt., 25 °C 30 ppt., 25 °C 35 ppt., and 30 °C 30 ppt. The relationships between ML and TSL in these different incubated conditions are shown in Table 1. On most occasions, the statolith size at hatching in these different conditions had a consistent trend (Table 2). The embryonic ring counts in the left statolith were the same as in the right one among all of these hatchlings ($p = 0.8137$, $df = 27$; $p = 0.2214$, $df = 45$; $p = 0.4383$, $df = 39$; $p = 0.2821$, $df = 191$; $p = 0.4208$, $df = 43$). Most of hatchlings in all of the cultured conditions had embryonic

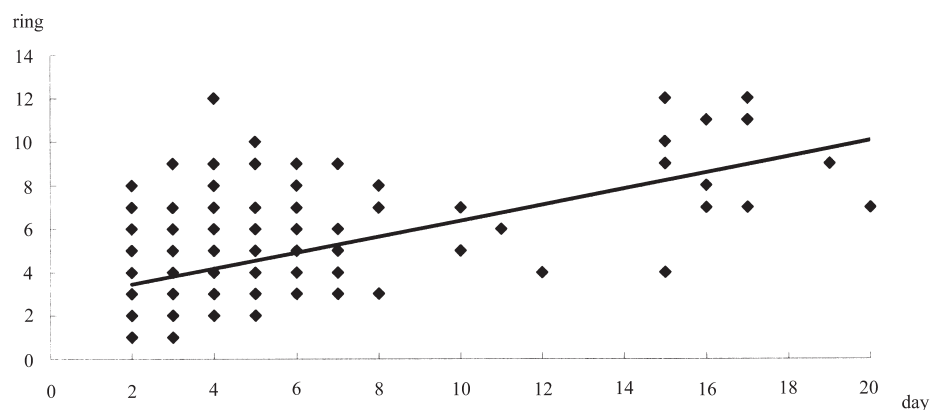
rings, concentrating from 0 to 5, but a part of hatchlings had nothing, especially before stage 26 (Fig. 2).

Experiment II (The effects of two different photoperiods on the larval ring formations):

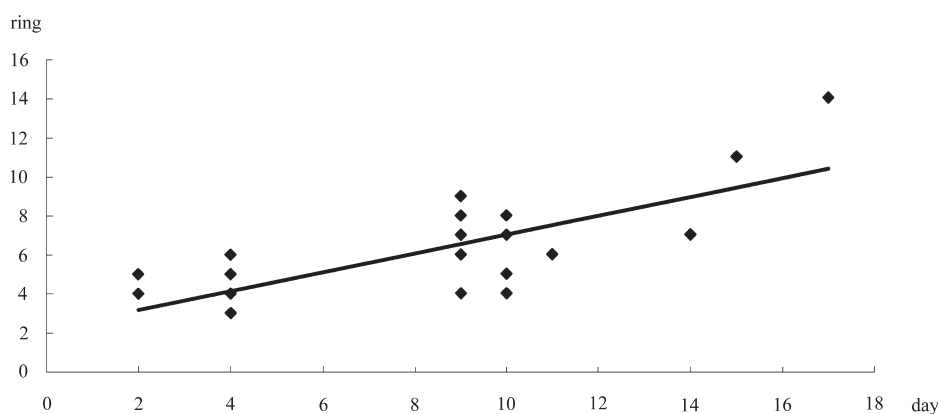
The rings on the rostrum in the hatchlings of *S. lessoniana* are as distinct as can be counted on the lateral dome, and numbers of rings are usually more than those of the lateral dome. It is most suitable for the increment reading in larval stages. The largest statolith, TSL = 830 μm , was transparent enough for reading the increments in this study. If the lateral dome is accumulated with calcium making the statolith thick, not transparent enough for reading, the statolith will need to be ground to read the increments.

The longest cultured duration was 20 days. The relationships between ML and TSL in the natural photoperiod regime, and in the constant 24 hr light region are shown with a linear equation in Table 1.

