Embryonic life of the loliginid squid *Loligo vulgaris*: comparison between statoliths of Atlantic and Mediterranean populations

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ABSTRACT: Egg masses of the loliginid squid *Loligo vulgaris* Lamarck, 1798 are attached to hard substratum or branched sessile organisms on the sea bottom. Embryonic development lasts from a few weeks to a few months, depending on the environmental water temperature. Because embryonic statolith growth of *L. vulgaris* is very sensitive to temperature under laboratory conditions, we analyzed the possibilities of determining past events in the squid's early life from analysis of the embryonic area of statoliths of wild squid populations. The relationship between egg-incubation temperature and daily growth of embryonic statoliths under laboratory conditions was determined by tetracycline markings at 10 incubation temperatures ranging from 12 to 24.7°C. In addition, the mean width of embryonic increments in statolith collections of wild *L. vulgaris* from the Eastern Atlantic (Saharan Bank and NW Iberian Peninsula) and the Mediterranean Sea (Central and Eastern) was calculated. The temperature inferred from the embryonic increment widths of the statoliths of wild squid indicates that embryonic development of *L. vulgaris* in the regions sampled is likely to occur at temperatures ranging from 12 to 17°C. Mediterranean squid have wider embryonic increments than Atlantic squid, reflecting the slightly higher water temperatures in the Mediterranean Sea during the development of the egg masses. Eggs of *L. vulgaris* spawned off the NW Iberian Peninsula were estimated, on average, to remain at sea for 47 d, 1 wk longer than Mediterranean eggs (nearly 1 mo longer when comparing minimum and maximum ranges). A longer incubation time for egg masses attached to the sea bottom increases mortality risks. Conversely, slow development at a lower temperature can improve yolk conversion, producing larger hatchlings, and increased hatching competence is expected from such squid. Therefore, a compromise between longer-versus-shorter incubation time and related characteristics does exist.

KEY WORDS: Cephalopods · Spawning sites · Embryonic development · Eggs · Larvae · Growth · Statoliths

INTRODUCTION

Loliginid squids are fast-growing coastal cephalopods. Their initial life cycle, i.e. embryonic development, lasts from a few weeks to a few months. The post-hatching life of most species is ≤1 yr. Embryonic development depends on the environmental water temperature which, for *Loligo vulgaris* Lamarck, 1798,

ranges from >3 mo at 10°C to ~1 mo at 20°C (von Bo-
letzky 1974). Studies on the age and growth of *L. vul-
garis* from statolith analysis have estimated the dura-
tion of their post-hatching life to be about 1 yr, i.e. 240
to 396 d (Natsukari & Komine 1992, Arkhipkin 1995,
Bettencourt et al. 1996, Raya et al. 1999, Rocha &
Guerra 1999). Statolith analysis has proved to be the
best tool for estimating loliginid age and growth of
wild populations, with nearly all studies focusing on
analysis of the post-hatching area of the statolith (see
the reviews Jackson 1994 and Jackson & O'Dor 2001).
Less attention has been paid to embryonic statolith
analysis. In statoliths of loliginid squids, the nucleus
area is outlined by the first increment. The natal ring,
which is formed at hatching, is the first prominent
growth increment outside the nucleus. A series of
growth increments are visible between the nucleus
and the natal ring. These growth increments have
been reported for the loliginid squids *Uroteuthis* (*Pho-
tololigo*) *edulis* (Hoyle, 1885) (Natsukari et al. 1988),
L. subulata Lamarck, 1798 (Lipinski 1986, Morris 1991,
1993), *Sepioteuthis lessoniana* Lesson, 1830 (Jackson
1990, Ikeda et al. 1999), and *L. forbesi* Steenstrup,
1856 (Collins et al. 1995, Martins 1997, Rocha & Guerra
1999). For *L. vulgaris*, these increments have been well
illustrated in photographs of published studies (see
Fig. 1 of Natsukari & Komine 1992, Fig. 1 of Arkhipkin
1995, Fig. 1 of Martins 1997, Fig. 2 of Rocha & Guerra
1999 and Fig. 3 of Villanueva 2000b). Egg masses of *L.
vulgaris* are laid on the underside of rocky overhangs
or on branched sessile organisms (von Boletzky 1998)
and fishing lines (Villanueva 2000b; see also 'Materials
and methods' of present study) and hang down in the
water. These egg masses comprise dozens to hundreds
of finger-like egg capsules, 60 to 160 mm in length,
each capsule containing an average of 90 eggs (Man-
gold-Wirz 1963). *L. vulgaris* is an interesting model for
study because its spawning period extends through-
out the year, both in the Atlantic (Coelho et al. 1994,
Guerra & Rocha 1994, Arkhipkin 1995) and the
Mediterranean Seas, and egg masses can be collected
year-round (Mangold-Wirz 1963, Villanueva 2000a).
Temperature and food are probably the most impor-
tant factors that affect cephalopod growth. Squid stato-
liths reflect the influence of temperature during pre-
hatching (Villanueva 2000b) and post-hatching life
(Durholtz & Lipinski 2000, Villanueva 2000a) as well as
food ration (Jackson & Moltschaniwskyj 2001). During
embryonic development, yolk reserves provide a con-
stant nutrient flow to the embryo, and it can be
assumed that embryonic statolith growth is not limited
by external nutrients as occurs in post-hatching squids.
Therefore, the environmental water temperature is
probably the most important influence on embryonic
statolith growth. Other factors such as egg position,

biofouling and (probably) salinity changes, are sources
of variability in embryonic development observed in
the field for the loliginid squid *Sepioteuthis australis*,
and should also be taken into consideration (Gowland
et al. 2002, Steer et al. 2002).

In *Loligo vulgaris*, daily deposition of statolith growth
increments has been validated under different condi-
tions and stages of development. In the field, as well
as in the laboratory, daily increment deposition in sta-
toliths of adult *L. vulgaris reynaudii* d'Orbigny, 1845
has been determined by tetracycline marking and
recapture (Lipinski et al. 1998, Durholtz et al. 2002). In
the laboratory, statolith increments of embryos and
paralarvae of *L. vulgaris* have been validated under
different temperature conditions by multiple tetracy-
cline marking, and have shown that cool temperatures
can affect statolith increment deposition (Villanueva
2000a,b). These laboratory results revealed a daily
increment deposition rate over 2 mo of paralarval rear-
ing at 11 and 19.5°C. However, at the lower tempera-
ture, only 21% of the statoliths were readable. For
embryonic statoliths the following observations were
made: (1) increases in statolith length (% d^{-1}) between
tetracycline marks were up to 5 times higher for
embryos incubated at 21°C than for those incubated at
12°C; (2) increment deposition was daily at 15.5°C, but
less-than-daily at 12°C; (3) when embryos incubated at
cool temperatures were transferred to warm condi-
tions, statolith growth rates increased, indicating the
plasticity of statolith growth.

