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AGE VALIDATION IN *OCTOPUS VULGARIS* BEAKS ACROSS THE FULL ONTOGENETIC RANGE: BEAKS AS RECORDERS OF LIFE EVENTS IN OCTOPUSES

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ABSTRACT This study demonstrates the daily deposition of increments in *Octopus vulgaris* beaks for both lateral wall surfaces (LWS) and rostrum sagittal sections (RSS). Forty-nine marked wild animals kept in aquaria (weight range, 158–3,521 g) and 24 captive-reared known-age individuals (paralarvae, 0–98 days old; adults, 200–734 days old) were studied, encompassing for the first time the full age range of the species, including known-age individuals older than 1 mo. The daily deposition of beak increments was validated in the LWS by injection of Calcofluor, and in the RSS by environmental marking (thermal, confinement, capture, and stress of the chemical marking process). A total of 111 successful validations (when beak increments corresponded precisely to days elapsed) were achieved, and the maximum validated periods were 57 days (LWS) and 112 days (RSS). In the pelagic stage and transition to the settlement stage, a new pattern of microincrements that record age was demonstrated in the lateral hood surfaces of upper jaws, where stress checks were observed. In the benthic stage, tip erosion in beak RSS results in some underestimation of age; however, the demonstration that RSS can record environmental stress renders it a potentially useful tool for documenting life events.

KEY WORDS: *Octopus vulgaris*, Cephalopods, octopus, age, growth, beaks, stress

INTRODUCTION

The common octopus *Octopus vulgaris* Cuvier, 1797 (Cephalopoda, Octopodidae) is one of the most important fished cephalopod species in the world, yielding a mean catch of ~ 41,000 t/y for the past 10 years (FAO 2014). As with other harvested species, sustainable management rests on an understanding of life history and modeling of the dynamics of wild populations, which in turn rest on age determination and knowledge of growth patterns. The identification and interpretation of growth increments in hardened structures, which can provide just such information, is thus important for exploited cephalopod species. These taxa have high and variable growth rates, short life cycles, and terminal postspawning mortality, which can prevent the use of other aging methods such as length frequency analyses (Jackson 1994, Boyle & Boletzky 1996, Perales-Raya 2001, Semmens et al. 2004).

Although much is known about the biology and ecology of *Octopus vulgaris* (e.g., Mangold 1983, Guerra 1992, Domain et al. 2000, Otero et al. 2008, Villanueva & Norman 2008, Otero et al. 2009), there are only a few studies determining age. Although statoliths have been used widely for this purpose in squid (e.g., Spratt 1979, Lipinski et al. 1998, Raya et al. 1999, Villanueva et al. 2003, Arkhipkin & Shcherbich 2011, Bilin et al. 2013) and cuttlefish (Raya et al. 1994, Bettencourt & Guerra 2001, Challier et al. 2002), *Octopus* statoliths lack growth rings (Lombarte et al. 2006, Perales-Raya et al. 2010) and hence cannot be used in this way. Relative lack of hard structures in soft-body animals such as octopuses requires exploration of new potential structures and the development of alternative aging techniques.

Current aging approaches for *Octopus* species are summarized in Table 1. Stylets have been widely investigated in this context (e.g., Sousa Reis & Fernandes 2002, Leporati et al. 2008a, Barratt & Allcock 2010, Hermosilla et al. 2010). The daily deposition of stylet increments has been validated using known-age, captive-reared specimens of raised *Octopus pallidus* (Doubleday et al. 2006, Leporati et al. 2008b), which is a holobenthic species, in which it is relatively straightforward to obtain known-age individuals because there is no pelagic stage. Quantification of the age–pigment lipofuscin as a potential tool for aging *O. pallidus* has also been demonstrated (Doubleday & Semmens 2011). In contrast, *Octopus vulgaris* has a merobenthic life cycle with small planktonic paralarvae, which greatly complicates the recruitment of known-age individuals for validation purposes. A recent validation study of stylet increments, using nonpermanent preparations of wild individuals marked in captivity, validated a periodicity of 1 increment/day in adults of *O. vulgaris* (Hermosilla et al. 2010). Some methodological problems exist, however, that may affect the accurate identification of increments and the interpretation of absolute ages in the stylets (Table 1).

Analysis of increments in beaks offers a potentially superior approach. Beaks are present in all extant species of cephalopods (not only in octopods), and are easily extracted and preserved, and their microstructures are not affected by freezing (Perales-Raya et al. 2010). Prior to this study, however, known-age specimens older than 1 mo have not been available for *Octopus vulgaris*, because high mortality rates experienced during the paralarval stage (Iglesias et al. 2007, Villanueva & Norman 2008, Prato et al. 2010) have thus far hampered the recruitment of juveniles and adults of known age for validation of absolute ages. Rostral sagittal sections (RSS) and lateral wall surfaces (LWS) are the most suitable beak regions/techniques for aging (Fig. 1). Analysis of RSS has been done by Raya and Hernández-González (1998), and of LWS by Hernández-López et al. (2001), Canali et al. (2011), and Cuccu et al. (2013). Perales-Raya et al. (2010, 2014) conducted comparisons of these approaches; we use both.

Beaks of *Octopus vulgaris* require marking for validation purposes, either chemically or via environmental stress. Oosthuizen

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TABLE 1.

Attributes, advantages, and disadvantages of current direct approaches for octopus aging.

