

Aging and Growth Determination of Common Cuttlefish (*Sepia officinalis*) using the Cuttlebone

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ABSTRACT: Determining the age and growth of aquatic animals was the main objective of scientists to study, evaluate, and conclude its biological aspects; since it was a cornerstone for fisheries management purposes. A new procedure was applied for aging *Sepia officinalis* from the cuttlebone by using 590 specimens; to realize its specific growth in its natural habitat in the southeastern Mediterranean Sea, North Sinai, Egypt. Dorsal mantel length – cuttlebone length, lamellae numbers, and thickness relationship were measured. The maximum age of the common cuttlefish found in this study was 1.25 years (5 seasons). The average numbers of lamellae formed in the first, second, third, fourth, and fifth seasons were 31,62,97,124, and 143 lamellae respectively. Results of the current research obtain a new approach for aging sepia from the cuttlebone when some information about the temperature over the year is available.

Key word: *Sepia officinalis*, Age determination, Cuttlebone, and the Mediterranean Sea

Received: May 8, 2024

Accepted: June 14, 2024

1. INTRODUCTION

Common cuttlefish is one of the most important species of cephalopod fisheries in many countries that are found abundant in eastern Atlantic and Mediterranean waters. It is probably close to its maximum sustainable production in several areas of its distribution; since negative trends in captures have been observed in recent years in some heavily fished areas, e.g., in the Mediterranean (Jereb and Roper, 2005).

Cuttlefishes are valuable commercial fish that are important in Egypt. In 2017, their recorded catch was 1,515 tones, which represents about 2.57% of the total catch value of the Egyptian Mediterranean yield (GAFRD, 2017). Among other mollusks caught along the North Sinai coast, common cuttlefish *S. officinalis* hasn't had any remarkable study, although, it presents about 4% of the total catch.

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Age studies on *Sepia* species are done by two different methods, firstly by the indirect length-frequency distribution method, and secondly by the direct technique with tagging and recapturing method and by the examination of hard parts, such as cuttlebone, statoliths, mandibles, eye lenses, and gladius (Young, 1960). Many authors focus their research on the direct examination of hard structure specifically on cuttlebone and statoliths (e.g., Richard, 1969; Ré and Narciso, 1994; Le Goff, *et al.*, 1998; Bettencourt and Guerra, 2001; Challier, *et al.*, 2002; Hall, *et al.*, 2007; Chung and Wang 2013). Using the cuttlebone in age determination is more appropriate than the statoliths; because the hypothesis 1 day = 1 increment has not yet been validated for the entire life cycle of this species (Bettencourt and Guerra, 2001). Cuttlebone was used for the age and growth estimation of cuttlefish by counting all septa in the cuttlebone. Since; it was observed in the cuttlebone, the formation rate depends on the temperature in the region, therefore, the age of the cuttlefish can be correctly assessed only if the animals grew under a known constant temperature (Richard, 1969).

A new procedure for aging common cuttlefish by reading the cuttlebones, also recommended for other *Sepia* species, was done in this study. That was proved by the fact cuttlebone formation changed by temperature, or there is a strong relation between cuttlebone formation and the prevailing temperatures in the region.

This work is dealing deals with a new approach to age and growth determination on the common cuttlefish *S. officinalis* collected from the southeastern Mediterranean Sea, El-Arish port, North Sinai, Egypt, by reading its cuttlebones.

2. MATERIALS AND METHODS

2.1. Sampling

Samples of common cuttlefish were collected monthly from El-Arish port and local fish market from 2016, 2017 to February 2018.

Overall, 181 cuttlebones were collected from fishes caught by trammel net in the Mediterranean offshore, and 409 cuttlebones were collected by trawlers gear in deeper waters of the western Mediterranean.

2.2. Biological measurements

Dorsal mantel length (DML) was measured nearest 0.1 cm; the mantle length (ML) is the standard size measure for cephalopods and is almost universally reported in the scientific literature. The dorsal ML and total weight of each sample were recorded, before the cuttlebone was extracted via a longitudinal incision along the mid-line of the dorsal mantle; total body weight was recorded to the nearest 0.1 gm.