These laboratory results demonstrated that embry-
onic statoliths are very sensitive to temperature and
suggested the possible usefulness of the embryonic
area of the statolith in determining past events of the
squid's early life cycle in wild populations. With this
aim, the present study had 2 objectives. The first was to
determine the relationship between egg incubation
temperature and embryonic statolith growth in *Loligo
vulgaris*. Accordingly, a series of 10 incubation temper-
atures (ranging from 12 to 24.7°C) were analyzed for
groups of independent egg capsules. The second ob-
jective was to measure the width of the embryonic in-
crements in statoliths from wild *L. vulgaris* individuals
(juveniles to adults) from different, well-differentiated
geographic regions, i.e. the East Atlantic and the
Mediterranean Sea. Statolith collections from previ-
ously published studies on *L. vulgaris* age and growth
(Arkhipkin 1995, Raya et al. 1999, Rocha & Guerra
1999) were analyzed, as well as newly collected mat-
erial. From these aged statoliths it was possible to
estimate by back-calculation the respective hatching
dates and the sea temperature ranges recorded for
each geographic region at hatching. Thus, a compari-
son was possible between the recorded sea tempera-
tures and the sea temperatures inferred from the rela-

tionship obtained in the laboratory between embryonic statolith growth and incubation temperature, opening the possibility of using these embryonic statolith characters to determine past events in the early life of squid.

MATERIALS AND METHODS

Embryonic statoliths of *Loligo vulgaris* incubated under laboratory conditions. The experimental procedure was as follows:

Collection of material: Two recently spawned egg masses of *Loligo vulgaris* were collected from fishing lines deployed 24 h before egg collection at depths of 15 to 30 m off Barcelona, Spain (NW Mediterranean). Thus, the time of collection period corresponded to the maximum age of the eggs. Egg capsules were transported to the laboratory on the same day and maintained in the open-circuit seawater system of the Institut de Ciències del Mar, Barcelona. They were incubated suspended near the surface in a 200 l tank by a nylon fishing thread passed through the mucilaginous mass at the base of the capsules. A high flow rate of 200 l h^{-1} ensured that the egg capsules remained in constant gentle motion with good ventilation. Egg Mass A was collected on 4 May 2000, and the whole egg mass was incubated for 15 d at a temperature of 15.6°C (range 15.3 to 15.8°C). Egg Mass B was collected on 24 April 2001, and the whole egg mass was incubated for 12 d at a temperature of 20.2°C (range 19.9 to 20.5°C).

Tetracycline staining and temperature regime between staining marks. The general procedures followed those of Villanueva (2000b). Egg masses A and B were both stained on 2 occasions (M_1 and M_2). The first staining (M_1) occurred 15 d (Egg Mass A) or 12 d (Egg Mass B) after collection by immersing the egg capsules for 3 h in a solution of 750 mg l^{-1} tetracycline in seawater in 5 l tanks with gentle aeration. Staining was always made between 10:00 and 13:00 h, after which the egg capsules were returned to the incubation tank. After M_1 staining, the egg capsules of the whole egg masses were divided into 4 groups (1, 2, 3, 4) for Egg Mass A and 3 groups (5, 6, 7) for Egg Mass B, with each group comprising 10 to 15 egg capsules. Group 1 was incubated at a mean temperature of 13.3°C (13.2 to 13.4°C), Group 2 at 17.5°C (17.4 to 17.6°C), Group 3 at 19.7°C (19.3 to 20.4°C), and Group 4 at 24.7°C (24.1 to 25.6°C). Group 5 was incubated at a mean temperature of 16.7°C (16.3 to 17.3°C), Group 6 at 22.4°C (22.2 to 22.5°C) and Group 7 at 23.5°C (22.9 to 23.9°C). The second staining (M_2) was performed 7 d later for all temperature groups, except Groups 1 and 3, for which the time between stainings was 14 and 13 d, respectively.

To avoid temperature shock, the egg capsules were gradually acclimatized to the new temperatures at a rate of 1°C h^{-1}. Incubations were carried out in separate 40 l tanks, using 250 l and 700 l closed seawater systems provided with automatic temperature control (Villanueva 2000b). Constant, low-intensity illumination was maintained at 1.5 µE m^{-2} s^{-1} at 1 cm below the water surface. M_2 staining was undertaken at the individual incubation temperatures. The temperature was then raised and maintained at 19.1°C (18.5 to 19.4°C) for all groups until hatching. Sets of data on embryonic statolith growth from tetracycline validation studies of *Loligo vulgaris* (Villanueva 2000b) have been included in the present research. These data correspond to 3 groups of egg capsules treated following the same protocol as above and incubated at mean temperatures of 12.0°C (11.4 to 12.5°C), n = 26 squid; 15.5°C (15.3 to 15.8°C), n = 26 squid; and 21.1°C (20.4 to 21.8°C), n = 25 squid (Villanueva 2000b).

Collection and preparation of marked statoliths: After M_2 staining, hatchling squid of each group were collected daily between 09:00 and 10:00 h. Squid were anaesthetized in 2% ethanol and the temperature was lowered to 1–4°C; they were then fixed in 95% ethanol and counted. For the statolith analysis of each group, we selected squid hatched during the day of maximum hatching intensity in that group. Statoliths were removed under the dissecting microscope at a magnification of ×40 and extracted with the tip of a scalpel. The statoliths were placed posterior-side up on a microscope slide and allowed to dry. They were then covered with a drop of Protexx synthetic mountant. The statoliths were not ground or polished, since tetracycline-stained bands are clearly visible under the fluorescent microscope. The statoliths were observed under a Nikon Diaphot 200 light microscope equipped with a 100 W Nikon HB-10101AF mercury lamp and neofluor ×10, ×20, and ×40 lenses. The microscope was connected to a Sony CCD-Iris colour video-camera, which was in turn connected to a video-recorder and a TV monitor. The statolith images were recorded on a video-cassette and analyzed in a personal computer equipped with a video-card using the image-analyzer program NIH Image, Version 1.61. Various measurements were made on the image of each statolith (definitions and terminology of Clarke 1978 and Lipinski et al. 1991). Statolith length (SL) at the level of the fluorescent stained bands M_1 and M_2 was recorded at a magnification of ×200 using neofluor lenses; the distance between the fluorescent bands was measured in 3 statolith areas, the dorsal dome, lateral dome and the rostrum. Measurements were made on the statoliths of 25, 26, 25 and 25 squid from Groups 1, 2, 3 and 4, respectively, and from 29, 27 and 29 squid from Groups 5, 6 and 7, respectively.