Method	Attributes	Advantages	Disadvantages
Stylet sections	Quantification of daily growth increments in thin, transverse stylet sections	Daily periodicity of increments validated in captivity for known-age *Octopus pallidus* (Doubleday et al. 2006, Leporati et al. 2008b) and for wild marked *Octopus vulgaris* (Hermosilla et al. 2010)	Previous freezing of the specimens (e.g., frozen onboard by industrial cephalopod fleets) and stylet inclusion in thermoplastic cement may cause cracking and further disintegration of preparations (Doubleday et al. 2006, Leporati et al. 2008b, Hermosilla et al. 2010) Caution needs to be applied to age data derived from stylets without validation of absolute ages (Doubleday et al. 2011) Not useful for squid and cuttlefish, which lack stylets
Beak surfaces	Quantification of daily growth increments in the inner surface of lateral walls (upper jaw)	Easy and quick preparation (Perales-Raya et al. 2010, current study) Daily periodicity of increments validated in captivity for *O. vulgaris*: in paralarvae up to 26 days old (Hernandez-Lopez et al. 2001), in certain adult sizes (160–610 g) (Canali et al. 2011), and in all sizes and ages (current study) Potentially applicable to any cephalopod species	Increments in the anterior and posterior borders of lateral walls are sometimes difficult to count (Perales-Raya et al. 2010)
Beak sections	Quantification of daily growth increments in the sagittal sections of the rostrum area (upper and lower jaws)	Daily periodicity of increments validated in captivity for *O. vulgaris* of all commercial sizes (current study) Suitable tool as life recorder of stress events Perales-Raya et al. 2014, current study) Potentially applicable to any cephalopod species	Some underestimation of absolute age has been reported in adults as a result of beak erosion in the tip (Perales-Raya et al. 2010, current study)
Age–pigment quantification	Quantification of age–pigment lipofuscin, which accumulates as a function of physiological age	Lipofuscin reported in histological sections of the optic lobes of wild-caught *O. vulgaris* (Sobrino & Real 2003) The relationship between age and lipofuscin has been validated for *O. pallidus* using known-age specimens (Doubleday & Semmens 2011) Potentially applicable to any cephalopod species	Requires specific equipment and histological techniques that are time-consuming Known-age specimens are necessary for validation purposes and it is still difficult to raise merobenthic octopuses such as *O. vulgaris* (Doubleday & Semmens 2011)

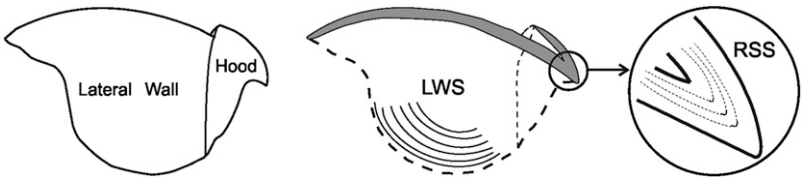

Figure 1. (Left) Drawing of adult *Octopus vulgaris* upper jaw (lateral view). (Middle) Sagittal section showing the lateral wall surface (LWS) bearing increments (lines), and the rostrum area (circle). (Right) Rostral area magnified, showing the increments across the rostrum sagittal section (RSS). Adapted from Perales-Raya (2001).

(2003) experimented with tetracycline as a chemical marker in the adult beaks of South African *O. vulgaris*, but reported that tetracycline marks were not visible evenly in the beaks. Other potential candidates for chemical markers are Calcofluor and Congo Red, because both have a specific binding affinity for chitin (Bartnickigarcia et al. 1994), one of the main components of cephalopod beaks (Hunt & Nixon 1981). Both markers have significant effects on the color, macroscopic texture, electron microscopic morphology, and crystal structure of the biosynthesized chitin, being red in presence of Congo Red and blue in the presence of Calcofluor (Bartnickigarcia et al. 1994). Neither of these two stains has been used previously for age validation. They are used primarily for fungi detection (Slifkin & Cumbie 1988, Matsuoka et al. 1995, Chioralia et al. 1998, Ruchel et al. 2004), and Congo Red is also widely used for the detection of amyloidal deposits (abnormal proteins) in human diseases such as Alzheimer's disease (Styren et al. 2000) or Creutzfeldt-Jakob disease (Goldfarb et al. 1993). Thermal environmental stress marks have been used successfully in otoliths of some fish species (e.g., Letcher & Terrick 1998, Volk et al. 1999), and also in beak LWS of *O. vulgaris* (Canali et al. 2011). In addition, as reported by Jackson (1989), the trauma of capture, collection and transfer to aquaria, and staining could all contribute the induction of a ''check'' in squid statoliths. According to Lipinski et al. (1991), these checks are conspicuously marked rings presumed to reflect specific ''stressful'' events or episodes in the cephalopod's life. Consequently, low-temperature exposure and brief confinement in restricted spaces were selected for this study to induce environmental stress marks in *O. vulgaris* beaks.

Recent studies in *Octopus* beaks have compared several techniques/areas and have enhanced the visualization of increments in upper and lower jaws (Perales-Raya et al. 2010). Subsequently, Canali et al. (2011) documented daily formation of beak growth increments for certain adult size classes, and Perales-Raya et al. (2014) analyzed maximum age and thermal stress marks in spent individuals from the wild. However, daily periodicity still needs to be confirmed in specimens of all sizes and known-age individuals to confirm absolute ages and to validate daily deposition throughout the entire life cycle of the species.

This study aims to validate the daily deposition of increments in *Octopus vulgaris* beaks across the full ontogenetic range. The exploited size range (~150–3,500 g as reported by Mangold and Boletzky [1973], Mangold [1983] and the FAO [2014]) was validated by chemical and environmental markings induced in wild adults kept in captivity. Moreover, despite the high mortality rates observed in *O. vulgaris* paralarval culture (Iglesias et al. 2007, Sánchez et al. 2013), some techniques (Iglesias et al. 2004, Carrasco et al. 2006) have been successful in rearing paralarvae. These approaches have provided us with sufficient numbers of known-age paralarvae, juveniles, and adults hatched under laboratory conditions for a study aimed at confirming, for the first time, absolute ages in *O. vulgaris* beaks.

MATERIALS AND METHODS

Wild Specimens

The beaks of 49 individuals (24 males and 25 females) of *Octopus vulgaris* caught from 2008 to 2010 in the waters of Tenerife (Canary Islands, central East Atlantic) were marked for age validation, as described later. The size range of individuals was between 158 g and 6,000 g in total body weight (Table 2). They were maintained in 1,000-L flow-through tanks in the aquaculture facilities of the Spanish Institute of Oceanography (IEO, Tenerife, Canary Islands). We used a maximum stocking density of 10 kg/m^3/tank, per Iglesias et al. (2007), with each tank containing individuals of similar sizes at a 1:1 sex

TABLE 2.

Marking treatments, number of individuals and weight range of the 49 adult *Octopus vulgaris* marked in captivity for age validation.