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A



B

Figure 2. Linear regression between survival duration and increments. A. in the natural photoperiod (12:12). $R = 0.3674 D + 2.705$, $r^2 = 0.2992$, $n = 444$; B. in the 24 hr constant light. $R = 0.4796 D + 2.2114$, $r^2 = 0.6397$, $n = 40$

Whether in the normal photoperiod or in the 24 hr light condition, the increments could be counted in all of these statoliths. The relationship between statolith increment number and actual squid age was variable. Relationships between increment number and living durations are shown with a linear equation. Although the trend of the older squids having more increments on the statolith than younger squids is consistent, the slope between the increment number and days lived was less than one (Fig. 3). The time required to form one ring on the statolith was more than one day. The total data set of the relationship between ML

and TSL in 25 °C, 35 ppt. contains 631 specimens, the original equation is $TSL = 44.706 ML + 115.13$ ($P < 0.0001$). The statolith growth rate has a high correspondence with the growth of the mantle length. The subsamples separated based on the numbers of rings on statoliths, were used to analyse the statolith growth rate. Frequency distribution of the statolith size at each ring, 1–9 rings, is bell-shaped, but the bell-shape is not formed in 10–12 rings because of smaller sample size. Intervals between rings are highly variable. The same size rank of statoliths has different rings, *i.e.* in the size rank of 326–350 μm having 1–8

Table 1. Results of t-test for slopes and intercepts among regressions between total statolith length (TSL) and mantle length (ML) of all rearing conditions.

Line 1 (field):	TSL = 60.1560 ML + 15.0448	$r^2 = 0.8671$ (n = 71)
Line 2 (20°C, 30‰):	TSL = 42.0672 ML + 79.7438	$r^2 = 0.4643$ (n = 28)
Line 3 (20°C, 35‰opsu):	TSL = 27.8353 ML + 158.9410	$r^2 = 0.2808$ (n = 46)
Line 4 (25°C, 30‰opsu):	TSL = 32.9875 ML + 145.2587	$r^2 = 0.5616$ (n = 40)
Line 5 (25°C, 35‰opsu):	TSL = 45.1122 ML + 97.9769	$r^2 = 0.7152$ (n = 192)
Line 6 (30°C, 35‰opsu):	TSL = 33.0115 ML + 153.7694	$r^2 = 0.5905$ (n = 44)
Line 7 (12:12, 25°C, 35‰opsu):	TSL = 44.5531 ML + 122.2147	$r^2 = 0.743$ (n = 554)
Line 8 (24, 25°C, 35‰opsu):	TSL = 67.7741 ML + 13.1897	$r^2 = 0.7507$ (n = 40)

Test I:

- a. Line 2 vs. Line 4
Slope t-test, Ho: $Sb_2 = Sb_4$ T = 0.8739, P = 0.1927
Intercept t-test, Ho: $Int_2 = Int_4$ T = 3.3627, P = 0.0006*
- b. Line 3 vs. Line 5
Slope t-test, Ho: $Sb_3 = Sb_5$ T = 2.8232, P = 0.0026*
- c. Line 3 vs. Line 6
Slope t-test, Ho: $Sb_3 = Sb_6$ T = 0.6622, P = 0.2548
Intercept t-test, Ho: $Int_3 = Int_6$ T = 4.2696, P < 0.0001*
- d. Line 5 vs. Line 6
Slope t-test, Ho: $Sb_5 = Sb_6$ T = 2.7254, P = 0.0035*

Test II:

- a. Line 2 vs. Line 3
Slope t-test, Ho: $Sb_2 = Sb_3$ T = 1.1254, P = 0.1321
Intercept t-test, Ho: $Int_2 = Int_3$ T = 3.3044, P = 0.0008*
- b. Line 4 vs. Line 5
Slope t-test, Ho: $Sb_4 = Sb_5$ T = 2.4692, P = 0.0071*

Test III:

- a. Line 1 vs. Line 3
Slope t-test, Ho: $Sb_1 = Sb_3$ T = 4.9177, P < 0.001*
- b. Line 1 vs. Line 5
Slope t-test, Ho: $Sb_1 = Sb_5$ T = 4.1095, P < 0.001*
- c. Line 1 vs. Line 6
Slope t-test, Ho: $Sb_1 = Sb_6$ T = 5.5610, P < 0.001*

Test IV:

- a. Line 7 vs. Line 8
Slope t-test, Ho: $Sb_7 = Sb_8$ T = 3.6114, P < 0.001*

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Table 2. The statolith size at hatching among different conditions had a consistent trend.