Analysis of embryonic statoliths from wild squid populations. Collection, measurements and data analysis were as follows:

Collection of material: Data and statolith collections from previously published studies on *Loligo vulgaris* age and growth were re-examined as well as unpublished data from new statolith collections. From all collections, only aged statoliths with some (n = 4 to 10) embryonic growth increments, clearly visible on the dorsal dome, were selected. This procedure was followed because the growth of the dorsal dome area in the embryonic statolith was highly correlated with the incubation temperature of the embryos under laboratory conditions (see 'Results'). Of the total number of aged statoliths of *L. vulgaris* (n = 582), only 238 (41%) showing clear increments in the dorsal dome region of the embryonic statolith were used in the present study. Statoliths used from previous published studies were: 101 squids ranging from 43 to 353 mm mantle length (ML) from Saharan Bank (Arkhipkin 1995), designated 'Saharan Bank-1'; 30 squids ranging from 65 to 508 mm ML from Saharan Bank (Raya et al. 1999), designated 'Saharan Bank-2'; 43 squids ranging from 92 to 400 mm ML from Galicia, NW Iberian Peninsula, NE Atlantic (Rocha & Guerra 1999). Detailed squid collection data are provided in the relative studies cited. Statoliths from unpublished collections (Table 1) were: 43 *L. vulgaris* (44 to 400 mm ML), collected from February to November 1995 by means of bottom trawling in the Strait of Sicily, Central Mediterranean; 21 *L. vulgaris* (77 to 366 mm ML), collected from September 1991 to April 1992 by bottom trawling and beach-seine in the Thracian Sea, Eastern Mediterranean. Table 1 summarizes the age of the squids examined and Fig. 1 indicates the geographical areas for all squids collected.

Statolith analysis, hatching dates and field temperatures: All statoliths were mounted posterior- or anterior-side up, and were ground and polished on both sides. Growth increments were counted from the natal ring according to techniques described by Arkhipkin 1995, Raya et al. 1999 and Rocha & Guerra 1999. Post-hatching statolith growth increments from all statolith collections were assumed to be daily, as their deposition has been validated by the use of tetracycline, both in the wild for subadult and adult *Loligo vulgaris reynaudii* (Lipinski et al. 1998), and for laboratory cultures of *L. vulgaris* paralarvae (Villanueva 2000a). In this way, the hatching date of each squid was estimated by back-calculation from the date of capture and increment counts. The years of hatching of each squid collection are indicated in Table 1. For each statolith collection, squid were grouped by month of hatching. Data for squid hatched in the same month but in different years were pooled.

The mean increment width (4 to 10 growth increments) was measured on the dorsal dome area of the embryonic statolith, using image-analysis systems
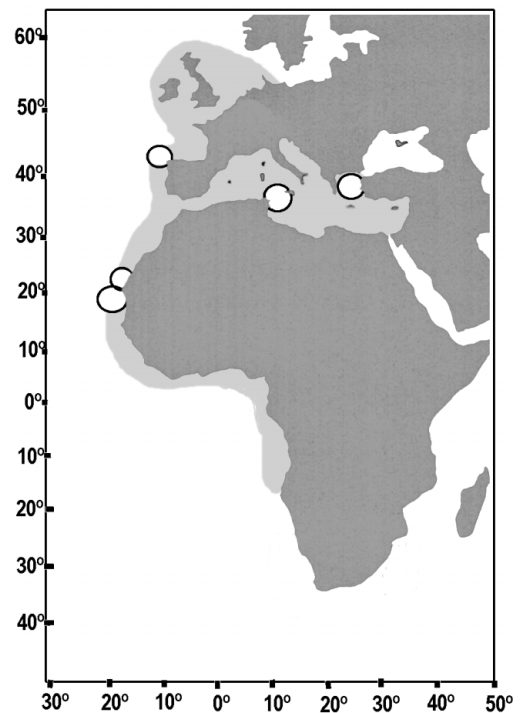

Fig. 1. *Loligo vulgaris*. Map of Atlantic showing distribution and origins of material used during the present study (○)

Table 1. *Loligo vulgaris*. Wild-collected squid from which embryonic statolith increment widths were obtained. For each geographic area, age range of squid (obtained from statolith growth-increment counts), year of hatching (estimated by back-calculation from date of capture and increment counts), number of squid used, and source of the material are indicated

Geographic area	Age range (d)	Hatching years	n	Source
Saharan Bank-1, NE Atlantic	135–342	1984–1987	101	Arkhipkin (1995)
Saharan Bank-2, NE Atlantic	129–249	1992–1993	30	Raya et al. (1999)
NW Iberian Peninsula, NE Atlantic	167–367	1990–1991	43	Rocha & Guerra (1999)
Central Mediterranean (Strait of Sicily)	108–315	1994–1995	43	Unpubl. data
Eastern Mediterranean (Thracian Sea)	137–357	1991–1992	21	Unpubl. data

(×400). The software packages used were VIDS for the statolith collection from the NW Iberian Peninsula (Rocha & Guerra 1999) and NIH Image (see earlier subsection) for the remaining statolith collections. Embryonic growth increments were assumed to be daily for temperatures exceeding 15°C (Villanueva 2000a,b). Mean monthly temperature profiles from 0 to 200 m depth from the Strait of Sicily (37 to 38° N, 12 to 14° E) and the Thracian Sea (40° 20′ N, 23° 38′ E to 41° 10′ N, 25° 58′ E), were obtained from the Mediterranean Oceanic Database (Brasseur et al. 1996) and MEDATLAS (2002). Mean monthly temperature profiles from the Saharan Bank in the upwelling areas of 22.5 to 25.5° N and 15.5 to 17.5° W, and also off Cape Blanco, at 20.5° N, 17.5° W, were obtained from Conkright et al. (1998); mean monthly temperatures from the NW Iberian Peninsula in front of the Ría de Vigo (42° 08.05′ N, 08° 57.5′ W), were obtained from the Centro Oceanográfico de Vigo, Instituto Español de Oceanografía database for the period 1994 to 2000.

