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Exploring the embryonic development of upper beak in *Octopus vulgaris* Cuvier, 1797: New findings and implications for age estimation

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ABSTRACT

The beak of cephalopods (in particular octopods, where statoliths are not suitable) is a useful tool for age estimation and the aging method provides essential information on cephalopod growth and life cycles. These parameters are valuable in the assessment of population dynamics and stock management. The embryonic development of cephalopod beaks is poorly known. The presence of pre-hatching increments on the reading areas (rostrum and lateral walls) is unclear and there are no data on temperature influence. In this study, egg clusters of *Octopus vulgaris* were reared at 16, 19, 21, 23, and 26 °C. The extracted upper jaws were observed in order to validate the age of first daily increment formation, assessing the accuracy of age inferred from the two reading areas. Jaw dimensions were also measured in order to explore the development at different temperature conditions. The growth rate was calculated for beaks of rearing condition 21 °C, and the overall dimensions were compared among all incubation temperatures. Three ad hoc developmental stages are proposed for the upper beak of *O. vulgaris* embryos. Increments on lateral walls appear during the second phase, whereas the first increment on the rostrum is visible only at hatching. Consequently, only the accuracy of age inferred from the rostrum surface is confirmed for the early stages. The growth rate of the rostrum region accounted for a drop in growth during the third phase. Conversely, the growth rate increased until hatching in lateral walls, suggesting that the heterogeneity of the growth rate could be due to the different role played by the beak areas. Temperature influenced beaks in terms of overall size, as embryos reared at a warm temperature (23 °C) were smaller than the others. These results confirm that the incubation environment could alter hatchling characteristics thus affecting the recruitment conditions.

1. Introduction

The knowledge of age, growth rates and feeding habits of fisheries' target species is essential in order to assess the population dynamics and its responses to a changing environment (Colloca et al., 2013; Hastie et al., 2016; Perales-Raya et al., 2010; Rodhouse et al., 2014). Length-frequency distribution analyses are used for estimating growth parameters in some cephalopod species, such as cuttlefish (Alemany et al., 2017), while the same methodology is unreliable for octopuses due to their soft-body and their intrinsic biological variability (Rodhouse et al., 2014; Semmens et al., 2004). More robust information is usually available in hard structures, such as statoliths (Villanueva, 2000) and beaks (Dawe and Beck, 1997; Clarke, 1965; Raya and Hernández-González, 1998). The microstructural pattern of the upper beak (jaw) has been analyzed to estimate the age mainly in Octopodidae species, as the lapse crystallization of their statoliths makes them unsuitable for age estimation (Raya and Hernández-González, 1998; Villegas-Bárcenas et al., 2014). In *Octopus vulgaris*, the daily periodicity of the increments observed in the lateral walls (Hernández-López et al., 2001; Perales-Raya et al., 2010) and in the Rostrum (Perales-Raya et al., 2014a, 2010) has been validated for the full ontogenetic range, using cultured and wild individuals (Perales-Raya et al., 2014b). In addition, cephalopod beaks have been acknowledged as good tools for a variety of studies in marine biology and ecology (Xavier et al., 2016). Indeed, the cephalopod beak consists of a very hard and flexible composite of proteins and chitin fibers (Clarke, 1986; Miserez et al., 2010, 2007;

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Table 1
Number of samples (n_i), Days from egg fecundation and mean values (μm) ± standard deviations of measurements, grouped by Temperature (T) and Naef stage (ST). Measurements (see details in Fig. 1) are: upper jaw width (UJW), lateral wall diagonal (LWD), upper jaw length (UJL), rostrum depth (RDE), teeth half width (THW), hood amplitude (HAM). SP I (spawning event 1); SP II (spawning event 2).