In the same salinity

temperature low → high
statolith small → large

In the same temperature

salinity low → high
statolith small → large

In the same salinity

temperature constant (lab.) → fluctuated (field)
statolith small → large

In the same salinity and temperature

photoperiod 12hr : 12hr → 24hr
statolith small → large

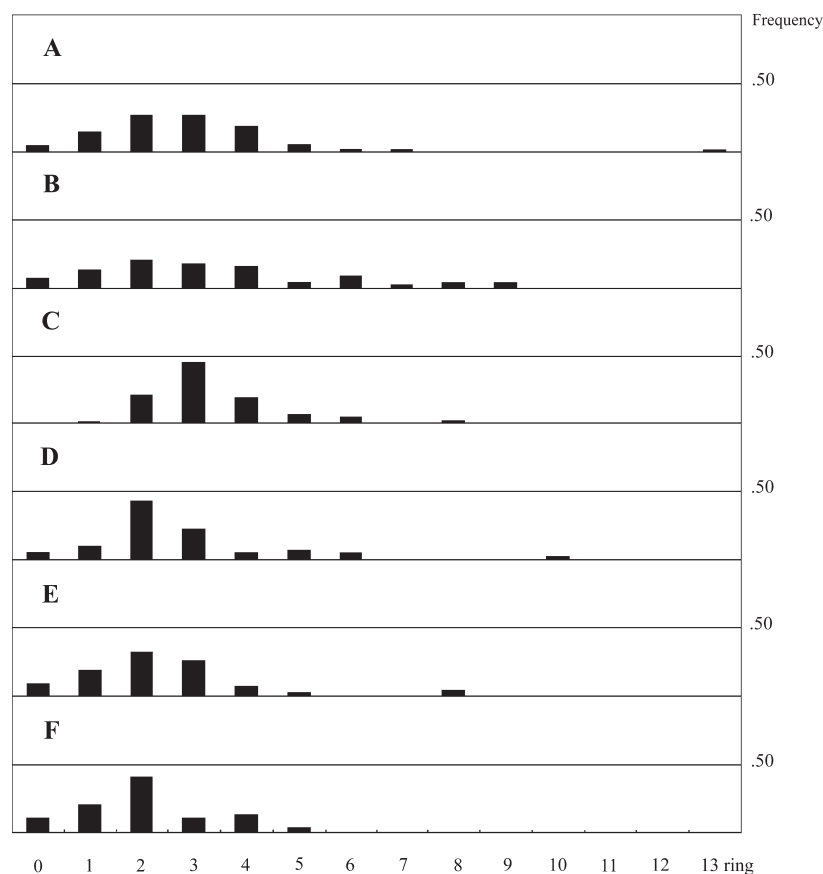


Figure 3. Frequency distributions of embryonic rings at hatching in the different conditions. A. in field; B. in 30°C, 35‰; C. in 25°C, 35‰; D. in 25°C, 30‰; E. in 20°C, 35‰; F. in 20°C, 30‰.

rings; in the size rank of 401–425 μm having 2–12 rings, *et al.*, presenting the bell-shape distribution (Fig. 4). Multiple comparisons are performed among slopes of these 10 equations. There is no significant difference among them. The statolith accumulative rate corresponds with the mantle length. Although rates of calcium accumulation on statoliths are similar, the interval between increments has high variations, the same size statoliths having different increment numbers. The ring formation rate cannot match with the rate of calcium accumulation. Intervals between rings are highly variable, and it is difficult to predict how many rings are in a known size statolith.

DISCUSSION

The majority of research on squid demography had taken place in temperate waters. Life spans for large temperate squids were generally believed to be annual (Natsukari, *et al.*, 1988). Daily increment formation in cephalopod statoliths had been validated for few species and it was of some concern that such a presumed pattern (*i.e.*, daily increment) had been extended to many species, without accompanying validation studies. Even for those species for which daily increment formation had been validated, nothing was known of the error associated with detecting daily increments.