2.3. Cuttlebone's preparation

The cuttlebone is composed of a dorsal shield and ventral phragmocone, the shield comprises three sheets of very hard and thin calcium carbonate (Naef, 1923); the external "periostracum", the intermediate "ostracum" and the "flat phragmocone".

The phragmocone is composed of successive calcareous lamellae deposited in each other during the growth of cuttlefish. Cuttlebones' length (bone length, BL) and width were measured to the nearest 0.1 cm and dried at room temperature.

To reveal the internal microstructure, the soft ventral phragmocone of the cuttlebone was sliced along the longitudinal axis with a scalpel, until the surface of the hypostracum (ventral surface of the dorsal shield) was reached. The phragmocone was carefully cut and scraped away from the surface of the hypostracum initially with a spoon and then with a scalpel, once the hypostracum was reached on one half of the cuttlebone posterior and anterior. Removed septa left a distinct line on the surface of the hypostracum, a growth increment was the width of a complete chamber or lamellae, i.e., the distance between two consecutive septa (Fig. 1).

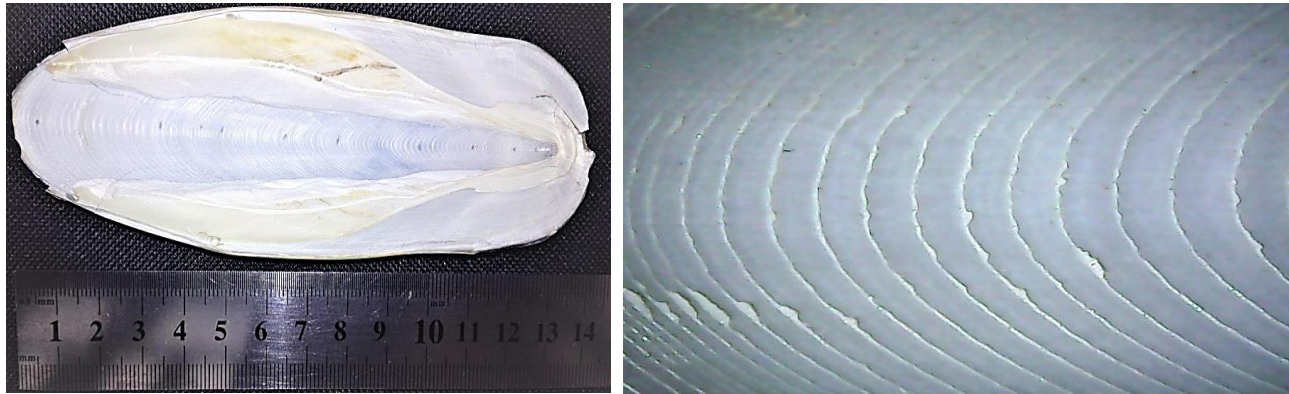


Fig. 1. Dissected and cleaned cuttlebone at the left and lamellae thickness measured by digital microscope on the right.

The lamellae number (the term streak is employed to determine the surface outcrop of lamellae) and the lamellae thickness were measured on the mid-sagittal surface of the hypostracum. Lamellae thickness (Increment widths) is measured to the nearest 0.1 mm, with a digital microscope equipped with an ocular micrometer along the length of the hypostracum from the posterior forked region to the anterior rim. The total number of increments/lamellae (LaNo) for each cuttlebone was calculated (Le Goff et al. 1998).

2.4. Data analysis

The dorsal mantle length – cuttlebone length relationship was measured according to (Whitney and Carlender, 1956) as $L = a + b x$ where, L: is the dorsal mantle length in cm, and X: is the cuttlebone length in cm.

Reading cuttlebone was done by the measurements of lamellae thickness (Increment widths or lamella width “LaWi”) to the nearest 0.01 mm, with a digital microscope equipped with an ocular micrometer along the length of

the hypostracum from the posterior forked region to the anterior rim. Furthermore, we discount the last lamella, as it didn’t entirely form. Also, the total number of increments/lamellae was calculated for each cuttlebone (LaNo).

