Journal of Experimental Marine Biology and Ecology 447 (2013) 132–137

Contents lists available at SciVerse ScienceDirect

Journal of Experimental Marine Biology and Ecology

journal homepage: www.elsevier.com/locate/jembe

Special issue: Cephalopod Biology

Age validation of the growth lamellae in the cuttlebone from cultured *Sepia pharaonis* at different stages ☆

Ming-Tsung Chung [a,b], Chia-Hui Wang [c,d,*]

[a] School of Ocean and Earth Science, University of Southampton, Southampton, UK
[b] Earth Dynamic System Research Center, National Cheng Kung University, Tainan, Taiwan
[c] Department of Environmental Biology and Fisheries Science, National Taiwan Ocean University, Keelung, Taiwan
[d] Center of Excellence for Marine Bioenvironment and Biotechnology, National Taiwan Ocean University, Keelung, Taiwan

ARTICLE INFO

Keywords:
Alizarin complexone
Cuttlefish
Hatchlings
Juveniles
Lamella width
Temperature effect

ABSTRACT

Here we examine growth rates of lamellae in the cuttlebones of *Sepia pharaonis* reared at different stages and temperatures (20, 25 and 30 °C). Juveniles showed a faster and more stable increase in the number of lamellae than hatchlings. Different temperatures affect the widths of increments; these decrease as the temperature increases from 20 to 30 °C. Our results suggest that in *S. pharaonis* the pattern of growth of the cuttlebone's structure does not follow a 'daily' increment, and that increments of lamellae vary between life stages. The growth of the cuttlebone is therefore considered to be affected by a combination of physiological and environmental factors.

1. Introduction

Age is the critical information required for understanding the life history and population dynamics of cephalopods, especially considering that these animals have short life cycles and fast growth rates (Boyle and Rodhouse, 2005; Lee et al., 1994; Pierce et al., 2008). Age determination is also critical to maintain stable and sustainable cephalopod stocks. However, research on age determination of cuttlefish is largely focused on *Sepia officinalis* and little research is conducted on other species such as the pharaoh cuttlefish, *Sepia pharaonis*.

S. pharaonis is a common commercial species, distributed in tropical coastal waters (at depths<100 m) in the Indo-Pacific (Nabhitabhata and Nilaphat, 1999). Although *S. pharaonis* has been successfully cultured and reared in the laboratory (Domingues et al., 2001; Minton et al., 2001), none of research validated the age from their hard structures. As such, the age validation of *S. pharaonis* through the examination of the cuttlebone is worth being conducted as part of culturing experiments.

Cephalopods possess several hard structures, such as beaks, statoliths and internal shells (see for example Neige, 2006). The periodic growth increments on these structures are currently utilized to estimate the age of animals. Hernández-López et al. (2001) found that the daily rings on the upper beak of the common octopus, *Octopus vulgaris*, correspond to the known age of the animals, and Canali et al. (2011) further supported this finding in samples taken from wild octopuses. Daily concentric rings in statoliths have been extensively analyzed and provide valid ways to assess the age of squids (Bettencourt and Guerra, 2000; Bettencourt et al., 1996; Jackson and Moltschaniwskyj, 1999; Perez et al., 2006; Raya et al., 1994). In addition, the gladius has been considered as an alternative age-recorder owing to the identifiable striae (Perez et al., 1996, 2006). Statoliths and cuttlebones have been utilized for age validation in *S. officinalis* (e.g. Bettencourt and Guerra, 2001). However, although the increments on the statolith of *S. officinalis* have been assumed to follow a daily deposition, it is difficult to make a definite determination of the age of the animal, due to the narrowness of increments corresponding to the early life stages of the animals that make difficult to discriminate growth rings in parts of the structure (Bettencourt and Guerra, 2001). As a consequence, statoliths are not considered good age-indicators for cuttlefishes. The cuttlebone, the internal shell, is a visibly layered structure of large size; it has a clear pattern of increments and has the potential to be utilized for age determination. Among cuttlefishes, it is only for *S. officinalis* that studies on age determination based on increment formation in hard structures have been carried out (Bettencourt and Guerra, 2001; Martínez et al., 2000; Ré and Narciso, 1994). Studies are not available for *S. pharaonis*, to the best of our knowledge.