T, ST	n_i	Day	UJW	LWD	UJL	RDE	THW	HAM
21°					**SP II**			
XV	6	21	250.32 ± 5.85	131.44 ± 2.59	137.09 ± 5.39	38.36 ± 1.67	81.14 ± 4.49	200.25 ± 10.49
XVI	6	23	275.58 ± 9.92	143.54 ± 6.6	155.69 ± 5.1	43.13 ± 4.04	105.47 ± 2.47	238.06 ± 9.21
XVII-XVIII	6	26	317.88 ± 12.96	170.74 ± 4.41	185.95 ± 6.73	46.29 ± 3.81	119.02 ± 2.25	258.77 ± 15.44
XIX-XX	6	27	338.02 ± 12.97	178.46 ± 4.41	197.19 ± 6.74	48.7 ± 3.80	123.76 ± 2.25	269.83 ± 15.44
Hatching	6	29	370.38 ± 9.71	203.43 ± 1.75	225.16 ± 9.85	47.12 ± 2.27	126.04 ± 1.59	290.83 ± 12.16
16°					**SP I**			
XVI	7	37	278.37 ± 2.28	142.6 ± 16.51	153.41 ± 6.01	49.89 ± 24.71	106.06 ± 7.98	227.38 ± 9.32
XVII-XVIII	7	43	318.7 ± 19.68	168.38 ± 10.96	185.3 ± 22.4	45.11 ± 2.67	115.08 ± 6.55	255.04 ± 8.25
XIX-XX	7	48	334.27 ± 12.22	177.48 ± 11.37	196.51 ± 10.05	47.36 ± 3.34	123.33 ± 9.44	267.90 ± 5.12
19°								
XVI	7	29	283.93 ± 12.23	144.74 ± 10.97	154.26 ± 9.33	41.93 ± 4.5	104.83 ± 6.51	232.23 ± 12.70
XVII-XVIII	7	32	330.83 ± 7.88	172.64 ± 5.24	187.19 ± 6.01	44.22 ± 5.48	114.66 ± 3.35	250.15 ± 6.29
XIX-XX	7	34	337.96 ± 19.15	175.94 ± 9.52	191.78 ± 9.84	49.08 ± 2.45	117.04 ± 7.09	266.88 ± 6.58
23 °								
XVI	7	19	251.80 ± 15.35	128.36 ± 12.33	138.42 ± 11.03	29.95 ± 3.61	93.01 ± 6.9	200.16 ± 13.03
XVII-XVIII	7	21	274.11 ± 10.24	133.46 ± 10.33	151.99 ± 12.55	35.75 ± 5.25	98.84.6 ± 4.68	216.55 ± 12.03
XIX-XX	7	23	311.07 ± 17.29	160.55 ± 11.92	181.84 ± 13.72	40.86 ± 7.6	117.08 ± 9.41	253.44 ± 12.26

Roper and Voss, 1983; Tanabe, 2012) that are continuously secreted during the lifespan of the animal (Dilly and Nixon, 1976), and the beak shape varies according to the feeding habits (Franco-Santos and Vidal, 2014; Hernańdez-García et al., 1998; Hu et al., 2018). The study of the microstructural pattern could also allow relating specific increments, or checks, to stressing events (Franco-Santos et al., 2016; Perales-Raya et al., 2014b).

In spite of a huge number of studies on cephalopod beaks at the adult stage, to our knowledge the paralarval stage has rarely been addressed (Franco-Santos et al., 2016; Franco-Santos and Vidal, 2014; Perales-Raya et al., 2018) and the embryonic development is still largely unexplored (Boletzky, 2007). Therefore, the embryonic ontogeny of the first increment and the different specialized parts of the beak have not been described. It would confirm the time of deposition of the first growth increment, which is essential in validating the age inferred from any hard structure (Campana, 2001). Moreover, it is well known that environmental conditions have a great effect on the rate and duration of embryonic growth (Boletzky, 1987; Nande et al., 2017; O'Dor et al., 1982; Repolho et al., 2014; Vidal and Boletzky, 2014; Sanchez-García et al., 2017), while influence on beak development is poorly explored. Extending the knowledge on early stages, by addressing those issues, could be useful to investigate the recruitment dynamics (Villanueva et al., 2016) and the feeding habits of the paralarval stage (Roura et al., 2016; Villanueva et al., 2017). In addition, for aquaculture purposes, the possibility to accurately assess the age of wild paralarvae is valuable for comparisons with rearing conditions, in order to understand biochemical and physiological features such as biochemical composition (Garrido et al., 2016) and gene expression (Gestal et al., 2015).

In the present study, egg clusters of *O. vulgaris* were reared at different temperature conditions and the upper beaks were extracted at various embryonic stages. The study focuses on the modifications occurring in the microstructure, the morphology and the morphometry of embryonic upper beaks, in order to explore the different specialized parts and to assess the presence of any pre-hatching increments. Moreover, the study addresses how temperature variability could affect beak formation. Based on a number of microstructural features, ad hoc developmental stages are proposed for the upper beak of *O. vulgaris* embryos.