Data treatment: Growth rates were calculated using the instantaneous growth coefficient (G) obtained from the equation $G = (\log_e Y_2 - \log_e Y_1) \times (t_2 - t_1)^{-1}$, where Y_2 = final length, Y_1 = initial length; t_1 = age in days at Y_1, and t_2 = age in days at Y_2. The relative growth rate (daily percentage gain in length) was calculated by multiplying G by 100. For comparison of the tetracycline-stained statoliths, values were transformed to meet the requirements of normality (Shapiro-Wilk method), after log-transformation of length data ($\mu m\ d^{-1}$) and arcsine-transformation of growth rate data ($\%\ d^{-1}$). Data were compared by Student's t-tests and ANOVA followed by the Tukey-Kramer HSD test, at 0.05 significance level. Tests were done using the JMP statistical package.

RESULTS

Embryonic statolith growth under laboratory conditions

Normal hatching success was observed in all groups of *Loligo vulgaris* embryos except for the group incubated at the higher temperature (24.7°C), where total mortality was observed in 11 egg capsules (73% of the total), and embryos from the remaining 4 egg capsules hatched prematurely, while still posessing large outer yolk sacs. After the first staining, growth occurred in all statoliths between the fluorescent stained bands M_1 and M_2. Incubation temperature clearly influenced the statolith growth, and this variable was strongly and positively correlated with all measurements recorded in the statoliths (Student's-t, p < 0.00001). A wide range of statolith growth rates was observed, depending on the incubation temperature (Table 2, Figs. 2 & 3). As a consequence, the growth in total statolith length of the embryos incubated at 23.5°C was more than 7 times that of embryos incubated at the lower temperature ($\mu m\ d^{-1}$). Moreover, 24.7°C squid had statolith growth rates ($\%\ d^{-1}$) that were 13 times greater than those of 12.0°C squid (Table 2). It should be noted that the growth as percentage per day ($\%\ d^{-1}$) for the statoliths incubated at temperatures of 12.0, 15.5 and 21.1°C was lower (Fig. 2b), the reason being that these groups of embryos were incubated at a higher temperature (22.3°C) before the first tetracycline staining (Villanueva 2000b), in comparison with the groups from Egg Masses A and B (incubated, respectively, at 15.6 and 20.2°C before the first staining). Therefore, statolith length at M_1 was longer at this time in the statoliths incubated by Villanueva (2000b). However, this difference was not observed for growth expressed as micrometers ($\mu m\ d^{-1}$) for the same statoliths, as embryonic statolith growth was fairly constant (Fig. 2a). As expected, mean growth in all statoliths was greater in the rostrum and least in the lateral dome. The highest correlation between incubation temperature and statolith growth was obtained for the dorsal dome region. In this region, growth rates differed by up to 7 times between the 2 most extreme temperatures used for
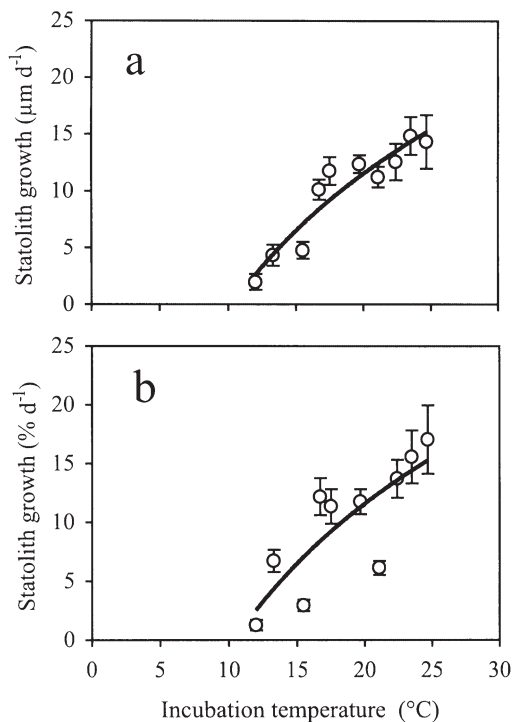

Fig. 2. *Loligo vulgaris*. Mean (±SD) daily statolith growth (a) in length and (b) in percentage, of embryos incubated under laboratory conditions at temperatures ranging from 12.0 to 24.7°C. Growth was recorded between 2 consecutive tetracycline marks. Logarithmic trend is indicated for each plot

Table 2. *Loligo vulgaris.* Mean ± SD (range) of daily growth in length of embryonic statoliths squid incubated in laboratory. Groups (G) 1–4 represent Egg Mass A, G5–7 represent Egg Mass B. Data for 12.0, 15.5 and 21.1°C are from Villanueva (2000b). Measurements shown for 3 statolith zones (dorsal dome, lateral dome, rostrum) and for total statolith length. Length was measured between 2 consecutive tetracycline marks; time elapsed between marks was 7 d, except for G1 and 3 (13.3 and 19.7°C), where time elapsed was 14 and 13 d, respectively. Means with same superscript letter for same measurement were not statistically different (p > 0.05)