The statistical analysis of *S. officinalis* cuttlebones was done during two fishing seasons from 2016 to February 2018, and a total of 590 specimens were examined. Aging *S. officinalis* from cuttlebones was done by two different methods, firstly, by the indirect length-frequency distribution method, and secondly, by the direct reading of *S. officinalis* cuttlebones. The length-frequency distribution is applied by determining the individual normal distribution method of (Bhattacharya, 1967) and also by the integrated method proposed by Pauly (1983). However, the direct method was done by arranging the lamella width (LaWi) into three different categories, according to its width, which is related to the dominant temperature in this region (table 1).

Table 1. The average monthly water temperature calculated based on the data over the past 10 years in El-Arish.

Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
18°C	17°C	18°C	19°C	22°C	26°C	28°C	29°C	28°C	26°C	23°C	20°C
64°F	63°F	64°F	66°F	72°F	79°F	82°F	84°F	82°F	79°F	73°F	68°F

Retrieved from <https://seatemperature.info/el-arish-water-temperature.html>

2.5. Statistical analysis

The analysis of variance test (ANOVA) was done to compare average lamellae widths (Avr.LaWi) between different seasons (the winter, spring/autumn, and summer).

Direct estimation of back-calculated length; is done by using the following equation of the correlation between dorsal mantle length and summation of lamella width (LaWi) at the previous age (season) as $L_i = a + b C_i$ where L_i is the length of fish at age "i", C_i is the summation of lamella width (LaWi) at age "i" and b constant.

Estimation of growth parameters was done by theoretical growth in length, by Von Bertalanffy's model parameters, L_∞ , K, and t_0 . The growth pattern can be defined by the infinite age plot, in which; length-at-age data is investigated according to the fact that *S. officinalis* has maximum 2 years life cycle (Mangold-Wirz, 1963, 1966; Guerra and Castro, 1988; Coelho and Martins, 1991; Gauvrit et al., 1997; Le Goff et al., 1998; Dunn, 1999; Domingues et al., 2006; Guerra, 2006; Gras et al., 2016) where $t_1=0.25$, $t_2=0.5$, $t_3=0.75$, $t_4=1$, $t_5=1.25$ $t_\infty=2$, therefore, the parameters K, t_0 , and L_∞ can be estimated from length-at-age data, by the linear regression between the age (t) as the independent variable (x) and the length (L) on the left-hand side as the dependent variable (y). The equation defines as linear, and hence K and L_∞ are computed as follows:

$y = a + b x$, or $L_t = a + b t$, a and b are constants, then $L_\infty = a + b t_\infty$

Slope = $(\Delta L / \Delta t)$ and $K = LN(\text{slope})$, then $K = LN(L_\infty / t_\infty)$ or $K = LN(b)$

$t_\infty = 2$ years or (1 season = 0.25 year, and $t_\infty = t_{\max} + 0.25$), where t_{\max} is the maximum age (season) that can be reached by sepia individuals. The age at length zero (t_0) can calculate by a direct method from the inverse of the infinite age plot model by applying the length at zero sizes ($L_0 = 0$) on the linear

equation for length on age against age at length zero (t_0) as:

$(L) = a + b (t)$, also $(L_0) = a + b (t_0)$, then $(t_0) = -a/b$

3. RESULTS

3.1. Biological relationships

In the present study, the dorsal mantle length (DML) and cuttlebone length (CL) of *S. officinalis* relationship were analyzed, the cuttlebone length (CL) varied between 2.1 and 17.3 cm, and (DML) measurement varied between 3.7 and 19.2 cm (Fig. 2a) The linear equation of the dorsal mantle length - cuttlebone length relationship was $DML = -0.1731 + 1.1007 X$ with $R^2 = 0.96$. The dorsal mantle length (DML) and the summation of cuttlebone lamellae widths (LaWi) relationship were measured to convert the summation of lamellae widths - at - back calculated season, to the dorsal mantle length (DML).