2. Materials and methods

2.1. Rearing conditions

The study was carried out in the aquaculture facilities of the Oceanographic Centre of the Canary Islands (Spanish Institute of Oceanography), based on the island of Tenerife (Spain). Eggs came from spawning events of two *O. vulgaris* specimens caught by artisanal fishermen off the coast of Tenerife Island, (Central-East Atlantic). Broodstocks were kept under conditions of natural photoperiod (14 h L – 10 h D), natural water temperature (19–21 °C) and salinity (36 PSU) and were fed *ad libitum* with a mix of frozen squids (*Dorytheuthis opalescens*) and prawns (*Parapenaeus longirostris*) until the spawning. In all experiments, the egg strings were collected from the female den using long forceps and 3–4 strings were attached in glassfiber cylindroconical 10 L tanks (3 tanks per treatment) during the embryonic development. The water flow was maintained by a semi-closed circuit at 2 L/min, and gentle aeration was provided in order to avoid bacterial disease and fungi (Iglesias and Fuentes, 2014). In Spawning event 1 (SP1, March 2012), four different rearing conditions (16°, 19°, 23°, and 26 °C) were tested, although the 26 °C batch was excluded because egg development was aborted after 12 days. In all other cases, the whole embryonic development lasted for 27 days at 23 °C, 41 days at 19 °C and 44 days at 16 °C. A cooling system (Teco mod. RA-200) was used to maintain seawater temperature at 16 °C. The temperature of 19 °C was the natural seawater T[a]. Several aquarium heaters (EHEIM JEGER - adjustable heater, thermal control 25 W) were used to warm water up to 26 °C and 23 °C. The tanks were maintained in the natural condition of salinity (36 PSU) and photoperiod. For each condition, three egg strings (≈800 eggs each) were reared. In Spawning Event 2 (SP2, April 2017), four strings (≈800 eggs each) where reared at natural environmental conditions of photoperiod, salinity (36 PSU) and temperature (21 °C).

In order to check the development of the beak reading areas, eggs were collected at different Naef stages (see Naef, 1928) together with the collecting of newly hatchling paralarvae (see details in Table 1).

All animal experiments were performed in compliance with the Spanish law 53/2013 within the framework of European Union adopted directive 2010/63/EU on animal welfare for the protection of animals employed for scientific purposes, following the Guidelines for the Care

and Welfare of Cephalopods in Research as proposed by Fiorito et al. (2015), and the present study was also approved (register document CEIBA2014-0108) by the Ethics Committee for Animal Research and Welfare (Comité de Ética de la Investigación y Bienestar Animal, CEIBA) at the University of La Laguna (Tenerife, Spain).

2.2. Beak extraction and preparation

All embryos were individually labeled and stored at −20 °C until their analysis. Beak extraction and manipulation were undertaken in accordance with Perales-Raya et al. (2014a, 2018). In addition, beaks were cleaned with dish soap (Ramulu et al., 2012) in order to remove all tissue residues. The methodology under stereomicroscope was as follows: (1) on each microscope slide a 0.5 cm diameter circle was drawn to facilitate beak detection; (2) the beak was placed in a soap drop of about 1 cm diameter and gently scrubbed using surgical needles; (3) the beak was moved to fresh and distilled-water drops consecutively for rinsing; (4) the beak was placed in a smaller drop of distilled water (about 0.2 cm diameter) in a clean slide, and gently pressed with the needle to slightly attach it to the slide; (5) the coverslip was placed keeping it tilted and moved to ensure the correct beak positioning with ventral side up. Only when a correct position was achieved the coverslip was fixed to ensure standardized images. Observation of samples and acquisition of images were carried out according to Perales-Raya et al. (2014a, 2018). Approximately 150 beaks were successfully extracted from both spawning events. All beaks were observed to construct the development table, and pictures of poor quality due to beak bending or damaging were not considered in the statistical analysis. The number of samples used in the analytical study for each temperature condition and developmental stage is shown in Table 1.

2.3. Description and analysis

The staging system for the developmental table has been established by combining the general embryonic development and stages described by Naef (1928) and our beak observations, while considering the most relevant parts and features as criteria. The analytical study relies on measures taken from the posterior area (lateral walls), namely upper jaw width (UJW), upper jaw length (UJL), lateral wall diagonal (LWD), and from the anterior area (rostrum), namely hood amplitude (HAM), rostrum depth (RDE), and teeth half width (THW). The set of measurements derives from previous studies carried out on adults and paralarvae of cephalopods (Clarke, 1986; Franco-Santos and Vidal, 2014) which have been adapted here to the embryonic beak (Fig. 1).

The morphometric analysis aimed to assess the dimension and proportion of the beaks through embryonic development. The Relative Growth Rate (GR, expressed as % days^{-1}) of beaks from SP2 was calculated by multiplying by 100 the instantaneous growth coefficient $G = \ln(\bar{Y}_2)–\ln(\bar{Y}_1)*(t_2–t_1)^{-1}$ (Forsythe and Van Heukelem, 1987), with $\bar{Y}_2$ = mean length at the foremost stage, $\bar{Y}_1$ = mean length at the lowermost stage; t_1 = days spent in ontogeny at $\bar{Y}_1$, and t_2 = days spent in ontogeny at $\bar{Y}_2$. Associated errors where calculated using the error propagation technique (Ciullo, 2014). The GR is presented by t_i = 23 days (Stage XVI), 26 days (Stages XVII-XVIII) and 29 days (Hatchlings). The significance of growth for every measurement was tested using a one-way analysis of variance (ANOVA) (Sandrini-Neto and Camargo, 2011) followed by a post-hoc Tukey HSD (honestly significant difference) test.