Treatment	Stain dose* (mg/kg)	Thermal marking (°C)	Exposure time†	*n*	Weight range (g)
Calcofluor	100			22	212–2,450
Congo Red injection	15			5	479–6,000
Congo Red feeding	30			2	782–1,430
Thermal		3–4	2–6 min	11	400–2,000
Confinement (pipes)			24–48 h	8	158–3,300
Confinement (tank volume)‡				1	3,521
Total individuals induced				49	158–6,000

* For chemical markers. † For thermal and confinement in pipes. ‡ Moved from 1,000-L to 100-L tanks.

ratio to avoid attacks and/or cannibalism. Each tank was provided with at least 2 PVC shelters per octopus. Individuals were maintained under similar environmental conditions of natural photoperiod and ambient seawater temperature (19–23°C), and were fed with defrosted squid (*Illex* spp.). At the end of the marking experiment, each individual was euthanized by chilling (to 1°C) until loss of activity/reaction occurred after 10 min (Moltschaniwskyj et al. 2007, Perales-Raya et al. 2011). All experiments conducted in this study follow Directive 2010/63/EU on animal welfare in cephalopod aquaculture research (Sykes et al. 2012).

Known-Age Specimens

The beaks of 24 known-age individuals were also analyzed (Table 3). The sample included 21 paralarvae in the pelagic stage and transition-to-settlement stage, and 3 juvenile and adult benthic specimens. Because of the multiple sources of the known-age specimens, and the possible impact of these differences on growth and beak formation, the culture details are given for those individuals or stages whose culture methodology is unpublished. The 17 individuals age 0–60 days and 98 days were reared in the IEO culture facilities at Tenerife. The experiment was carried out in a 500-L cylindrical–conical tank (black walls and bottom) with an initial density of 3 individuals/L, a temperature of 24.12 ± 1.30°C, and a natural photoperiod. Paralarvae were fed on *Grapsus adscensionis* zoeae (0.1/mL) and *Artemia* juveniles (0.3/mL). To obtain *Artemia* juveniles, *Artemia nauplii* (EG Type, INVE AQUACULTURE, Belgium) were on-grown with lyophilized phytoplankton (*Tetraselmis chuii*) for 7 days in 100-L tanks with a density of 10 *Artemia*/mL and 2 g *T. chuii* each day. Final *Artemia* enrichment was carried out for 24 h in 50-L tanks with a density of 2 *Artemia*/mL and 10^7 cells/mL *Nannochloropsis gaditana*. The 4 paralarvae at 70 days old were provided by the IEO research group in Vigo (Spain), and were cultured by the methods described by Viciano et al. (2011). The other 3 specimens were a known-age juvenile (200 days old) and 2 adult individuals (560 days old and 734 days old) supplied by the research group at The Fisheries Research Center (CEP) (Gijón, Spain). The paralarvae culture protocol for these specimens is described by Carrasco et al. (2006). When they reached benthic stage (60 days old, 22 mg dried body weight), frozen mysids and

TABLE 3.

Analysis of known age specimens of *Octopus vulgaris* maintained in captivity and sourced from different locations in the East Atlantic.

(A) Pelagic and transition to settlement individuals.

Individual	Location	VML (mm)	Age (days)	NI LHS	CV (%)
PL-C1	Canaries	1.57	0	0	0.00
PL-C2	Canaries	1.86	0	0	0.00
PL-C3	Canaries	1.69	0	1	0.00
PL-C4	Canaries	1.90	15	14	0.00
PL-C5	Canaries	2.03	15	14	0.00
PL-C6	Canaries	1.84	15	15	4.88
PL-C0	Canaries	1.95	21	19	7.44
PL-C7	Canaries	2.21	29	26	13.86
PL-C8	Canaries	2.04	29	29	2.48
PL-C9	Canaries	1.93	29	24	0.00
PL-C10	Canaries	3.34	44	44	0.00
PL-C11	Canaries	3.27	44	43	1.66
PL-C12	Canaries	2.83	44	42	1.70
PL-C13	Canaries	2.80	60	57	2.48
PL-C14	Canaries	3.84	60	58	2.44
PL-C15	Canaries	3.09	60	59	4.79
PL-V1	NW Spain	3.50	70	70	4.04
PL-V2	NW Spain	3.64	70	72	0.99
PL-V3	NW Spain	3.07	70	69	2.05
PL-V4	NW Spain	3.39	70	67	9.57
PL-C16	Canaries	4.00	98	96	0.74

CV, coefficient of variation; LHS, lateral hood surface; NI, mean number of increments of 2 counts; NW, northwest; VML, ventral mantle length.

(B) Benthic individuals.

Individual	Location	Sex	Weight (g)	Age (days)	NI LWS	CV (%)	NI RSS	CV (%)
EC3	North Spain	—	1,010	200	182	3.89	—	—
EC2	North Spain	F	3,250	560	532	0.27	508	3.06
EC1	North Spain	M	5,000	734	716	1.28	688	2.47

CV, coefficient of variation; F, female; LWS, lateral wall surface; M, male; NI, mean number of increments of 2 counts; RSS, rostrum sagittal section; — undetermined sex.

sea fleas (*Talitrus saltator*) were added to the diet; from the juvenile stage (10 g wet body weight) until the end of the experiment, the diet consisted of frozen fish and crabs.

Chemical Marking

Calcofluor and Congo Red were tested as staining fluorescent chemical markers in 29 wild individuals with a body weight between 212 g and 6,000 g (Table 2). To increase the mark sample but not the number of specimens, those we considered better recovered from the first marking ($n = 13$) were marked for a second time. Thus, the total number of chemical marks induced was 42. To avoid any toxicity (Burg et al. 1977), Calcofluor doses of 100 mg/kg body weight were prepared using a solution of Sigma Fluorescent Brightener 28 (Calcofluor White M2R) in 30°C filtered seawater (25 mg/mL), adding 2 drops NaOH (1 M)/100 mg Calcofluor to increase solubility (Chioralia et al. 1998). A 0.2-M phosphate buffer solution was added at a concentration of 0.25 mL/mL Calcofluor solution. The Calcofluor solution was kept in the dark (4°C) until injection. Using the concentration ranges of Frid et al. (2007), Congo Red doses of 15 mg/kg body weight were prepared using a solution of Sigma Congo Red in filtered seawater (50 mg/mL) at room temperature. Preys (squids) injected with a dose of 30 mg/kg body weight were used for marking 2 octopuses. They were fed ad libitum with the stained preys during 1 day.