The summation of cuttlebone lamellae widths (LaWi) was found to vary between 3.3 to 18.1 cm, and the linear equation was as follows: $DML = 2.2084 + 0.9879 C$ with $R^2 = 0.95$ (Fig. 2b).

3.2. Length frequency distribution

By applying the normal distribution proposed by (Bhattacharya, 1967) and the integrated method of age determination suggested by (Pauly, 1983). *S. officinalis* males and females had five ages autumn, winter, spring, summer, and last in the autumn or winter at the end of the reproductive season. The average dorsal mantle lengths in cm were obtained in (table 2).

3.1. Cuttlebone's analysis

From table 3, the average lamellae number (LaNo) were 31, 62, 97, 124, and 143 lamellae for both males and females for 1st, 2nd, 3rd, 4th and fifth seasons respectively .

The Maximum (LaNo) in the first season was 53 lamellae and the minimum was 13 lamellae. On the other hand, the cuttlebone lamellae widths (LaWi) were found to vary between 0.2 to 1.9 mm.

The summation of lamellae widths (LaWi) was calculated for each season and represented by the end of each growing season. The average number of lamellae width (Avr.LaWi) is considered by the next season to the embryo/hatchling stage (season of beginning) to compare the three types/groups of the cuttlebones and to comprise the different *S. officinalis* life stages. From table 4, the

temperature shows some effects on the cuttlebone by the differences between lamella deposition and periodicity along with the time or the season of growing; it was about 1.3 mm during the winter, 1.1 mm during the spring/autumn, and 0.9 during the summer. Overall data from cuttlebone analysis shows that the *S. officinalis* lasted for 1.25 years during its life cycle in the eastern Mediterranean Sea.

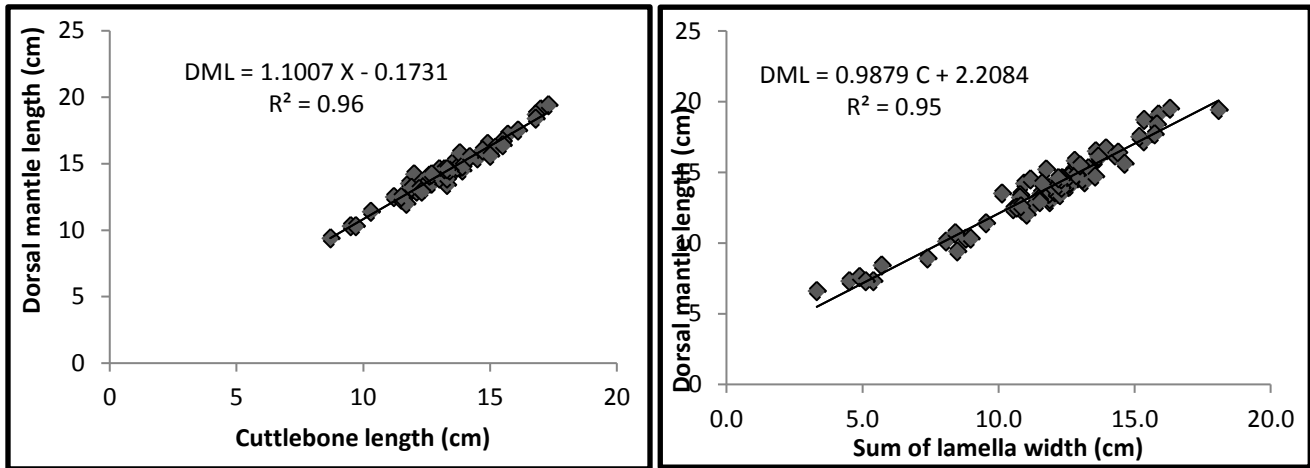


Fig. 2. The relationship between dorsal mantle length (DML) and A: is the cuttlebone length; B: is the summation of lamella width.

Table 2. Observed length, direct back-calculated length, Bhattacharya, 1967, and Pauly, 1983, on length-at-age data.