To assess the effect of temperature on beak growth, the overall size of beaks from SP1 and SP2 were compared. A sample consisted of the combination of all measures in a single beak, normalized by applying a Log10 transformation. A Two-Way Permutational multivariate analysis of variance (PERMANOVA) (9999 permutations, Oksanen et al., 2016) was applied to the matrix of Euclidean distances between replicates, using as factors: Temperature (four levels: 16, 19, 21, 23 °C; embryos reared at 26 °C are excluded) and Period (Three levels of Naef stages: XVI; XVII-XVIII and XIX-XX). A pairwise analysis (Arbizu, 2017) between levels of temperature factor was planned and implemented using the Bonferroni correction to reduce errors related to multiple comparisons (Bland and Altman, 1995). All statistical analyses were carried out in the R 3.4.4 environment (R Core Team, 2018).

3. Results

Embryos developed without abnormalities, except for clusters reared at 26 °C which were aborted after 12 days. The beak of *O. vulgaris* develops early in embryogenesis and it was possible to extract it successfully after the Naef stage XIV. Morphological development of the beaks was not affected by the experimental temperature. The phases described below were the same at all the rearing conditions. Nonetheless, the phase duration was influenced by the temperature (Table 1). Considering the events observed during the beak ontogeny, three main phases were identified:

a Early phase (phase 1), Naef stages XIV to XVI (Fig. 2a): the upper jaw is a rudiment, as it completely lacks the Hood. Two layers are visible: one close to the tip and one comprising the lateral walls.

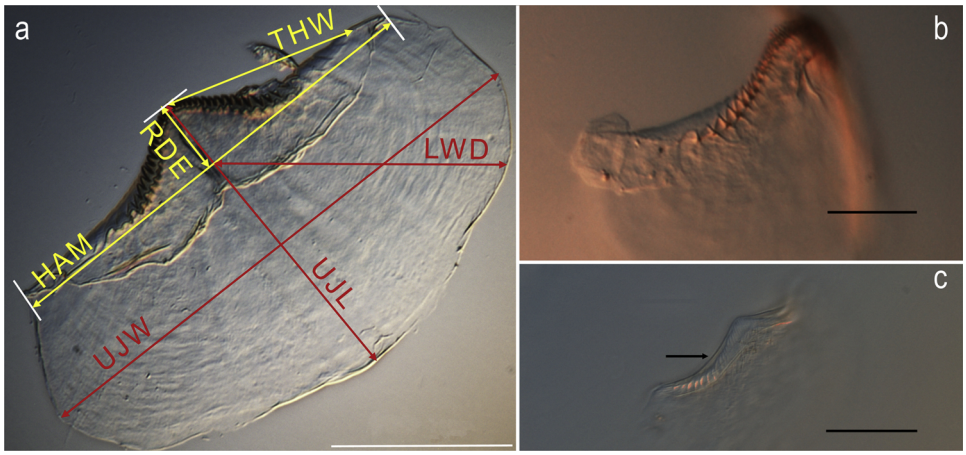

Fig. 1. Measurements proposed and details: (**a**) upper jaw from phase 3 showing measurements taken from beaks. Anterior part: teeth half width (THW): line joining the rostrum apex and the beginning of dentition; rostrum depth (RDE): Line parallel to anteroposterior axis of the beak, joins the rostrum apex and the baseline of the dentition; hood amplitude (HAM): straight line joining the most external points were the hood begins. Posterior part: upper jaw length (UJL): straight line joining the rostrum apex and the posterior edge of the lateral walls; upper jaw width (UJW): straight line joining the side edges of the lateral walls; lateral wall diagonal (LWD): line oriented at -30° with respect to the lateral axis of the beak, joining the baseline of the dentition and the edge of the lateral walls, bar = 100 µm; (**b**) beak from phase 2, detail of dentition, bar = 50 µm; (**c**): beak from phase 2, arrow showing the layer forming the hood and the shoulder, bar = 50 µm.