Before injection, octopuses were anaesthetized by immersion in cold seawater (3–4°C) (Andrews & Tansey 1981) until sucker suction and mobility ceased (1 min 30 sec–7 min 15 sec). Individuals were then marked by subcutaneous injection at the dorsal arm base. After injection, the individuals were roused immediately from anesthesia in seawater at a mean room temperature of 21.5°C. Each individual was maintained in a 300-L aerated recovery tank to control animal mobility and response to stimuli. This procedure was performed before moving the animal to a separate tank. We considered the animal recovered when both suction activity and normal mobility (1 min 15 sec–11 min 40 sec) were regained. The marked individuals were kept in separate tanks and fed *ad libitum* until the next injection, euthanasia, or natural death.

Environmental Marking

Exposure to low temperatures and confinement were tested as environmental stress markers in 20 wild individuals with a body weight between 158 g and 3,521 g (Table 2). Thermal marking was used for 11 individuals (body weight, 400–2,000 g) and was undertaken by exposing specimens to temperatures of 3–4°C until no sucker suction or mobility were observed (2–6 min). The recovery procedure and times were the same as described previously for chemical marking. Confinement in closed PVC refuges (pipes with several holes to ensure proper water renewal) for 24–48 h was used for 8 individuals (body weight, 158–3,300 g). The beaks of another individual, a large male weighing 3,521 g, were analyzed for confinement marks as this octopus was moved from a 1,000-L to a 100-L tank for 10 days; this event registered clearly in the microstructures of its beaks. We included the capture from the wild as an environmental marker after observation of a darker stress increment corresponding to the day of capture in the RSS of some individuals. The day of capture was known in 38 individuals. In addition, the day of chemical injection was also used as environmental mark after observation of a corresponding check in some individuals, probably caused by thermal shock and handling.

Comparison of Growth Rates Between Treatments

To investigate the relative effects of different marking treatments (capture, Calcofluor marking, and thermal marking) on subsequent growth of the animals, we calculated in each individual the daily specific growth rate (SGR; expressed as percent body weight per day). Such effects were not studied for other treatments because insufficient individuals with weight data were available before and after the treatment. Although all experiments were performed in similar culture conditions, there were differences in body size, animal stage, and time of capture among individuals. The SGR was calculated as follows:

$$\mathrm{SGR} = 100 \times \frac{\ln BW_f - \ln BW_i}{T_f - T_i},$$

where BW_i and BW_f are weight on the day of the treatment and weight at death (or next treatment), respectively, and $T_f - T_i$ is the number of days elapsed between treatment and death (or between treatments). Mean SGR ± SD was calculated for each treatment. Data were analyzed for statistical differences among groups (treatments). Because data were presented as percentages (Fowler et al. 1998), an arcsin transformation was carried out prior to analysis. In view of the different sizes of samples ($n = 7$ for thermal marking, $n = 15$ for Calcofluor marking, and $n = 13$ for capture), nonparametric statistics were preferred. The Kruskal-Wallis and Games-Howell *a posteriori* tests were used, with significance set at $P < 0.05$ in all cases.

Beak Analysis

After euthanasia, the beaks of marked individuals were extracted, cleaned (in distilled water), and preserved in distilled water at approximately 4°C, according to the procedure of Perales-Raya et al. (2010). When beaks were not examined within 1 day of death, the individual was frozen at –20°C until observation. The upper jaw LWS, and the RSS of both upper and lower jaws (Fig. 1) were analyzed for all adult individuals according to the methodology described in Perales-Raya et al. (2010), using a Nikon Microscope Multizoom AZ100 with an epifluorescent attachment and different magnifications (50–400×). Fluorescent marks were observed by epi-fluorescent light. Nikon light excitation filters UV2A (330–380 nm) and B2A (450–490 nm) were used for observing the Calcofluor and Congo Red stains, respectively. Calcofluor showed a fluorescent blue ring under UV epi-illumination of the external area of the LWS (Fig. 2A). For an accurate observation of increments in the nonpigmented border of the LWS, both episcopic UV light and transmitted light with Nomarski differential interference contrast (DIC) were used.

Known-age individuals were preserved in ethanol 70% after natural death. Paralarvae LWS were usually broken during beak extraction, because of the fragility of these structures after long exposure to ethanol. The analysis of RSS from the paralarvae of known-age was also discarded because of the very small size and thickness of the beak rostrum, which prevented sections from being obtained. Nevertheless, an alternative and new counting area of the paralarvae upper

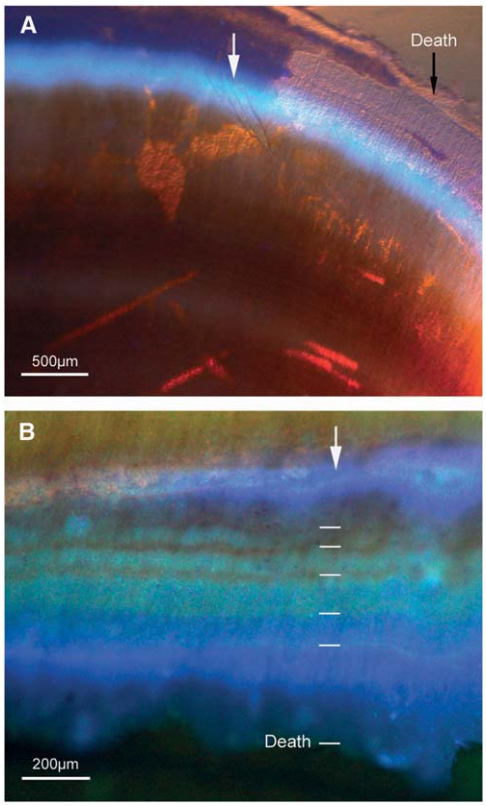

Figure 2. (A) Calcofluor mark (white arrow) under UV light in the lateral wall surface of an *Octopus vulgaris* upper jaw marked 14 days before death. (B) Octopus specimen marked 6 days before death, showing 6 increments (white lines) formed after the Calcofluor mark (white arrow).

jaw, which we named the lateral hood surface (LHS), was identified in the lateral surface of the hood, where parallel and thin increments were observed. Because paralarvae beaks are almost transparent, their increments were observed using transmitted light, a Nikon microscope (AZ100), and 400× magnification with Nomarski DIC, which creates a 3-dimensional image in which the microincrement sequence was revealed in the microstructure of the hood. Two counts were made in each known-age individual, and the final number of increments considered was the mean value of both counts. In adult specimens, 4 counts were obtained for each individual: 2 for the LWS and 2 for the RSS. Age "precision" (*sensu* Campana 2001) was assessed with the coefficient of variation (CV; SD divided by the mean number of increments in each sample) (Chang 1982, Campana 2001).