Age (season)	Observed length (cm)	Mean back-calculated length (by current study)	Bhattacharya, 1967	Pauly, 1983
1	3.92	3.84	4.5	4
2	7.42	6.77	8.7	8
3	10.21	10.19	11.9	12
4	14.25	13.91	14.8	15.5
5	17.98	17.57	17.9	17.2

Accordingly, the lamellae width (LaWi) was measured and analyzed by analysis of variance (ANOVA) to test the effect of temperature on lamellae formation. Significant statistical

differences (ANOVA, $F < F_{crit}$: $66.96 < 3.001$, $P < 0.05$) were found in the cuttlebones of *S. officinalis* that were caught during different seasons.

Table 3. Average, maximum, and minimum lamella numbers (LaNo) and the summation of lamella widths (LaWi) by season

Sum of lamella width of <i>S. officinalis</i>				
Season no. 1	Season no. 2	Season no. 3	Season no. 4	Season no. 5
1.65	1.65	2.24	1.68	1.44
	4.83	5.93	5.65	5.29
		8.53	8.94	8.71
			12.22	12.35
				15.72
Average, maximum, and minimum lamellae numbers of <i>S. officinalis</i>				
Season no. 1	Season no. 2	Season no. 3	Season no. 4	Season no. 5
31	62	97	124	143
Min. 13	46	73	106	130
Max. 53	68	108	147	151

3.3. Estimation of back-calculated length

In the present study, the dorsal mantle length (DML) and the summation of lamellae widths (LaWi) relationship were measured to convert the widths to a back-calculated DML of the *S. officinalis* (Fig. 2b). The linear equation of the dorsal mantle length - cuttlebone length relationship was: $DML = 0.9879 C + 2.2084$, with $R^2 = 0.96$.

Results in the table (2) show more compatibility between the observed dorsal length and the new direct estimation of back-calculated dorsal length than indirect length-frequency distribution analysis.

The highest increment in length occurred in the first 1st season of life at 3.84 cm, after which the seasonal increment in length is gradually fluctuating in the growth rate during its life. The back-calculation in length was 3.84, 6.77, 10.19, 13.91, and 17.57 cm at the first, 2, 3, 4, and 5th seasons respectively (Fig. 3). The back-calculated weight was 8.75, 42.23, 131.48, 311.99, and 597.11 gm for the same ages respectively.

3.4. Theoretical growth in length

Results of the von Bertalanffy growth model constants a , b , L_{∞} , K , t_0 , and W_{∞} calculated by infinite age plot were represented in table (5). The growth coefficient "K"- related to the longevity of the organism - is higher for short-lived animals and lower for long-lived animals. In the present study, the value of "K" ≈ 2.63 , which means the *S. officinalis* growth curve is near a sharp peak, and it is growing faster to approach its asymptotic length L_{∞} .

3. DISCUSSION

Cuttlebones analysis

To determine the age of *S. officinalis* by examining Sepia's cuttlebone, we suggest some rules that also recommended for other Sepia species. These rules describe growth patterns and validate age based on the season.

According to dependable sea temperature data over 10 years on study area in the South-eastern Mediterranean Sea (El-Arish), there are three growing seasons during the year, starting in the winter (The first season) at months from December to April as temperatures are between 18-20 °C.

Table 4. Average lamellae widths (Avr.LaWi) of *S. officinalis* cuttlebone, distributed according to growing season.

Season of growth	Embryo and	Juvenile		Adult		Width (mm) at the season of catch
	Season of beginning	Second season	Third season	Fourth season	Fifth season	
Winter	0.6					0.6
	0.7	<u>1.3</u>				1.3
	0.7	<u>1.4</u>	0.6			0.6
	0.7	<u>1.3</u>	1.1	1.0		1.0
	0.7	<u>1.3</u>	1.2	1.2	1.3	1.3
Spring/ Autumn	0.6					0.6
	0.6	<u>1.0</u>				1.0
	0.6	<u>1.0</u>	1.2			1.2
	0.6	<u>1.1</u>	1.0	1.1		1.1
	0.6	<u>1.1</u>	1.3	1.1	1.2	1.2
Summer	0.6					0.6
	0.6	<u>0.9</u>				0.9
	0.5	<u>0.9</u>	1.1			1.1
	0.6	<u>0.9</u>	1.1	1.2		1.2
	0.6	<u>0.9</u>	1.0	1.4	1.0	1.0