In the cuttlebone outer and inner cones are distinguishable. The outer cone, also referred to as the dorsal shield, is a thick and non-porous calcified cover that seals off the lamellae (Birchall and Thomas, 1983). Lamellae are basic units that make up the inner cone. It is this layered structure that is used for age determination. A lamella is composed of one septum with numerous perpendicular pillars; different components 'built-up' using an organic 'framework' filled with calcium carbonate (Florek et al., 2009). The structure consists essentially of carbonate, while organic matters constitute about 9.5% of the total weight of a

☆ This article is part of a special issue on Cephalopod Biology published under the auspices of CephRes-ONLUS (www.cephalopodresearch.org).

* Corresponding author. Tel.: +886 2 24622192x5029.
E-mail address: chwang99@mail.ntou.edu.tw (C.-H. Wang).

0022-0981/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jembe.2013.02.020

cuttlebone (in *S. officinalis*, Birchall and Thomas, 1983; Florek et al., 2009). The cuttlebone is 'embedded' in the dorsal sac of the mantle, and is distinguishable in embryos. For example, in *Sepiella japonica* the cuttlebone is observable starting from stage 25 (Yamamoto, 1982). After hatching, deposition of carbonates is continuous (Denton and Gilpin-Brown, 1961b), and a newly formed lamella is located at the outermost portion of the cuttlebone. The ever increasing number of lamellae plays an important role in supporting the body of the cuttlefish and regulates its buoyancy (Denton and Gilpin-Brown, 1961a,b).

In a classic study, Choe (1963) found that lamellae represent daily deposition events, and that the frequency of their formation is constant at temperatures ranging between 19 and 30 °C. Indeed, Ré and Narciso (1994) suggested that in *S. officinalis* the periodicity of increments is not daily, but correlated to the growth rate of individuals. This has been further supported by Bettencourt and Guerra (2001) who estimated the formation of one lamella requiring between 3.1 (18–20 °C) and 8 days (13–15 °C). Food stress and temperature have been reported to affect muscle and cuttlebone growth in *Sepia elliptica* (Martínez et al., 2000); the rate of formation of lamellae is higher in conditions with warm waters and abundant food, whereas the situation is reversed with scarce available food (Martínez et al., 2000). The magnitude of these phenomena on the growth of the cuttlebone remains an unaddressed problem.

The aim of this work is to examine the periodicity of formation of the lamellae in the cuttlebone at two distinct life stages: *i.* hatchlings, and *ii.* three-month-old juveniles. The study was carried out in rearing experiments under different controlled temperatures (20, 25 and 30 °C), also to assess the influences of temperature on the periodicity of lamellae growth.

2. Materials and methods

2.1. Hatchlings

Eggs were collected off the south-western coast of Taiwan in two instances (October 2009 to February 2010) and cultured at 25 and 30 °C. Once cuttlefishes hatched, they were immediately immersed in sea-water containing alizarin complexone (25 ppm, 3 h). No-one of the hatchlings died during staining procedure. In order to evaluate the potential effects of exposure to alizarin complexone on the growth of cuttlefishes, some individuals were not stained (25 °C) and served as control. After exposure, 20 stained or unstained hatchlings were placed in a 10 L tank with circulating seawater. Tanks are part of a closed system running artificial seawater and equipped with filter beds, ultraviolet sterilizer and protein skimmers. Temperature was regulated and the photoperiod was kept constant (light/dark: 10/14 h). White shrimps (body length<1 cm) were offered as food to the hatchlings twice a day, ad libitum. Uneaten food was removed from the tanks within 2 h after preys were presented. Water quality was checked daily for ammonia, pH and salinity. Rearing was extended for one month (31 days, 25 °C; 30 days, 30 °C). After this time individuals were sacrificed by slow continuous cooling (till freeze).