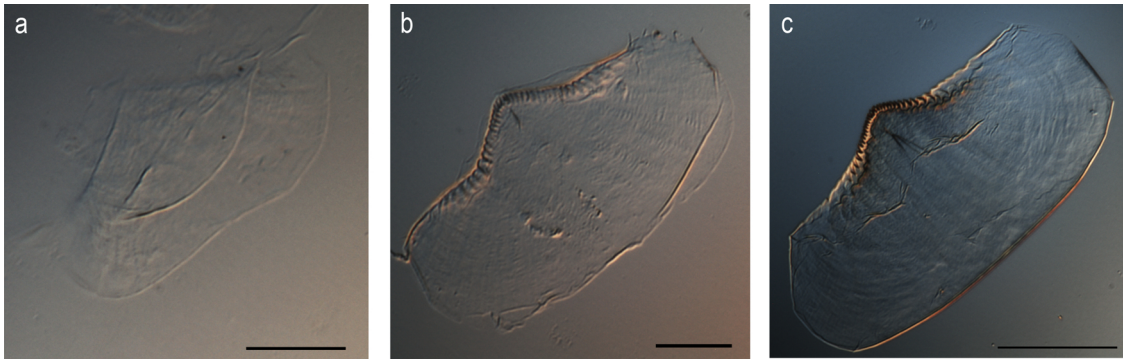

Fig. 2. Developmental stages proposed: (**a**) phase 1, Naef Stage XV, bar =50 µm; (**b**) phase 2, Naef Stage XVII-XVIII, bar =50 µm; (**c**) phase 3, hatching, bar =100 µm.

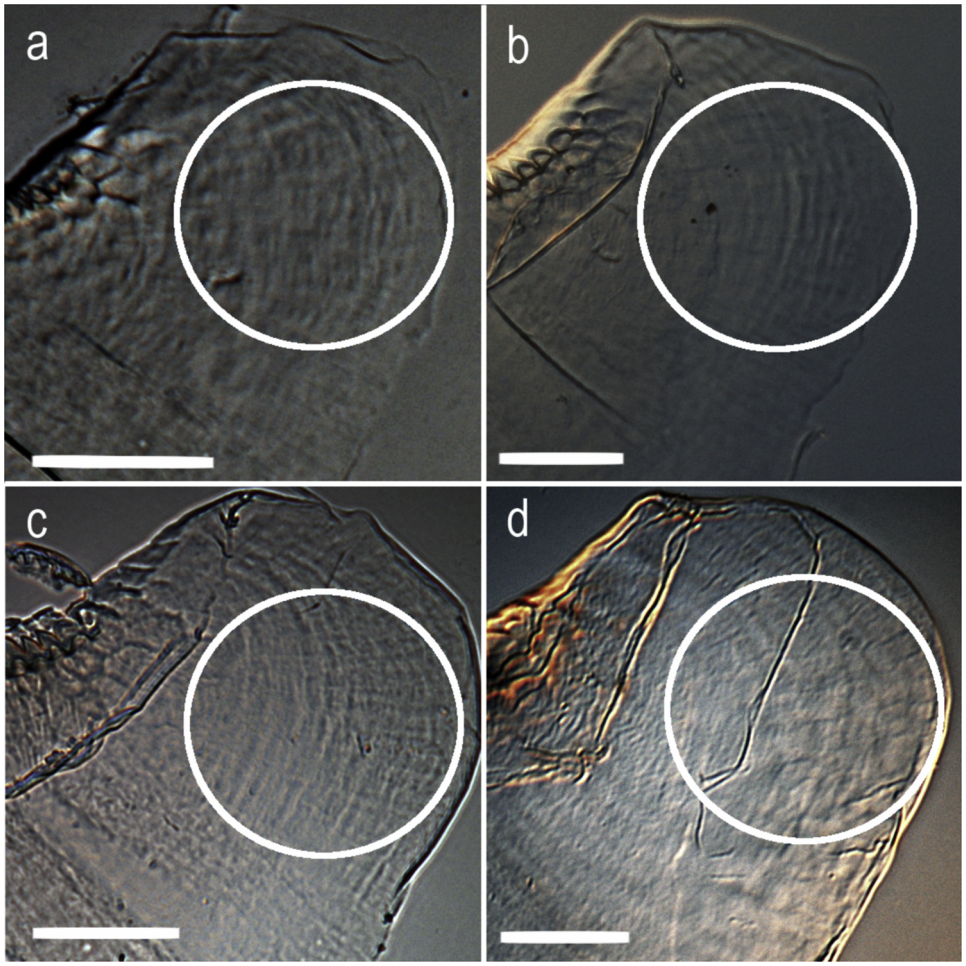

Fig. 3. Follow-up of pre-hatching increments (see the area surrounded by the white circle) on the lateral walls: (**a**) phase 2; (**b**) beginning of phase 3; (**c**) hatching; (**d**) paralarva of 7 days. Bars =50 µm.