RESULTS

We treated a beak as containing a "positive mark" when (1) the fluorescent ring or the darker stress increment was visible either in LWS or RSS and (2) the number of days elapsed to the next mark or to death were equal (±2) to the number of increments observed. Deviation of ±2 increments is arbitrary, but was considered a practical degree of error with which to work. The number of positive marks obtained in beaks of *Octopus vulgaris* was 27 in LWS and 84 in RSS, as shown in Table 4. Details of the positive marks are given in Table 5 for LWS and Table 6 for RSS. "Nonpositive marks" are the absence of a mark, difficulty in counting increments after the mark, or when a deviation greater than 2 was obtained between the number of increments and elapsed days. This last situation (mismatched marks) occurred in 6 individuals (6 marks of the 153 induced marks; i.e., 3.9%). One of these mismatches is a thermal mark induced 13 days before death when 10 increments were counted. The other 5 mismatches were capture events that occurred 47–49 days before death, with a mean deviation of 5 (absolute value) between the number of increments and elapsed days.

Chemical Marking

Calcofluor marks were very well marked in the lateral wall of the beak, although they were less visible when the beak was examined away from the border, making them more difficult to locate. This happened in oldest Calcofluor marks (located in a less pigmented chitinous area of the lateral wall), which lost fluorescence as a result of the longer elapsed time to examination. In the border area, the increments formed after the mark were usually also blue (Fig. 2B), probably because Calcofluor still stained the new chitinous and nonpigmented material synthesized a few days after the marking injection. A total of 14 positive Calcofluor marks were obtained in the LWS of 12 individuals (body weight range, 212–2,450 g; Tables 4 and 5). The mean validated period from Calcofluor markings in LWS was 14 days, with a maximum value of 57 days for an individual weighing 1,300 g (Table 5).

TABLE 4.

Summary of age validation results of marking in *Octopus vulgaris* beaks.

Treatment	Induced marks	Positive marks* in LWS	Positive marks* in LWS (%)	Positive marks* in RSS	Positive marks* in RSS (%)
Calcofluor	33	14	42	0	0
Congo Red	9	0	0	0	0
Thermal	14	3	21	11	79
Confinement	17	7	41	17	100
Capture†	38	0	0	27	71
Marking process‡	42	3	7	29	69
Total	153	27	18	84	55

LWS, lateral wall surface; RSS, rostrum sagittal section. * When number of days elapsed to death or to next mark equal the number of increments ±2. † Not induced directly to produce marks, but the event had known dates. ‡ The check resulting from the chemical marking process.

TABLE 5.

Positive marks (see Table 4) in lateral wall surfaces of *Octopus vulgaris* beaks.

Individual	Sex	Weight (g)	Mark 1	Days	Mark 2	Days to death	No. of days validated
CF15	M	212	CF			6	6
CF17	M	282	CF			4	4
CF24	F	400	CF			13	13
CF25	H	500	CF			13	13
CF21	M	747	CF	11	MP	6	17
CF23	F	900	CF			13	13
CF12	F	1,200	CF			12	12
CF9	M	1,300	CF	37	CF	20	57
CF27	M	1,450	CF			13	13
CF22	M	1,470	CF			4	4
CF6	F	2,000	CF	21	CF	14	35
CF8	M	2,450	CF			5	5
C3	M	158	CO			5	5
C4	F	402	CO			10	10
C5	M	702	CO			10	10
C7	M	1,046	CO			10	10
C8	F	1,091	CO	20	CO	10	30
P8	M	3,521	CO			19	19
CF5	F	1,348	MP			3	3
CF26	M	1,600	MP			13	13
T5	F	400	T			13	13
T7	F	900	T			13	13
T8	F	1,500	T			13	13
Total = 27 positive marks			23		4		mx = 57

Individuals grouped by mark categories (dashed lines). CF, Calcofluor mark; CO, confinement mark; F, female; M, male; MP, mark resulting from the chemical marking process; T, thermal mark. * Days elapsed between marks.

Congo Red stained 5 beaks, but none of the marked individuals produced clear positive marks (Table 4), either because the stained area was too diffuse or because the increments around the mark were not clear enough to be counted. It seems that Congo Red stains the nonpigmented chitinous border of the beak, although the high dispersion of Congo Red in the beak microstructure did not produce a clear ring but a diffuse stained area. Because better results were obtained with Calcofluor, we did not continue to mark individuals with Congo Red.

Environmental Marking

Thermal, confinement, capture, and chemical marking stress were the 4 kinds of environmental markings that produced checks in RSS and/or LWS (Tables 4–6). Our results indicate that environmental checks are better recorded by the RSS (average positive marks, 80%) than by the LWS (average positive marks, 17%), mainly because counting the outer increments of the LWS border was usually difficult.

Thermal stress was recorded as a darker increment, but less so than for marks caused by confinement and capture. Thermal marking produced 11 positive marks in the RSS, whereas only 3 positive thermal marks were obtained in LWS, mainly as a result of the absence of an identifiable check. Confinement for 24 h caused a check in the RSS, whereas 48-h confinement produced 2 checks (Fig. 3) in 5 of the 6 individuals induced. Confinement produced 17 positive marks in all the analyzed RSS, and 7 positive marks in the LWS (Tables 5 and 6). The day of capture was usually strongly marked as a check identifiable in the RSS microstructural pattern (Fig. 4). It was also the darkest mark of all the positive environmental marks analyzed. However, no capture marks were observed in the LWS of the sampled octopuses. The check formed on the day of stain injection (chemical marking stress) was marked clearly as an RSS check in 29 (69%) of the 42 injections. These checks were useful to increase the number of marks for periodicity validation. It remains unclear, however, which aspects of the chemical marking process (thermal anesthesia, handling, injection, or the entire process) were responsible for the growth check.