| Incubation temp. (°C)	n	Dorsal dome (μm d^{-1})	Lateral dome (μm d^{-1})	Rostrum (μm d^{-1})	Statolith length (μm d^{-1})	($\%$ d^{-1})
12.0	26	0.9 ± 0.2^a (0.6–1.3)	0.8 ± 0.2^a (0.3–1.1)	1.4 ± 0.3^a (0.8–2.2)	2.0 ± 0.7^a (0.5–3.5)	1.3 ± 0.4^a (0.3–2.3)
13.3 (G1)	25	1.8 ± 0.5^b (1.2–3.6)	1.4 ± 0.3^b (0.9–1.9)	2.3 ± 0.5^b (1.1–3.7)	4.3 ± 0.9^b (2.2–7.3)	6.7 ± 0.9^b (4.3–9.1)
15.5	26	2.2 ± 0.4^c (1.3–3.1)	1.5 ± 0.3^b (0.8–2.1)	3.5 ± 0.5^c (2.6–4.5)	4.8 ± 0.8^b (3.3–6.2)	3.0 ± 0.5^c (2.2–4.1)
16.7 (G5)	29	3.9 ± 0.5^d (3.2–5.1)	2.7 ± 0.4^c (1.8–3.5)	5.4 ± 0.7^d (5.5–8.7)	10.1 ± 0.9^c (8.6–11.4)	12.2 ± 1.6^d (9.3–15.0)
17.5 (G2)	25	5.0 ± 0.5^e (4.3–6.2)	3.9 ± 0.6^d (3.0–5.4)	7.1 ± 1.0^e (5.2–8.5)	11.8 ± 1.2^c (8.6–14.1)	11.3 ± 1.5^d (8.0–13.6)
19.7 (G3)	25	4.7 ± 0.5^e (3.6–5.7)	3.6 ± 0.3^d (3.1–4.2)	7.6 ± 0.8^e (5.5–8.7)	12.4 ± 0.8^d (10.5–13.5)	11.8 ± 1.1^d (9.3–13.3)
21.1	25	4.4 ± 0.3^d (3.8–4.9)	2.8 ± 0.5^c (1.7–3.5)	7.0 ± 0.9^e (4.9–8.2)	11.2 ± 0.9^c (8.5–12.3)	6.1 ± 0.6^b (4.6–7.2)
22.4 (G6)	27	5.3 ± 0.8^e (3.9–7.1)	3.7 ± 0.4^d (2.8–4.4)	7.1 ± 1.1^e (5.0–9.5)	12.6 ± 1.6^d (10.0–16.7)	13.7 ± 1.6^e (10.6–17.0)
23.5 (G7)	29	5.9 ± 0.7^f (3.8–7.0)	4.0 ± 0.6^d (2.6–5.0)	7.9 ± 1.3^e (4.8–10.4)	14.9 ± 1.7^e (10.5–18.0)	15.6 ± 2.2^f (12.4–20.6)
24.7 (G4)	25	6.4 ± 1.3^f (3.8–9.3)	5.0 ± 1.1^e (2.3–6.7)	8.2 ± 1.7^e (4.9–13.1)	14.4 ± 2.4^d (9.2–18.8)	17.0 ± 2.9^g (11.4–22.6)
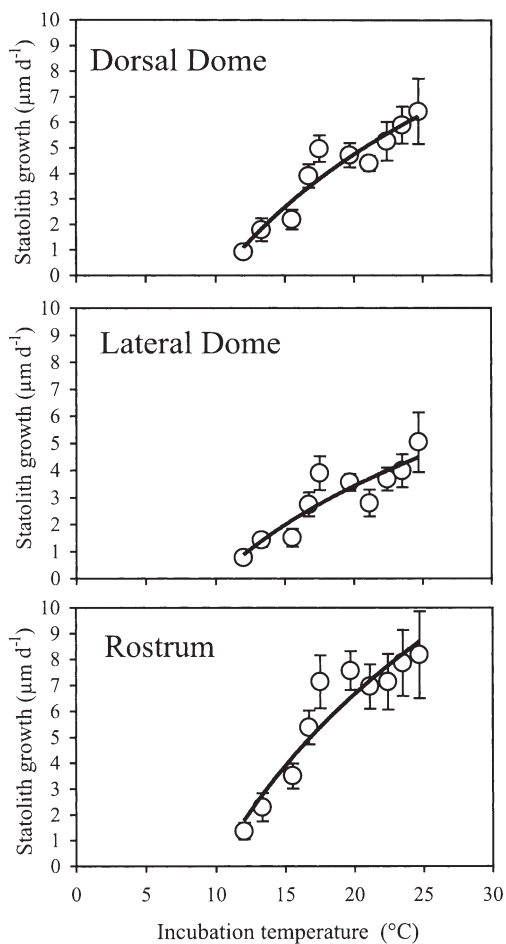
Fig. 3. *Loligo vulgaris.* Mean (±SD) daily statolith growth in 3 areas of statolith of embryos incubated under laboratory conditions at temperatures ranging from 12.0 to 24.7°C. Growth was recorded between 2 consecutive tetracycline marks. Logarithmic trend is indicated for each plot

incubation (12 and 24.7°C: Table 2). The logarithmic function fitted this temperature versus growth relationship well for all measurements obtained (equations in Table 3).

Embryonic statolith of wild squid and temperatures inferred for spawning sites

The width of the embryonic growth increments of *Loligo vulgaris* ranged from 0.97 µm for squid hatched during April and collected off the NW Iberian Peninsula, to 3.64 µm for squid hatched in September and collected from the Central Mediterranean. Table 4 shows the embryonic increment widths for all geographic areas for each month of hatching. No differences in the mean increment width were found between sexes (*t*-test, p > 0.6). In general, embryonic increment widths of Mediterranean squid were wider than those of Atlantic squid. To enable comparisons between the mean increment width of the statoliths from the different collections, data from all individuals for each collection were pooled ('All' in Table 4). The 5 collections differed in mean increment width (ANOVA, p < 0.0001). However, no differences were found between the 2 Saharan collections or the 2 Mediterranean collections (p > 0.3). The NW Iberian Peninsula collection differed from the others (p < 0.05), having lower mean increment widths. The mean width for all statoliths from the Saharan Bank was 1.90 µm (±0.33 SD), and for all the Mediterranean statoliths was 2.31 µm (± 0.39 SD). No differences were found (p > 0.2) between increment widths in the cold (winter-spring) versus warm (summer-autumn) months of the year for each collection, except for the Eastern Mediterranean group (p < 0.05).
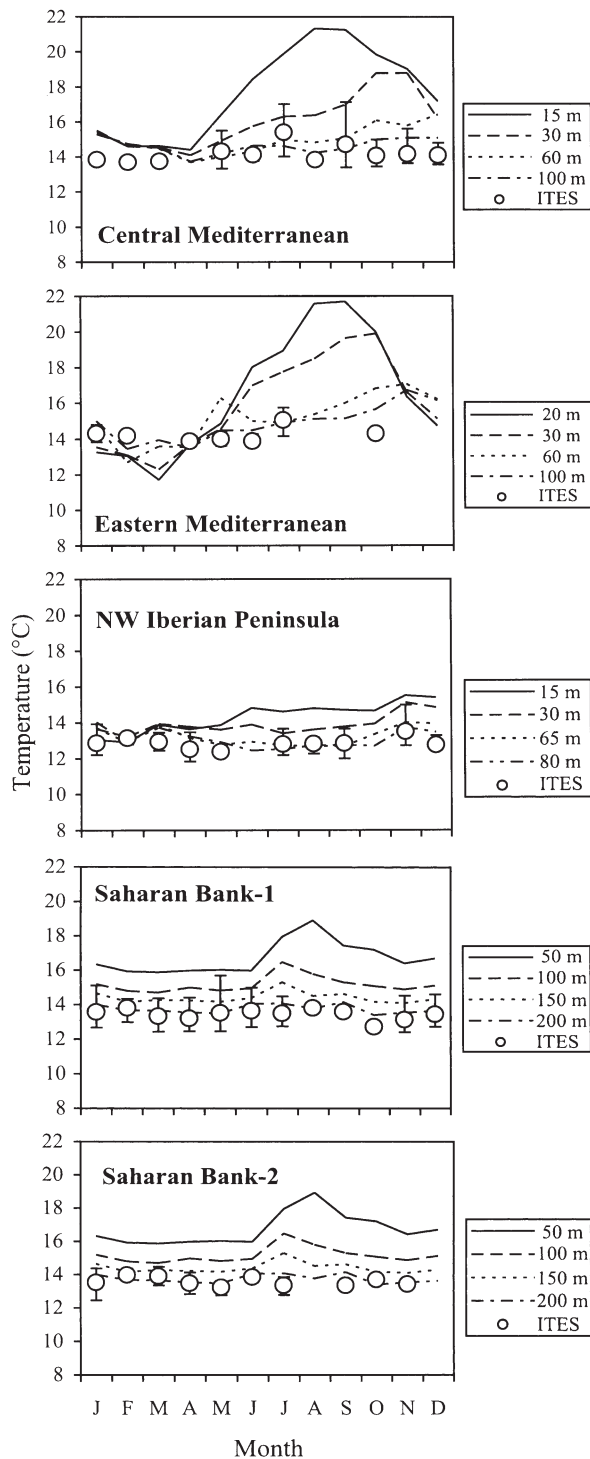
Fig. 4. *Loligo vulgaris*. Temperatures inferred from embryonic statolith (ITES) on a monthly basis for statolith collections of wild squid from Central and Eastern Mediterranean, NW Iberian Peninsula, and 2 collections from Saharan Bank. Bars indicate minimum and maximum ranges. Mean monthly temperature profiles at sea, for different depths (in m), are also indicated for the respective areas. Temperature at sea for Saharan Bank corresponds to that in the area of Cape Blanco (20.5° N, 17.5° W)