Other hatchlings (N = 10) were reared at 20 °C (30 days) and not exposed to any staining, acting as further control.

2.2. Juveniles

Cuttlefish eggs were collected off the south-western coast of Taiwan in September 2007. Hatchlings were reared in an open system (two tanks, 1 m in diameter), and fed live white shrimp, *Litopenaeus vannamei*, twice a day ad libitum. Seawater was drawn from the coastal seawater of south-western Taiwan, and was kept at a temperature of 24–26 °C. The photoperiod was controlled by the natural light. Three-month-old juveniles were immersed in a 25 ppm solution of alizarin complexone for 3 h. No mortality was observed after treatment. After staining, the juveniles were reared in the same environmental conditions and were sacrificed after 35 days by slow cooling down of seawater temperature (up to freeze).

2.3. Preparation and examination of cuttlebones

Cuttlebones were dissected from the mantle, cleaned and dried (30 °C, overnight). The cuttlebones of the three-month-old juveniles were cut longitudinally by an IsoMet low speed saw (Buehler). The lamellae were observable to the naked eye and the alizarin complexone marker was visible as a purple band (Fig. 1A, B). Counting the lamellae of the juveniles was straightforward and reliable. Due to their fragility, the cuttlebones of hatchlings were embedded in epoxy resin and cut into thin sections (Fig. 1C, D, E). Cut-line was arranged along the longest profile extended from the cuttlebone spine; thin sections were mounted on a glass slide. In order to make the image of the lamellae as clear as possible under microscopic examination, sections were ground using P320 to P2500 grit papers and polished with 9 μm alumina powder. Profiles were examined under a stereo microscope (Nikon SMZ800) and photographed by a QImaging Micropublisher RTV camera. ImageJ (NIH, USA) was utilized to measure the number of lamellae. Average width of lamellae was estimated by dividing the length of the cuttlebone by the total number of lamellae, observed in one month of growth.

2.4. Data analysis

The influence of ALC on the growth of cuttlefish was examined by *t*-test comparing stained (25 °C hatchlings) and control groups. Chi-square test was used to examine the differences of survival between groups. Another *t*-test was utilized to evaluate differences in the frequency of lamellae formation between three-month-old juveniles and 25 °C hatchlings. One-way ANOVA was applied to compare increments' width between three life stages: 25 °C embryo (based on the analysis of the lamellae formed before hatching from the 25 °C hatchling group), 25 °C hatchlings, and three-month juveniles; Scheffé's test was applied for *post-hoc* comparisons. Finally, one-way ANOVA (and Scheffé's *post-hoc* test) was utilized to evaluate the effects of temperature on the rate of formation and average width of lamellae. All statistical analyses were carried out by SPSS (IBM).

3. Results

3.1. Effects of ALC staining

The alizarin complexone does not cause a difference in growth or survival and therefore does not seem to affect deposition of the cuttlebone (Table 1). No significant difference emerged considering the growth of individuals cultured at 25 °C and stained or not with alizarin complexone (Mantle length: $t = 0{,}186$, df = 73, P = 0.853; body weight: $t = -0.035$, df = 73, P = 0.972) as their survival ($\chi^2 = 0.036$, df = 1, P = 0.850).

3.2. Formation of lamellae

Before hatching, 5–6 lamellae are already formed in *S. pharaonis*. Once hatched the frequency of formation of lamellae varies with the growth; hatchlings require more time than three-month-old juveniles to add lamellae to the cuttlebone (25 °C, $t = -16.83$, df = 76, P<0.001; Fig. 2).

The average distance between lamellae at the embryo, hatchling and juvenile stages appears to be different (ANOVA: F = 480.5, df = 2, P<0.001; Fig. 3). Embryos and the three-month juveniles all have relatively higher values when compared to hatchlings (Fig. 3).

3.3. Effects of temperature

A positive correlation of mortality with temperature was observed from the hatchlings (Table 1). Hatchlings at 20 and 25 °C have a higher survival rate (100% and 65.8%, respectively); when the temperature