The increments on embryos of *Loligo subulata* were significantly fewer than the number of elapsed days of the experiment (Morris, 1991b; Morris, 1993). The evidence was reported that differential daily growth rates existed in embryonic statoliths of the squid *Loligo vulgaris* incubated at lower temperature. Non-daily increment deposition observed in embryos of *L. vulgaris* corresponded to periods of low temperature during which statolith growth was substantially lower than at higher temperatures (Villanueva, 2000b). It was consequently very likely that the low incubation temperatures experienced by the embryos in the study of Morris (1993) caused reduced statolith growth, resulting in narrow increments, less than 1 μm , which were difficult to detect and count and hence contributing to an underestimate of the number of increments in

the statoliths. In this study, intervals among rings were always more than 2.5 μm , good enough for counting under microscope resolution. Results of increment numbers were less than elapsed days in embryos and larvae of *S. lessoniana* in this study.

Daily periodicity in statolith increments of *S. lessoniana* had been previously validated for tropical Australian (Jackson, 1990; Jackson and Moltschaniwskyj, 2001), Japanese (Jackson, *et al.*, 1993), and Philippine populations (Balgos and Pauly, 1998). Among these studies, the daily rings were checked with juveniles and subadults stained with tetracycline or Alizarin red. Jackson and Moltschaniwskyj (2001) found that there was a reduction-trend in the increment widths of the statoliths of squids after tetracycline staining. In another study, the mortality in the days following each tetracycline staining session may have been caused by handling of the squids during this procedure; this may also have been the source of the possible stress marks on the *L. vulgaris* statoliths analyzed (Villanueva, 2000a). Tetracycline-regulated gene express systems are widely used to allow temporal and quantitative control of transgene expression in cultured cells and transgenic animals (Rennel and Gerwins, 2002). There are more and more studies about tetracycline on the gene or cell level that pathways are known in detail. Microinjection of tetracycline to regulate tTA mRNA binding could cause different degrees of tetracycline-regulated gene expressions in different developmental stages, oocyte and embryo, in *Xenopus laevis* (Ridgway, *et al.*, 2000). The effect of tetracycline on cephalopods during different life stages is little known. Although tetracycline is an excellent marker for statoliths, tetracycline may cause different degrees of effects on the cephalopod's physiology in different life stages. Tetracycline staining was not performed in this study, and it might be the reason for the different result, non-daily ring, to the early studies.

Different ageing methods may also cause different results of increments and counting biases. Lipinski and Durholtz (1994) found that significant differences in increment counts for