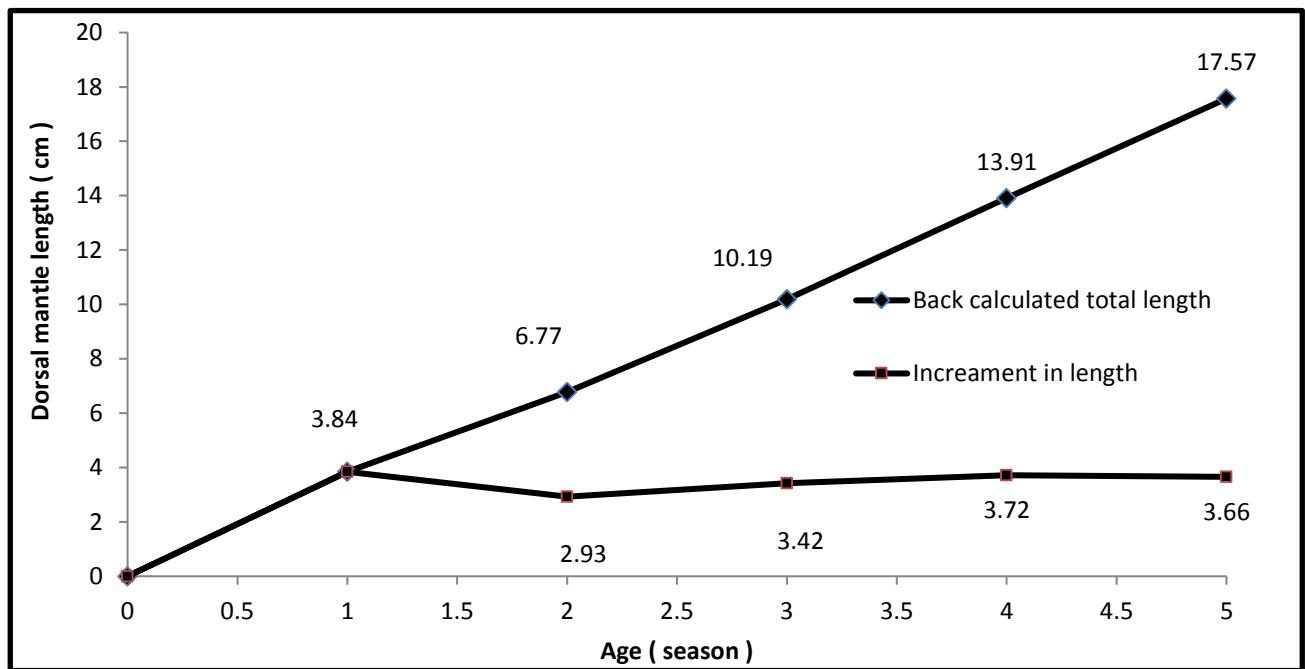


Fig. 3. Back-calculated dorsal mantle length (DML) and increments in the length of *S. officinalis*.

Table 5. Von Bertalanffy's growth parameters of *S. officinalis*, at different geographic locations.

Author and date	M/F	L_{∞}	K	t_0	Location
Challier, <i>et al.</i> (2005)	M/F	87.09	0.735	-	English Channel
AL Marzouqi, <i>et al.</i> (2009) for <i>S. pharaonis</i>	M/F	46.5	1.04	-0.13	Arabian Sea, Oman
Mehanna and Haggag (2011)	M/F	24.35	0.61	-	Southeastern Mediterranean
Kennouche and Nouar (2016)	M/F	26.93	0.38	0.704	Center of Algeria
Saddikioui, <i>et al.</i> (2017)	Males	75	0.29	-0.69	Oran Bay, Western Algeria
	Females	60	0.16	-0.35	
	Total	24.64	0.68	-0.97	
Present study	Males	27.47	2.62	- 0.016	Eastern Mediterranean
	Females	27.64	2.63	- 0.017	
	Total	27.75	2.63	- 0.006	

Moreover, the second season includes (spring/autumn) in which temperatures are the same, starting with two months from May and June, and a separate one in November as temperatures are between 20-25 °C. Besides, the third season involves (The summer) which starts from July to October when temperatures are between 26-30 °C.