During this phase, these layers gradually merge. The outline of the teeth row is already visible, but it is unpigmented.

b Middle phase (phase 2), Naef stages XVII to XVIII (Fig. 2b): a band-shaped layer attached over the teeth row arises (Fig. 1b and c). Subsequently, the attached surface expands, strengthening the rostrum and creating protrusions at front and back sides, forming the shoulder and hood respectively. From the inner side, it is no longer possible to observe a separation between layers. Small but regular undulations are visible on the developing lateral walls, and this is more evident when using polarized light (Fig. 3).

c Pre-hatching phase (phase 3), from Naef (1928) stages XIX-XX onwards (Fig. 2c): after the second inversion (Boletzky, 1971a), the pigmented dentition erupts from a posterior unpigmented layer, bringing out the reading area on the rostrum (Fig. 4). When embryos reach the hatching competence, the dentition keeps erupting, forming the apical part of the rostrum. The thin line at the base of dentition, identified as 'day 0 mark' in rostrum surface, is observed only when embryos empty the outer yolk sac. On the lateral walls several undulations are visible at this point, but it is not possible to associate them with the time of hatching (Fig. 3).

In the morphometric study, the analysis of variance conducted on

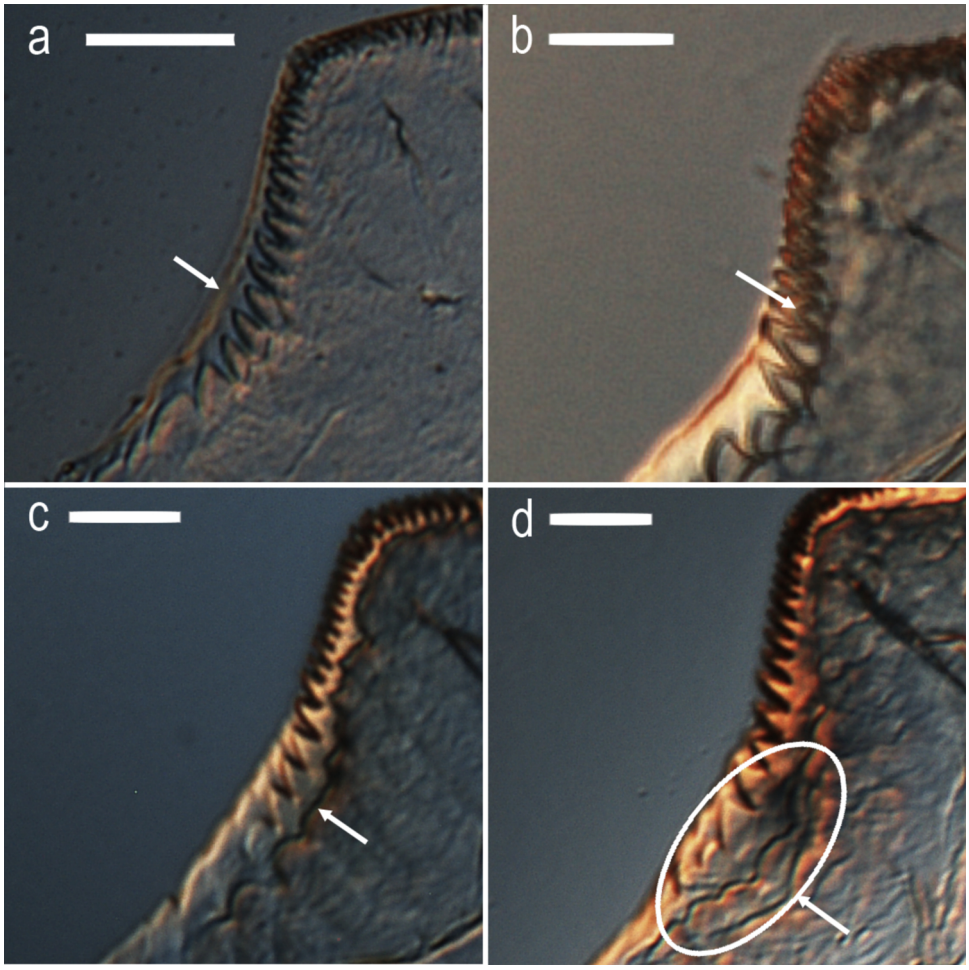

Fig. 4. Follow-up of the rostrum surface onset: (**a**) unpigmented dentition and arrow showing the layer forming the hood and the shoulder, phase 2; (**b**) inception of rostrum eruption (arrow), beginning of phase 3; (**c**) the process lead to the exposure of the rostrum surface, hatching mark (arrow); (**d**) rostrum surface with growth increments in the area marked with the arrow, paralarva of 7 days. Bars = 25 µm.

Table 2
p-values of Tukey HSD test conducted on measurements from spawning event II. Measurements are: upper jaw width (UJW), lateral wall diagonal (LWD), upper jaw length (UJL), rostrum depth (RDE), teeth half width (THW), hood amplitude (HAM). Factor: Time (Days); Levels: 23, 26, 29 days from egg fecundation. α = 0.01, significant results in bold.