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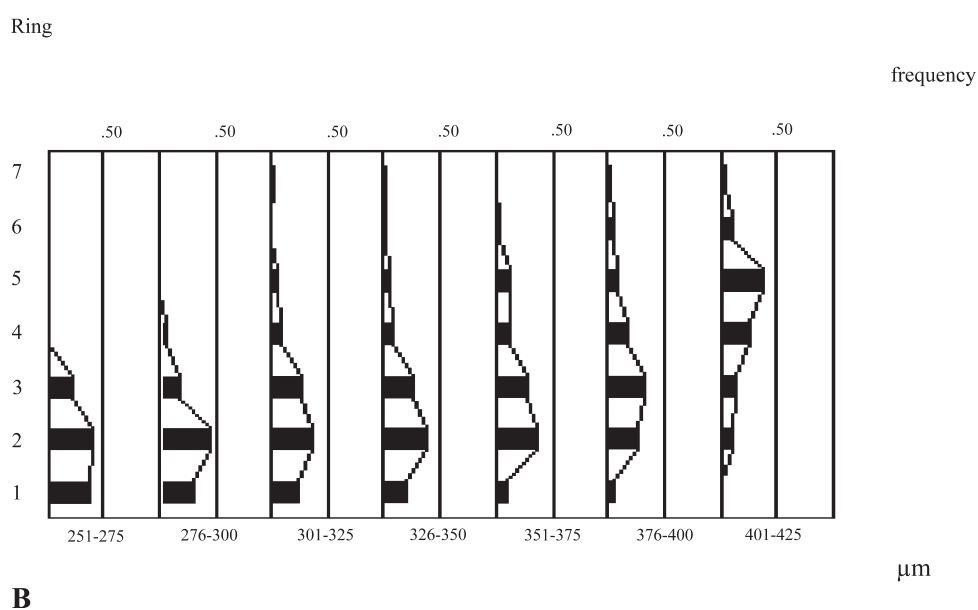
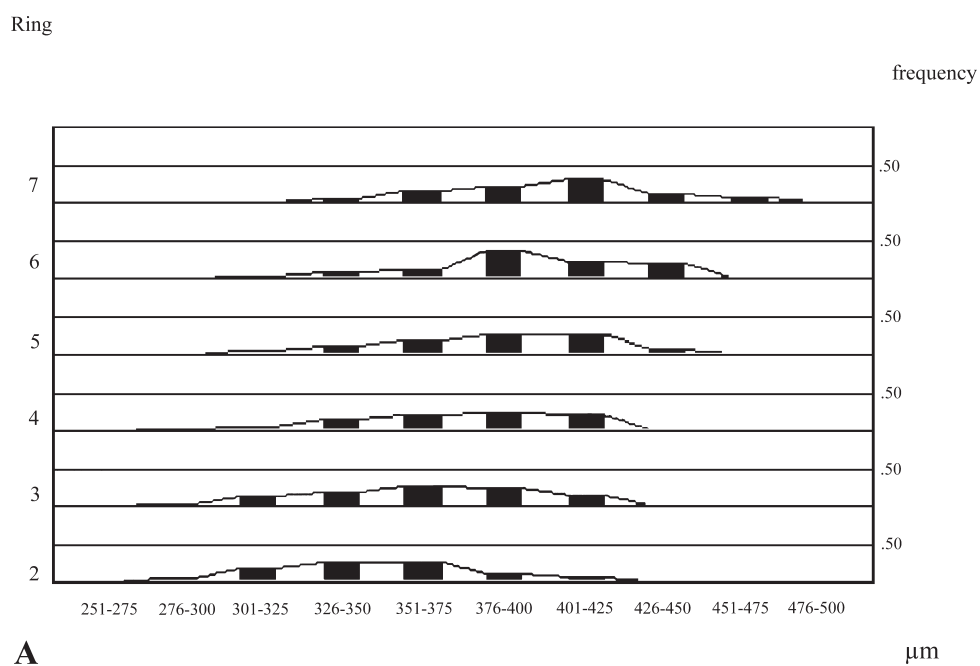


Figure 4. The bell-shaped distribution. A. Different sized statoliths with same number of rings (n= 574); B. Statoliths of the same size rank with different numbers of rings (n = 568)

Loligo vulgaris reynaudii between a light microscopy method and a scanning electron microscopy method. Gonzalez *et al.*, (2000) found that both the mean number of increments and variance from the camera lucida method were lower when statoliths ground on two sides were examined than those ground on only one side were examined. Systematic bias between methods had a great effect on estimated growth rates. Bell-shaped distributions can be found in several analyses in this study, especially with large sample size in 25 °C at 35 ppt. In the regression between elapsed days and rings, bell-shaped distributions were presented from 2 to 7 elapsed days (n = 422). Frequency distributions of the statolith size at each ring, for the range 2–7 rings (n = 574), and numbers of statoliths with different rings at each size class 276–300 µm to 426–450 µm (n = 568) also were bell-shape distributions. If the sample size was small, the bell-shape distribution would be obscure. This may result in oversimplification in interpreting the result. Sometimes this kind of problem could be inevitable because collecting a large enough sample size was difficult or even impossible.

Abiotic and biological events could cause the formation of peripheral checks on the statolith. Factors such as low temperature, tetracycline marking, stress, and counting bias would influence the result of increment counting. All these events would cause the result of increment counting to present a wide distribution instead of a concentrated distribution. Unfortunately, results presented in this study only apply to a narrow size range of larval *S. lessoniana*. In any validation exercise, it would be difficult to obtain data for the whole life cycle. This study indicates that the hypothesis of daily-increment for the whole life history, at least in *S. lessoniana*, would need to be verified with larger sample size and wider size ranges of the tropical squids in future.

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