During this study, cuttlebone reading shows bone patterns mainly three “cuttlebone pattern” types/groups of successive calcareous lamellae widths deposited in each other during the growth of cuttlefish, the first is when the measurement of lamellae widths are (≤ 0.9 mm.), the second is between (1.0 and 1.2 mm.) and the third when lamellae width are (≥ 1.3 mm.).

Current results showed every season contains at least 18 successive lamellae, Chung and Wang (2013) found that the average time necessary for the formation of one lamella deposition is about 3.1 days for *S. pharaonis* reared at 25 °C. Furthermore, the maximum numbers of

lamellae that belong to the same season are between 48-54 successive lamellae, Bettencourt and Guerra (2001) found that the average time necessary for the formation of one lamella deposition is about 3.1 days for *S. officinalis* reared at 18-20 °C. Also, Chung and Wang (2013) found that the average time necessary for the formation of one lamella deposition lasted 2.2 days for *S. pharaonis* reared at 30 °C.

Season is confirmed at cuttlebone only when successive calcareous lamellae width deposition belongs to the same season measurement.

Some cuttlebone includes, additionally to the previous groups, another group (type no. 4th) that consists of calcareous lamellae with widths ranging between <0.9 , and >1.3 mm, and was called the “irregular group. The relationship between dorsal mantle length (DML) and cuttlebone length (CL) of *S. officinalis* shows some variation in measurements, which may be to the fact established by Bandel and von Boletzky 1979, which the cuttlebone is enclosed in a circular tissue called “shell sac”. This thin tissue sheet, consisting of one cell layer, fulfills

the functions of mineralization and gas-liquid exchanges necessary for buoyancy regulation. On the other hand, the cuttlebone length (CL) is larger than the summation of cuttlebone lamellae widths (LaWi) due to the organic membranes, which present a thin sheet coating the lamellar and pillar surfaces, as freely suspended sheets running parallel to the septa (Le Pabic *et al.*, 2016). Several types of growth curve functions have been applied in cephalopod growth studies, perhaps reflecting the fact that these organisms show large inter-individual growth variations (Challier *et al.*, 2002).

Richard (1971) and Boletzky (1979) proved that reared *S. officinalis* growth was sigmoid. Forsythe and Van Heukelem (1987) described a two-phase curve; with an exponential phase followed by a logarithmic phase. Therefore, the indirect age determination methods cannot describe this oscillating growth of sepia species. Since 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 (Chung and Wang, 2013). Additionally, many authors declared that aging cuttlefishes using the cuttlebone is still a problem owing to the unclear mechanisms underlying their formation. In addition, the observed periodicity responds differently to biotic and abiotic factors; the interaction between physiological and environmental factors makes this issue even more complicated. However, once the formation mechanism of the microstructure can be understood, this may allow examining the life history of the cuttlefish based on the analysis of the cuttlebone (Chung and Wang, 2013).

Also, Natsukari *et al.* (1991) concluded that “it might be possible to evaluate lamellar numbers as an aged character of the sepiids”. Nevertheless, other authors indicated that the lamellar deposition rhythm of the cuttlebone in *S. officinalis* individuals was strongly dependent

on temperature (Richard, 1969; Bettencourt and Guerra, 2001). Moreover, Chung and Wang, (2013) for *S. pharaonis*.

Many authors found that lamellar deposition is negatively temperature-dependent. 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) were estimate the formation of one lamella required between 3.1 (18–20 °C) and 8 days (13–15 °C).