	26 UJW	29	26 UJL	29	26 LWD	29
23	**1.12E-05**	**1E-16**	**2.2E-06**	**1E-16**	**2E-07**	**1E-16**
26		**4.8E-06**		**9E-07**		**2E-07**
	HAM		THW		RDE	
26	**2.34E-2**	**7.8E-06**	**1E-09**	**1E-16**	2.7E-1	1.3E-1
23		**1.81E-3**		**2.69E-04**		8.9E-1

SP2 beaks showed a significant increase of size for the selected measurements during the embryonic development, except RDE (p-value = 0.13), which was consequently excluded from the GR analysis (Table 2 for details on pairwise analysis). The measurements considered showed different growth patterns, resulting in two Growth Rate (GR) trends (Table 3). Hence, all the measurements taken from the posterior area (UJW, UJL, LWD), as well as one measurement for the anterior area (HAM), accounted for the highest GR value during the last part of the ontogeny. Conversely, THW, from the anterior area, accounted for a sharp dimensional increase between the Naef stage XVI and the stages XVII-XVIII, followed by a slowdown in late ontogeny. The rearing temperature did not change the gross morphology of the beaks.

Table 4
Two-Way Permanova conducted on Euclidean distance among beaks reared at different temperature. Factors considered: temperature (T°) and stages. S1 = days 23; S2 = days 26; S3: days 29 (days from egg fecundation) α = 0.01.

Source	df	Sum of sqrs	Mean square	F	p	Significance
T°	3	0.45	0.15	20.06	**0.0001**	***
St	2	0.57	0.29	37.95	**0.0001**	***
Interaction	6	0.08	0.01	1.68	0.0962	ns
Residual	69	0.52	0.01			
Total	80	1.62				

Table 3
Growth Rates trends (%d^{-1}) ± errors of Measurements from SP II (spawning event II). T_i (days from egg fecundation): S1 = days 23; S2 = days 26; S3: days 29.

T_i	UJW	LWD	UJL	RDE	THW	HAM
S1-S2	2.07 ± 0.79	2.51 ± 0.76	2.57 ± 0.71	1.02 ± 1.80	1.75 ± 0.44	1.20 ± 1.03
S2-S3	2.21 ± 0.70	2.54 ± 0.39	2.54 ± 0.82	0.26 ± 1.38	0.83 ± 0.33	1.69 ± 1.06

Table 5
Pairwise contrast conducted on Euclidean distance among beaks reared at different temperatures. P-value after Bonferroni correction on lower diagonal, significance on upper diagonal. α = 0.01.

T	16°	19°	21°	23°
16 °C	–	ns	ns	***
19 °C	1	–	ns	***
21 °C	1	1	–	***
23 °C	**0.006**	**0.006**	**0.006**	–

Notwithstanding, it demonstrates that it does have an influence on the dimensions of the beaks. In fact, results of the Two-Way PERMANOVA (Table 4) showed that both factors, time and temperature, caused highly significant differences ($p < 0.001$) in overall sizes of beaks, whereas no significant interaction was observed between factors. Considering the factor of temperature alone (Table 5), beaks of embryos reared at 23 °C resulted in being significantly different (smaller) to replicates at 16, 19 and 21 °C, whereas no significant differences were observed between these temperatures. In addition, the highest variability was observed in beaks from embryos reared at 23 °C.

4. Discussion

Based on the onset of characteristic features, a developmental table divided into three phases was proposed, spanning from the Naef stage XIV to the hatching stage. During the 'Early phase', the upper jaw is barely more than a rudiment and the Hood is completely absent. Nonetheless, the outline of the dentition is already visible and could be seen as the beginning of the secretion of the proteinaceous backbone, fundamental for the sclerotization and the darkening process (see Miserez et al., 2007, 2010 for details). The 'Middle phase' is characterized by the formation of the hood and of the shoulder, as well as the undulations in the lateral walls. The shift toward the third phase, 'Pre-hatching', is marked by the second inversion, a turning point in the ontogeny of the whole digestive system (López-Peraza et al., 2014) and also a breakthrough for the development of the beak. At this point, the gradual exposure of the dentition and of the rostrum surface begins. The latter has been previously recognized as the area showing daily growth increments at the paralarval stage, and was named by Perales-Raya et al. (2014a) as 'Lateral Hood Surface', later by Franco-Santos et al. (2016) as 'Anterior Coloured region', and finally confirmed as 'Rostrum Surface' by Perales-Raya et al. (2018).