The temperatures inferred from the increment width, using the statolith growth equation obtained in the laboratory (Eq. 3 in Table 3) represent the estimated incubation temperatures at sea, ranging from 11.8°C for the NW Iberian Peninsula squid hatched during April to 17.1°C for the Central Mediterranean squid hatched in September (Table 5). The mean inferred temperature for all statoliths from the Saharan Bank was 13.5°C (±0.6 SD), and for all Mediterranean statoliths it was 14.3°C (±0.8 SD). Comparisons between the estimated and the observed seawater temperatures in different months and depths for all geographical areas investigated were also made (Fig. 4).

DISCUSSION

Embryonic statoliths and spawning areas

The present results indicate that embryonic statolith growth under laboratory conditions is dependent on the incubation temperature, which directly affects both metabolic rates and growth in squids (O'Dor & Wells 1987). The temperature inferred from the embryonic increment widths of statoliths indicates that embryonic development of *Loligo vulgaris* in the geographic regions sampled occurs at temperatures from 12 to 17°C. Therefore, at sea, these squid select a temperature range to spawn, avoiding high temperatures. As spawning takes place throughout the year, the depth selected to spawn changes as a function of the ambient temperature. Villa et al. (1997) corroborated our laboratory results with field data. Based on monthly egg sampling and associated hydrographic parameters throughout the year on the south coast of Portugal, egg masses of *L. vulgaris* were recorded between temperatures of 13 and 19°C. The Portuguese eggs were collected at depths between 2 and 35 m (mostly between 20 and 30 m) by SCUBA diving (Villa et al. 1997). These field results indicated that the temperatures inferred from embryonic statolith analysis follow the temperature ranges recorded at sea. As mentioned earlier, Mediterranean squid have wider embryonic increments than Atlantic squid, indicating slightly higher sea temperatures during the development of the egg masses for the former. This difference may be related to the fact that the shelf waters of Mediterraean Sea are warmer than the coastal Atlantic regions sampled during the present study (Brasseur et al. 1996, Conkright et al. 1998).

For the Saharan Bank area, the inferred incubation temperatures indicate the possible existence of spawning areas of *Loligo vulgaris* at or below 200 m depth (a depth that may be close to the limit for the species). Our results inferred from statoliths may thus be under-

Table 3. *Loligo vulgaris*. Growth equations for embryonic statoliths of squid incubated under laboratory conditions at temperatures from 12.0 to 24.7°C. Length measured between 2 consecutive tetracycline marks. Growth equations were logarithmic: $y = b \ln(x) - a$. SG: statolith growth (μm or % d^{-1}); T: temperature (°C); SE: standard error of b; n: number of hatchling squid for which statoliths were measured

Equation no.	Equation	r^2	SE	n
Statolith total length				
(1) Growth (μm d^{-1})	SG = 17.450 ln(T) − 40.768	0.891	0.031	263
(2) Growth (% d^{-1})	SG = 17.650 ln(T) − 41.302	0.654	0.050	263
Statolith zones				
(3) Dorsal dome (μm d^{-1})	SG = 7.1673 ln(T) − 16.730	0.908	0.013	263
(4) Lateral dome (μm d^{-1})	SG = 4.9834 ln(T) − 11.507	0.812	0.012	263
(5) Rostrum (μm d^{-1})	SG = 9.5911 ln(T) − 22.067	0.891	0.020	263

estimates. Despite the heavy fishing effort in the area (Balguerías et al. 2000), no spawning areas or egg masses of *L. vulgaris* have been collected or reported for the Saharan Bank. It is therefore difficult to check the theoretical results obtained in the present study. The hatching peak of *L. vulgaris* from the Saharan Bank has been suggested to occur in the month of May (Raya et al. 1999), indicating that embryonic development occurs mainly in April. On the Saharan Bank (25° 37' N, 14° 51' W) during April over a bottom depth of 50 m, the water temperature (at 40 m depth) was 13.3°C; over a bottom depth of 70 m, the temperature (at 50 m depth) was 13.6°C (Manríquez et al. 1976). These temperatures match our results for the temperatures inferred from embryonic statoliths incubated in April and for both statolith collections examined from that area (Table 5, Fig. 4). Further research is needed to confirm these possible spawning areas. In this connection, the existence of deep spawning at depths of 115 to 125 m has been recorded for *L. v. reynaudii*, indicating that spawning occurs in 2 contrasting bottom environments, the warm inshore and cold mid-shelf off the south coast of South Africa (Roberts & Sauer 1994, Roberts et al. 2002). Guerra & Rocha (1994) indicate that the spawning season for *L. vulgaris* off the NW Iberian Peninsula can continue throughout the year, but mainly falls between December and April, with a peak in February. These authors reported 3 egg masses collected in January at depths of 30 to 65 m, and an egg mass collected in June at 15 m. The temperature inferred from NW Iberian Peninsula statoliths indicates that peak spawning can take place in relatively shallow water during the period of water-mixing. Some squid may spawn deeper than 30 m during the following warmer months (Fig. 4). We have no data with which to compare the egg mass reported for June.