Besides, the cycle of deposition of lamellae in *S. officinalis* is assumed to be dependent upon the temperature effect (Bettencourt and Guerra, 2001). Richard (1969) highlighted the temperature influence on the rhythm of formation of the *S. officinalis* shell and the nutrition influence on the structure of newly formed chambers, both parameters being strongly linked. However, the mean increment widths of the cuttlebone lamellae were at a higher correlation with temperature (negative relationships). *S. pharaonis* reared at 20 °C has the widest increments compared to other temperature groups (Chung and Wang, 2013).

During this study lamella deposition required 3.12 days in the summer, 3.61 days in the winter, and 2.70 days in the spring/autumn on wild *S. officinalis* in the southeastern Mediterranean Sea. The *S. officinalis* life cycle was found to last 5 seasons \approx 1.25 years in the eastern Mediterranean Sea before the cohort became extinct. Bettencourt and Guerra (2001) obtained similar results when they cultured *S. officinalis* under controlled conditions (individuals were cultured in two different temperature regimes: 13–15 °C and 18–20 °C) and found that the maximum number of days in captivity (420 days) was about 80% of the life span estimated for this species in the study area

that study. Le Goff *et al.* (1998) stated that *S. officinalis* has a maximum 2-year life cycle. Alternatively, various authors mention that *S. officinalis* has a life cycle lasting between 1 and 2 years depending on the latitude at which it lives (Domingues *et al.*, 2006; Guerra, 2006).

From West Africa to the Portuguese coasts, the entire population has a 1-year life cycle (Mangold-Wirz, 1963, 1966; Guerra and Castro, 1988; Coelho and Martins, 1991). Gras *et al.* (2016) declare that within the English Channel, the common cuttlefish is a semelparous species for which a 2-year life cycle was exclusively described in the 1980s.

In the 1990s, new research indicated that a 2-year life cycle was still evident for females and the majority of males; a small proportion of males were maturing at only 1 year of age. Gauvrit *et al.* (1997) found that the life cycle of *S. officinalis* was described as lasting exclusively 2 years. Dunn (1999) found that the entire female population and 96% of male specimens had a 2-year life cycle, but the remaining males (4%) were found to be mature at the age of 1 year.

The back-calculation of the total body length, at the end of each season, was carried out by a new direct estimation. The cuttlebone length is directly proportional to the dorsal mantle length in the family Sepiidae, so we can define the beginning and end of the season on the cuttlebone for age determination. Additionally, we can estimate the relationship between dorsal mantle length (DML) and the average summation of lamellae widths (LaWi) in cuttlebone for every season. Le Goff and Daguzan (1991) studied the *S. officinalis* cohort from hatchling to death and found in South Brittany, the average length for males and females was 0.7, 7.6, 10.4, 19.4, and 22 cm. In the current study, we used the infinite age plot to describe the von Bertalanffy growth parameters; the results were compared with

those obtained by other researchers and are given in table (5).

The current asymptotic length (L_{∞}) was similar to many authors (Mehanna and Haggag, 2011; Kennouche and Nouar, 2016; Saddikioui, *et al.*, 2017).

On the other hand, in the present study, the maximum asymptotic weight is more than that estimated by (Mehanna and Haggag, (2011), which realized who mentioned that $W_{\infty} = 1496$ gm., and (Kennouche and Nouar, 2016), which found $W_{\infty} = 1775$ gm. From these results for different authors, we can notice that von Bertalanffy's growth parameters values show a variance among different geographic localities for the same species.

ACKNOWLEDGMENT

The authors, thank the Fish Farming and Technology Institute (FFT) for holding this study. Special thanks to Prof./ Alaa El-dien El-Haweet, professor of Fisheries Management, College of Fisheries Technology and Aquaculture, Arab Academy for Science, Technology and Maritime Transport, Egypt. And Prof./Saad Zakaria, professor of Invertebrate, Marine Biology, Marine Science Department at Suez Canal University., Ismailia, Egypt for their supports.

CONFLICT OF INTERESTS

This manuscript has not been published and is not under consideration for publication elsewhere. We have no conflicts of interest to disclose.

Funding

This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors

Author contributions

All authors are responsible for the experiment design, collection of the samples, in-field data records, laboratory examination, data statistical analysis, and general design of the manuscript.

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