For all environmental markings, a total of 84 and 13 positive marks were registered in the RSS and the LWS, respectively (45 individuals, body weight range, 158–3,521 g). The mean validated period for increment deposition in the RSS was 43 days, with a maximum value of 112 days for an individual of 2,450 g (Table 6).

Comparison of Growth Rates Between Treatments

The SGR varied between treatments, decreasing from 2.05 ± 0.78 ($n = 7$) for the thermal marking to 1.1 ± 0.84 ($n = 15$) for the Calcofluor marking, and 0.87 ± 1.1 ($n = 13$) for the capture event. Significant differences were found between the SGR after thermal marking and SGR after capture, according to Kruskal-Wallis ($P = 0.034$) and Games-Howell ($P = 0.033$) tests. These results suggest that capture events promote a reduction of SGR to a greater extent than that caused by thermal marking, whereas Calcofluor marking shows intermediate effects.

Known-Age Specimens

Table 3 summarizes the results from known-age individuals that were hatched and reared in captivity. At the pelagic stage and

TABLE 6.

Positive marks (see Table 4) in rostral sagittal sections of *Octopus vulgaris* beaks.

Individual	Sex	Weight (g)	Mark 0	Days*	Mark 1	Days*	Mark 2	Days to death	No. of days validated
C3	M	158			CO			5	5
C4	F	402	C	13	CO	26	CO	10	49
C5	M	702	C	19	CO	19	CO	10	48
C6	F	762			CO	19	CO	10	29
C7	M	1,046	C	17	CO	21	CO	10	48
C8	F	1,091			CO	20	CO	10	30
C2	M	2,800			CO	46	CO	21	67
C1	M	3,300			CO	16	CO	15	31
P8	M	3,521			CO	10	CO	19	29
CF16	F	273	C	7	MP	11	MP	6	24
CF19	F	321			MP	11	MP	5	16
CF24	F	400	C	36	MP			13	49
RC7	M	479	C	14	MP			9	23
CF18	F	494	C	7	MP	11	MP	6	24
CF10	F	651			MP			43	43
CF21	M	747	C	7	MP	11	MP	6	24
CF23	F	900	C	50	MP			13	63
CF12	F	1,200			MP	20	MP	12	32
CF9	M	1,300			MP	37	MP	20	57
CF27	M	1,450	C	24	MP			13	37
CF26	M	1,600	C	50	MP			13	63
CF11	M	1,600	C	20	MP	19	MP	12	51
CF14	F	1,602	C	20	MP	20	MP	55	95
CF13	M	1,750	C	28	MP	20	MP	12	60
RC3	F	1,750			MP			13	13
CF6	F	2,000			MP			35	35
CF8	M	2,450	C	107	MP			5	112
RC2	M	2,920			MP			22	22
T5	F	400	C	34	T			13	47
T2	F	700			T			46	46
T7	F	900			T			13	13
T11	M	915	C	34	T			9	43
T9	F	1,050	C	36	T			13	49
T10	M	1,400	C	36	T			13	49
CF22	M	1,470	C	47	T	3	MP	4	54
T3	M	1,800			T	15	T	46	61
T1	F	2,000			T	15	T	46	61
CF25	F	500	C					49	49
MCH3758	F	708	C					27	27
T6	M	750	C					49	49
RC8	F	838	C					31	31
CF7	F	950	C					40	40
S1	M	1,129	C					23	23
T8	F	1,500	C					49	49
Total = 84 positive marks			27		37		20		mx = 112

Individuals grouped by mark categories (dashed lines). C, capture mark; CO, confinement mark; F, female; M, male; MP, mark resulting from the chemical marking process; T, thermal mark. * Days elapsed between marks.

at the transition toward settlement (age range, 0–98 days), increment counts were made in our newly identified region of the LHS—a beak region already being formed at the youngest ages (Fig. 1B). Analysis of the LHS showed that microincrement count (Fig. 5) aligned with chronological age (Table 3A and Fig. 7), with a high "precision" (*sensu* Campana 2001) between counts (i.e., showed a high degree of repeatability; mean CV, 2.82%) for the 21 pelagic and in-transition-to-settlement individuals.

Three benthic specimens 200 days old, 560 days old, and 734 days old were validated (Table 3B). Readings were taken in the LWS (Fig. 6) and in the RSS. In the LWS of old individuals, a narrowing of increment width was observed in later growth stages, reflecting the slowing down of lateral wall growth (Fig. 6B). The onset of these thinner increments occurred 84 days before death in a female that was 560 days old, and 496 days before death in a male that was 734 days old. The mean aging precision (CV) was 1.81% for the LWS and 2.76% for the RSS. Accuracy was greater in the LWS, for which absolute counts were better aligned to chronological age (mean underestimation, 12 days) than the RSS (mean underestimation, 40 days).

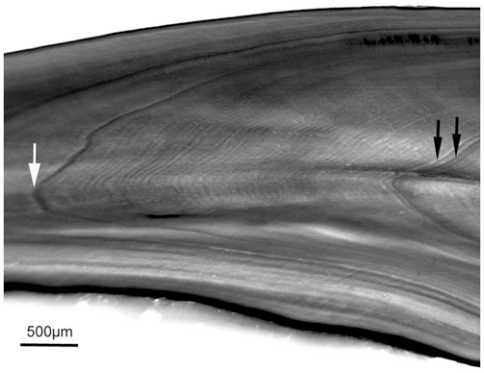

Figure 3. Confinement stress marks in the rostrum sagittal section (RSS) of an *Octopus vulgaris* upper jaw. (Left, white arrow) Stress mark formed after 24 h of confinement. (Right, black arrows) Two stress marks corresponding to the 48-h confinement.

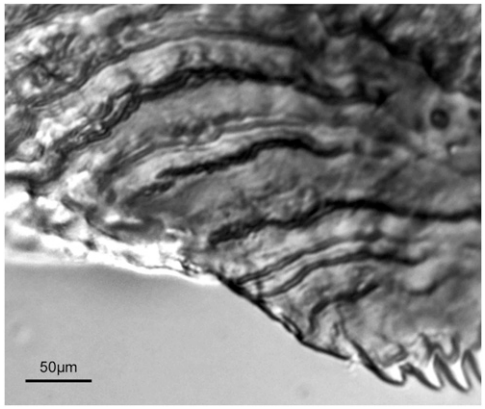

Figure 5. Microincrements in the inner lateral surface of the upper jaw hood. Paralarva, 98 days old.