It is known that light influences the hatching period in *Loligo vulgaris* (Paulij et al. 1990). Light may influence embryonic statolith growth according to different bottom environments, as light intensity decreases exponentially with depth (Jerlov 1968). Light and photoperiod effects on embryonic squid statoliths are practically unknown, and should be studied in the future. For example, in embryos of Atlantic salmon *Salmo salar*, the effect of photoperiod has been known to influence the rate of increment deposition of their otoliths (Geffen 1983). However, Jackson et al. (1993) found a ratio of 1 increment d^{-1} in *Sepioteuthis lessoniana* squid reared under constant 24 h d^{-1} lighting throughout their entire life span, indicating a possible major endogenous component in the rate of post-hatching statolith growth. It should be taken into account that during the present study all incubations in the laboratory were done under constant, low-intensity illumination. Additional sources of variation are also suspected, as irregularities were found during the present study at the extreme egg incubation temperatures of 12 and 24.7°C. These temperatures seem to be unusual for *L. vulgaris* egg incubation at sea, producing irregular statolith growth or low embryo survival in the laboratory. The low incubation temperature of 12°C produced a less-than-daily increment deposition rate in embryonic statoliths of *L. vulgaris* (Villanueva 2000b). This effect could be a source of error when inferring low temperatures, as we assumed a daily deposition rate when counting and measuring all statolith increment widths from wild squid. Loliginid embryos are very sensitive to temperature, and for *L. v. reynaudii*, the occurrence of morphological abnormalities in embryos increases to 50% at laboratory incubation temperatures of 9 and 21°C (Oosthuizen et al. 2002).

Embryonic life and paralarval variability

Temperature is the main factor that regulates the duration of embryonic development in cephalopods (von Boletzky 1987). For *Loligo vulgaris*, differences in this duration can be up to 50 d (the incubation period in the laboratory ranges from 70 d at 10°C to 25 d at 20°C; von Boletzky 1974). The temperatures inferred at sea from embryonic statolith growth during the present study ranged from 11.8°C for squid hatched during April and collected off the NW Iberian Peninsula, to 17.1°C for the squid hatched in September and collected from the Central Mediterranean. Using the relationship between temperature and duration of embryonic development in *L. vulgaris* provided by von

Table 4. *Loligo vulgaris*. Width (µm) of embryonic growth increments in dorsal dome region of the statolith grouped by month of hatching, for wild squid collected from different geographic areas

Month	Saharan Bank-1			Saharan Bank-2			NW Iberian Peninsula			Central Mediterranean			Eastern Mediterranean		
	Mean ± SD	(Range)	n	Mean ± SD	(Range)	n	Mean ± SD	(Range)	n	Mean ± SD	(Range)	n	Mean ± SD	(Range)	n
Jan	1.95 ± 0.29	(1.48–2.73)	18	1.93 ± 0.42	(1.35–2.27)	4	1.56 ± 0.39	(1.20–2.15)	8	2.1	–	1	2.34	(2.10–2.57)	2
Feb	2.08 ± 0.24	(1.65–2.36)	7	2.16 ± 0.18	(2.05–2.37)	3	1.73 ± 0.17	(1.53–1.91)	4	2.03	–	1	2.28	–	1
Mar	1.82 ± 0.33	(1.33–2.36)	15	2.13	(1.84–2.42)	2	1.61 ± 0.23	(1.33–1.89)	7	2.05	–	1	0	–	–
Apr	1.76 ± 0.31	(1.35–2.40)	12	1.9	(1.55–2.25)	2	1.37 ± 0.34	(0.97–1.90)	5	0	–	–	2.12	(2.05–2.19)	2
May	1.91 ± 0.68	(1.35–3.00)	5	1.77 ± 0.23	(1.52–1.94)	3	1.29	–	1	2.33 ± 0.54	(1.84–2.91)	3	2.18 ± 0.16	(1.98–2.38)	6
Jun	1.99 ± 0.37	(1.48–2.67)	13	2.11 ± 0.17	(1.94–2.27)	3	0	–	–	2.25 ± 0.16	(2.06–2.36)	3	2.12 ± 0.15	(1.97–2.33)	5
Jul	1.91 ± 0.32	(1.52–2.43)	9	1.84 ± 0.24	(1.53–2.10)	6	1.53 ± 0.31	(1.19–2.00)	6	2.85 ± 0.60	(2.20–3.58)	4	2.70 ± 0.33	(2.26–3.03)	4
Aug	2.09		1	0		–	1.54 ± 0.28	(1.22–1.73)	3	2.10 ± 0.13	(2.00–2.30)	4	0	–	–
Sep	1.97		1	1.84 ± 0.12	(1.71–1.94)	3	1.54		2	2.52 ± 0.58	(1.88–3.64)	8	0	–	–
Oct	1.49	(1.34–1.65)	2	2.03	(1.88–2.18)	2	0	–	–	2.22 ± 0.29	(1.91–2.68)	8	2.34	–	1
Nov	1.72 ± 0.41	(1.32–2.45)	7	1.88	(1.84–1.93)	2	1.89 ± 0.67	(1.50–2.67)	3	2.26 ± 0.48	(2.00–2.97)	4	0	–	–
Dec	1.89 ± 0.36	(1.50–2.48)	11	0	–	–	1.51 ± 0.22	(1.33–1.81)	4	2.23 ± 0.25	(1.97–2.59)	6	0	–	–
All	1.89 ± 0.35	(1.32–3.00)	101	1.94 ± 0.26	(1.35–2.42)	30	1.57 ± 0.34	(0.97–2.67)	43	2.33 ± 0.43	(1.84–3.64)	43	2.28 ± 0.29	(1.97–3.03)	21

Table 5. Inferred temperature (°C) at sea during embryonic development of wild *Loligo vulgaris* collected from different geographic areas. Data for each month of hatching inferred from the relationship between incubation temperature in laboratory and width of the embryonic growth increments in the dorsal dome region of the statolith (see Eq. 3 in Table 3)