These results are relevant in the context of age estimation, since they confirm the age of first increment formation proposed in previous validation studies focused on rostrum microstructure (*i.e.*, Perales-Raya et al., 2014a, 2018; Franco-Santos et al., 2016). To determine the age of first increment formation is a practice considered to be the first step for a powerful validation method according to Campana (2001). The process leading to the emergence of the hatching mark was observed in octopus beak in our study for the first time, to our knowledge. In particular, the mark considered as 'Increment 0′ was detected during phase 3 and subsequently until emptying the outer yolk sac, which is considered the optimal moment for hatching in normal conditions (Boletzky, 1987). On the other hand, increments on the lateral walls were observed from the Naef stage XVII, phase 2, and the identification of an unambiguous hatching mark was not feasible. Therefore, our results support validation of the aging method based on the rostrum microstructure (Raya and Hernández-González, 1998; Perales-Raya et al., 2010, 2014a, 2018) and suggest it as the appropriate methodology for aging paralarvae, evidencing that age estimated in the early stages using increments of the lateral walls in upper jaws could be overestimated. However, differences in number of increments counted in the two areas may be reduced during the animal life, as the observed pre-hatching increments in lateral walls could be reabsorbed in later post-hatching stages. In fact, Perales-Raya et al. (2014a) counted in known-age adults a similar number of increments in the lateral walls than the number of days elapsed from hatching (mean difference was 5% less increments than days). These contrasting results might also indicate a link between increment deposition and sclerotization process (Dilly and Nixon, 1976; Miserez et al., 2010): true increments may be observable only when the darkening process starts to occur, and those noted on the lateral walls during embryonic phase could be just false increments. In any case, determining the age of first increment formation in the lateral walls is still necessary.

The morphometric analysis evidenced that the transition from the second to the third phase is accompanied by a 'shift' in the growth pattern. The THW measurement, taken in the front part, accounted for a drop in the Growth Rate, whereas the trend for the posterior area was more stable. These resultant changes in proportion are likely to be of functional significance. The rostrum is a slicing and grabbing element, which requires increasing hardness (Boletzky, 1971b; Hernández-López et al., 2001; Nixon and Mangold, 1998; Tanabe, 2012). On the other hand, the lateral walls are concerned with muscular attachment and their dimensions are related to the size of the ingested food (Franco-Santos and Vidal, 2014; Franco-Santos et al., 2014; Hernańdez-García et al., 1998), indeed they are expected to grow continuously.

According to literature, the incubation temperature significantly affected the duration of embryogenesis (Boletzky, 1987). The temperature of 26 °C was demonstrated to be over the tolerance limits, as the development had not been successfully completed. Higher temperatures are known to accelerate growth and yolk consumption (Vidal et al., 2002; Uriarte et al., 2012), resulting in shorter development time and smaller hatchlings. In a similar way, beaks were significantly smaller at warm rearing temperature (23 °C). Moreover, a larger amount of variability was observed in the beak size from warm rearing conditions (23 °C), suggesting that sub-optimal developmental conditions could enhance the individual response of embryos. Hence, higher rearing temperatures might reduce the duration of embryonic development meaning a saving of time for aquaculture purposes, but this will not necessarily represent an advantage. In fact, having reduced yolk reserves could affect the survival of hatchlings that have not initiated feeding (Boletzky, 1994), and in this scenario a reduced beak dimension is likely to be an additional unfavorable condition for the feeding opportunities of hatchlings. As reported by Iglesias et al. (2006), due to the poorly developed arms and inefficient predatory strategy of young paralarvae, the denticulate beak is an essential tool to catch and retain prey during the first days of life (Nixon and Mangold, 1996). In a similar manner, Nande et al. (2017) suggest that lower incubation temperature (around 18 °C) improves paralarval viability.

5. Conclusions

The development of *O. vulgaris* beak in late ontogeny (Naef stage XIV onwards) was structured into three phases. Increments on lateral walls appear during the second phase (Naef stage XVII-XVIII), while the "Increment 0″ on the rostrum area was detected during the third phase (Naef stages XIX-XX onwards) and only once the embryo consumed the outer yolk reserves. Indeed, the accuracy of the aging method using the increments in the rostrum is confirmed. The growth rate of the rostrum area (THW) registered a drop during the third phase of ontogeny, whereas the growth of the lateral walls was more constant, suggesting that the heterogeneity of the growth rate could be due to the different role played by beak areas. Regarding temperature, at 26 °C the embryogenesis has not been completed, and beaks of embryos reared at a warm temperature (23 °C) were significantly smaller than the other tested temperatures. This finding adds implications on the well-known thermal sensibility of octopus eggs and could influence the recruitment success in scenarios of ocean warming and also in the field of the common octopus culture.

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CRediT authorship contribution statement

Enrico Nicola Armelloni: Conceptualization, Investigation, Formal analysis. **María Jesús Lago-Rouco:** Investigation. **Aurora Bartolomé:** Methodology. **Beatriz C. Felipe:** Investigation. **Eduardo Almansa:** Supervision, Validation. **Catalina Perales-Raya:** Methodology, Validation, Supervision, Project administration, Conceptualization, Writing - review & editing.

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