Figure 7 shows the strong positive linear relationship between age and mean number of increments (in the LHS and the LWS for pelagic and benthic octopuses, respectively) for the known-age individuals. It was highly significant ($r^2 = 0.999$, $P < 0.001$, $n = 24$), with a slope of 0.965 increments/day and an intercept of –0.251 increments at age 0.

DISCUSSION

Daily formation of beak growth increments in *Octopus vulgaris* has been documented for paralarvae up to 26 days old (Hernández-López et al. 2001), and for a period of 30 days before death in certain adult size classes (body weight, 160–610 g) by Canali et al. (2011). Our results on known-age individuals extend the validated age to pelagic/transition stages up to 98 days old using the LHS of the upper beaks. Furthermore, for the first time in *O. vulgaris*, 3 known-age benthic specimens 200 days old, 560 days old, and 734 days old were validated using the LWS and the RSS. The current results of chemical and environmental marking extend the validated size range to 158–3,521 g body weight, validating the daily formation of beak increments across the full ontogenetic range of the species.

Before the current study, neither Calcofluor nor Congo Red had been used for age validation purposes. Gohel et al. (2005) showed that Calcofluor produced clear bands in the chitinase when compared with Congo Red and other staining dyes. Our results also support the superiority of Calcofluor over Congo Red as a stain for chitin formation. The lack of Congo Red staining in the newly deposited beak material is probably a result of the masking of red color of Congo Red by the natural red-brown pigmentation of the beak. Both chemical markers performed better in the nonpigmented border of the LWS, where there is more chitin and less protein than in the pigmented region of the beaks (Miserez et al. 2008). Rostrum sagittal sections were not marked with either Calcofluor or Congo Red

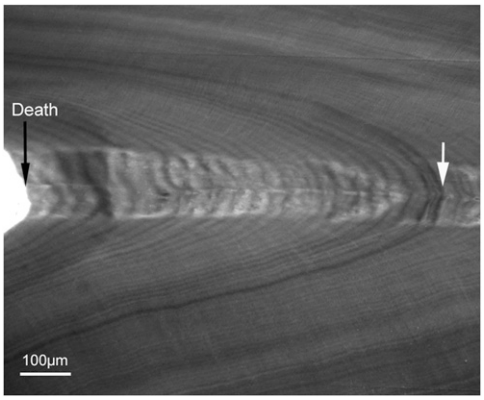

Figure 4. Capture stress mark (white arrow) in the rostrum sagittal section of an *Octopus vulgaris* upper jaw.

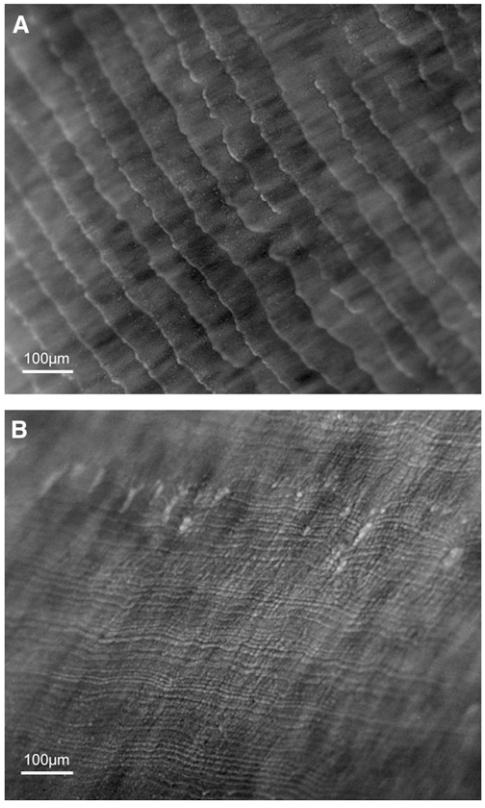

Figure 6. Lateral wall surface used for counting increments in a known-age female weighing 3,250 g. (A) Beak increments in the medial area of the lateral wall. (B) Thin increments near the posterior nonpigmented border.

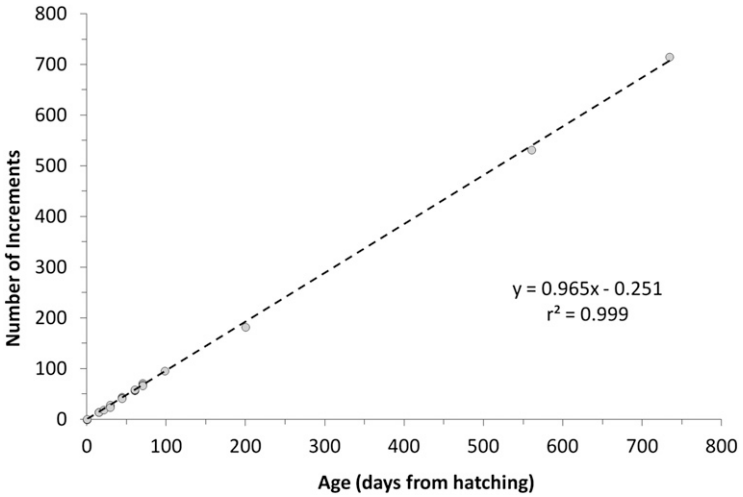

Figure 7. Relationship between age (number of days after hatching) and the mean number of increments (in the lateral hood surface for pelagic specimens and in the lateral wall surface for benthic specimens) in known-age individuals ($n = 24$) of *Octopus vulgaris*.

stains, probably because of the lower chitin-to-protein ratio in the rostrum area of cephalopod beaks, as reported by Miserez et al. (2008).

The environmental (thermal) markings carried out by Canali et al. (2011) in *Octopus vulgaris* beaks were of a similar percentage of positive marks in the LWS (85%) as the current study in the RSS (79%). The RSS were not tested by these authors. From our results, only 21% of the induced thermal marks were positive in the LWS. The cause of this discrepancy is unknown, but may be related to different responses to thermal shock among populations, experimental methods, or different body sizes used in both thermal marking experiments (160–610g by Canali et al. [2011] and 400–2,000 g by us).