Month	Saharan Bank-1			Saharan Bank-2			NW Iberian Peninsula			Central Mediterranean			Eastern Mediterranean		
	Mean ± SD	(Range)	n	Mean ± SD	(Range)	n	Mean ± SD	(Range)	n	Mean ± SD	(Range)	n	Mean ± SD	(Range)	n
Jan	13.6 ± 0.6	12.7–15.1	18	13.5 ± 0.8	12.5–14.4	4	12.8 ± 0.7	12.2–13.9	8	13.8	–	1	14.3	13.8–14.8	2
Feb	13.8 ± 0.4	13.0–14.3	7	14.0 ± 0.4	13.7–14.4	3	13.1 ± 0.3	12.8–13.5	4	13.7	–	1	14.2	–	1
Mar	13.3 ± 0.6	12.4–14.4	15	13.9	13.3–14.5	2	12.9 ± 0.4	12.4–13.4	7	13.7	–	1	0	–	–
Apr	13.2 ± 0.6	12.5–14.4	12	13.5	12.8–14.1	2	12.5 ± 0.6	11.8–13.5	5	0	–	–	13.9	13.7–14.0	2
May	13.5 ± 1.3	12.5–15.7	5	13.2 ± 0.4	12.8–13.5	3	12.4	–	1	14.3 ± 1.1	13.4–15.5	3	14.0 ± 0.3	13.6–14.4	6
Jun	13.6 ± 0.7	12.7–15.0	13	13.9 ± 0.3	13.5–14.2	3	0	–	–	14.1 ± 0.3	13.8–14.3	3	13.9 ± 0.3	13.6–14.3	5
Jul	13.5 ± 0.6	12.8–14.5	9	13.3 ± 0.4	12.8–13.8	6	12.8 ± 0.6	12.2–13.6	6	15.4 ± 1.3	14.0–17.0	4	15.1 ± 0.7	14.2–15.8	4
Aug	13.8		1	0		–	12.8 ± 0.5	12.2–13.1	3	13.8 ± 0.3	13.6–14.2	4	0	–	–
Sep	13.6		1	13.3 ± 0.2	13.1–13.5	3	12.8		2	14.7 ± 1.2	13.4–17.1	8	0	–	–
Oct	12.7		2	13.7	13.4–14.0	2	0	–	–	14.1 ± 0.6	13.5–15.0	8	14.3	–	1
Nov	13.1 ± 0.8	12.4–14.5	7	13.4	13.3–13.5	2	13.5 ± 1.3	12.7–15.0	3	14.2 ± 1.0	13.7–15.6	4	0	–	–
Dec	13.5 ± 0.7	12.7–14.6	11	0	–	–	12.7 ± 0.4	12.4–13.3	4	14.1 ± 0.5	13.6–14.8	6	0	–	–
All	13.5 ± 0.7	12.4–15.7	101	13.5 ± 0.5	12.5–14.5	30	12.9 ± 0.6	11.8–15.0	43	14.3 ± 0.9	13.4–17.1	43	14.2 ± 0.6	13.6–15.8	21

Boletzky (1987), it can be estimated that the duration of the embryonic development for both extreme temperatures inferred from statoliths ranged from 55 to 30 d, respectively. Likewise, the mean inferred temperature for all statoliths from the NW Iberian Peninsula (12.9°C) and the Mediterranean (14.3°C) gave estimates of mean duration of embryonic periods of 47 and 40 d, respectively. These estimates indicate that the NW Iberian Peninsula egg masses remained at sea 1 wk longer (18%) than the Mediterranean egg masses (nearly 1 mo longer [83%] when comparing minimum and maximum ranges).

The differing durations of the incubation periods of *Loligo vulgaris* in different geographic areas is a source of paralarval variation and differentiated recruitment. Loliginid squids do not care for their eggs after spawning, and egg masses attached to a substratum and remaining for a long period at sea are exposed to 2 major risks. First is the probability of encountering strong periods of turbulence. Moving sand after periods of strong wind has been observed to cover egg masses of *L. v. reynaudii.* Large egg beds of the same species can be detached and presumably displaced by strong turbulence (Sauer et al. 1993). A second risk is predation. Polychaetes of the families Capitellidae, Syllidae, Lumbrinereidae and Sabellidae have been observed on the egg masses of *L. vulgaris*. They do not prey on the embryos, but eat the gelatinous mass, swelling the structure and making perforations which are used by Nematoda, Harpacticoidea and Ciliata (von Boletzky & Dohle 1967). Predation by sea stars *Patiria* sp. and sea urchins *Lytechinus* sp. on egg masses of *Loligo opalescens* (Hixon 1983) and predation by scyliorhinid sharks *Poroderma pantherinum* and sparid fish *Spondyliosoma emarginatum* on egg masses of *L. v. reynaudii* (Sauer & Smale 1991, 1993) has been reported. In addition, the impact of fishing damage to egg beds has been described for *L. v. reynaudii* (Sauer 1995), and egg masses of *L. forbesi* are incidentally caught in demersal trawls (Lordan & Casey 1999, Salman & Laptikhovsky 2002).

Conversely, slow development at a lower temperature can improve yolk conversion in cephalopods producing larger hatchlings, as occurs in the cuttlefish *Sepia officinalis* (Bouchaud 1991) and larval fishes (Blaxter 1992). Embryonic incubation in the laboratory at cool temperatures (mean 12.2°C) produced *Loligo vulgaris* hatchlings that were significantly heavier (8%) and longer (7%) than embryos incubated at warmer temperatures (mean 19.5°C) (Villanueva 2000a). Larger *L. vulgaris* hatchlings probably have an initial competitive advantage due to their greater swimming power, which may enhance food-searching and prey-capture capacities (Packard 1969, Chen et al. 1996, Villanueva et al. 1996), making them less vulnerable to small

predators. Thus, a compromise between the risks of long versus short embryonic incubation duration, and the resulting hatchling size and hatchling competence, probably exists. From the results of the present study on inferred differential incubation temperatures, Atlantic *L. vulgaris* hatchlings would be expected to be larger in size than those from the Mediterranean. Future studies are necessary to test this hypothesis and to investigate the respective advantages for each paralarval population by determining what other developmental factors may be involved. As an example, in hatchlings of the lolignid squid *Sepioteuthis lessoniana* originating from maternally identical egg strings, incubated under constant temperature in laboratory, body size and statolith size are larger in hatchlings hatching later, with embryonic duration ranging from 22 to 27 d (Ikeda et al. 1999). During the present study, for each region and month, egg masses of *L. vulgaris* developed in a relatively small range of water temperature. However, in some extreme cases, differences in the inferred temperature for the same month can be up to 3.2°C (e.g. May, Saharan Bank-1) and 3.7°C (e.g. September, Central Mediterranean: Table 5, Fig. 4). In such extreme cases, paralarval squid with a different embryonic developmental history and a different degree of competence at hatching can co-exist in the same squid recruitment area. The dynamics and degree of co-existence of loliginid hatchlings with different embryonic developmental histories is an unexplored field that needs further research.

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