Stress has been shown to reduce brachial uptake of calcium in fish, resulting in a calcium carbonate-poor check structure in scales and otoliths that is visually prominent compared with the surrounding daily increments of the calcified pieces. The visual intensity of a check is often proportional to the magnitude and duration of the stress that caused it (Campana 1983). Thermal and other environmental marking methods have been used in the mass marking of certain species of fish (e.g., Letcher & Terrick 1998, Volk et al. 1999, Campana 1999). They are normally created using short-term temperature fluctuations to induce distinctive structural marks on the otoliths (Hagen et al. 1995). Rapid temperature reduction causes dense bands to be formed, and recognizable marks may be generated by maximizing differences between normal patterns and artificially generated thermal patterns (Durholtz & Lipinski 2000). In cephalopod statoliths, stress marks or checks (*sensu* Lipinski et al. 1991) result more typically from a short-term decrease in growth rate, and may reflect stressful events. It is also possible that some environmental stimuli, such as storms, temperature shocks, or unsuccessful attacks by predators, provide sufficient stress to induce check formation within the statolith microstructure (Arkhipkin 2005). As noted earlier, the trauma of capture and confinement at low-oxygen conditions during collection, combined with the subsequent staining and temperature shock associated with the transfer to aquaria, can induce a statolith check (Jackson 1989). Checks in stylet sections of *Octopus vulgaris* have also been observed after chemical marking (tetracycline injections [Hermosilla et al. 2010]). The use of environmental rather than chemical markers in cephalopods may have animal welfare advantages, because the markers generated in this way may be less detrimental to or dangerous for the individual.

Comparison of SGR between treatments suggests that thermal marking is less stressful (in terms of effect on body weight growth) than capture events. Calcofluor marking produces intermediate stress levels, although it involves thermal anaesthetizing, to which half the individuals were exposed twice. We treat these inferences as provisional in view of differences in the date of capture, and the size and condition of individuals. However, the day of capture was also the darkest of all the positive environmental marks analyzed in the current study, and the visual intensity of a check is probably proportional to the magnitude of the stress that caused it (Campana 1983). Therefore, our combined results strongly suggest that capture was the most stressful event studied.

According to Burke et al. (1993), restriction of fish growth in adverse conditions (e.g., polluted waters) usually produces otolith stress indices, which result from changes in the process of increment deposition and are measured by marginal otolith increment widths. In our experiments, however, no irregularity in deposition of increments was observed after treatment. On occasion, we observed other checks in the beak microstructure, probably caused by stressful events that were neither controlled nor induced by our experiments. These were, in no cases, located near enough to the day of marking to create any confusion with the induced mark. No checks were observed in young paralarvae (0–15 days old), but in oldest paralarvae (29–98 days old), some checks occurred in the LHS, possibly related to stress situations during captivity. We observed that specimens older than 44 days still had oral denticles in their beaks, but older individuals had lost some of the denticle tips, presumably worn by feeding on zoea larvae, as reported by Villanueva and Norman (2008).

For benthic known-age individuals, the underestimation of age that was observed in this study may reflect the loss of a number of increments located near the rostrum. Although both the RSS and the LWS showed daily deposition of increments in

our marking experiments, the absolute increment count in the LWS was closer to chronological age than that in the RSS. The counting area of RSS is subject to more loss of material by erosion because it includes the most anterior region of the rostrum tip (Fig. 1A), which is most exposed during predation on armored prey such as bivalves or crustaceans. A recent study carried out with adult known-age specimens of *Octopus maya* fed an artificial diet (without hard parts such as shells) showed a precise coincidence between the number of RSS increments and chronological age (Bárcenas et al. 2014). To minimize any underestimation in the RSS, Perales-Raya et al. (2010) proposed counting increments in the dorsal area of the rostrum, which should be less affected by erosion, despite the likelihood of some underestimation resulting from the narrower deposition of increments in this area.

The thinner increments at the LWS posterior region of the known-age female (Fig. 6B) may relate to reproduction because spawning started 84 days before death. Senescence, cessation of feeding, and the slowing of growth after reproduction could be the cause of thin-increment deposition. Senescence in females is especially apparent in those that are brooding eggs, have survived after the eggs hatched, or are removed from their eggs (Anderson et al. 2002). Senescence in males is less obvious. In 774-day-old male, the greater number of thin increments observed in the posterior region of the LWS ($n = 496$) might support the hypothesis that longevity in *Octopus vulgaris* is not much longer than 1 y in the wild, and that benign captivity conditions extended its senescence stage artificially. A recent study (Perales-Raya et al. 2014) analyzed the age of wild spent octopuses from central East Atlantic waters, and the maximum age obtained from the LWS was 303 days and 322 days for males and females, respectively. However, both individuals were reared in northern Spain, where relatively low temperatures likely promote longer life cycles and lower growth rates (temperature is highly correlated to growth and longevity in shallow-water or benthic octopuses [Van Heukelem 1979, Semmens et al. 2004, Leporati et al. 2008b, André et al. 2009]).

In conclusion, results from known-age specimens demonstrate daily deposition of beak increments obtained from markings in the RSS and the LWS of the beaks. Although the known-age sample does not cover the full age range, missing some of the benthic stage (because of the difficulty of obtaining known-age adults), the positive linear relationship between age and mean number of increments strongly supports the 1 increment/day hypothesis in *Octopus vulgaris* beaks. We consider that daily deposition in beaks has now been validated across the full ontogenetic range of this species, and can be used as a reliable aging tool. Furthermore, because these structures are present in all living cephalopods, they are potentially suitable for aging any cephalopod species.

Additional analysis of stress checks observed in the LHS of paralarvae beaks is encouraged to identify stressful events across the pelagic stage. This could be crucial to understanding the causes of the low survival rate of the common octopus in captivity and the reasons preventing the settlement stage. In the benthic stages, the RSS have also been shown to produce quality checks as a response to stressful conditions; it may find utility in future research to ascertain the effects of environmental and other potentially stressful factors during the life cycle of both wild and reared octopuses.

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