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Comparison of fluorescent bands during staining of statoliths of the squid *Loligo forbesi* (Cephalopoda: Loliginidae)

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The statoliths of squid show growth rings that are commonly used for direct age estimation.[1] Although a hypothesis of daily ring deposition has been widely assumed and disseminated in squid growth studies,[2,3,4] some controversy persists, as controlled experimental validation studies are still lacking.

For validation studies of ageing, several methods have been used to produce marks in the ageing structures. Among the panoply of methods applied for otolith marking in fishes ([5]for a review) induced chemical markers, especially fluorescent compounds, have been widely used. The marks produced by these compounds remain detectable for a variable period after application.[6] In age validation studies of squid some of these fluorescent compounds have been used in a variety of ways (administration methods and concentrations—Table 1). However, comparison of results produced by the different markers in statoliths of squids have never been carried out. The ageing of *Loligo forbesi* using statoliths has been based on non-validated criteria,[2,7] with the assumption of the daily increments.

A total of 16 adult and sub-adult squid *Loligo forbesi* were caught by hand jigging near Faial Island, Azores, at depths of 200–300 m. The animals were kept in a floating cage, in Horta harbour. Each individual was measured (dorsal mantle length, DML −mm) and individually identified by a plastic T-tag placed in the antero-dorsal part of the mantle muscle. The compounds OTC (Terramicina-100 ®Pfizer), AC (®Sigma A-3882) and CA (®Sigma C-0875) were injected on the squid antero-lateral mantle muscle in two experiments, using a 5 ml syringe.

The first experiment was made during May of 1993 using 8 individuals (Table 2), which were injected with OTC at concentrations ranging from 0.5 to 10 $mg.ml^{-1}$. In the second experiment carried out on April of 1994 (Table 2), 3 individuals were injected with OTC (0.1–0.5 $mg.ml^{-1}$), 2 with AC (0.1–0.25 $g.ml^{-1}$) and 3 with CA (0.1–0.5 mg/ml^{-1}).

After death of the squid (all the animals died, rather than being killed experimentally), the statoliths were removed and, after cleaning, were mounted, ground and polished on both sides, using the method described previously.[22] Each slide was wrapped in aluminium foil to avoid loss of fluorescence. Statoliths were viewed under UV light, using ®Olympus BH2 microscope with blue and green filters, under a 200 × magnification. The sex and the maturity stage were also assessed.[23]

Relationships between survival (in days) and compound concentration and between survival and DML were analysed using Spearman rank correlations. Correlations are significant at $P \leqslant 0.05$([24]).

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Table 1. Summary of methods used for staining statoliths and otoliths of Teuthoidea and fish species, respectively.

Chemical Compounds	Concentrations	Methods	Species, References
TC	250 mg/l of sw	**Im** – in the solution for 2 h.	*Lolliguncula brevis*, [3]
TC	250 mg/l of sw	**Im** – in the solution for 2 h.	*Idiosepius pygmaeus*, [8]
TC	6 mg/ml of sw	**Ij** – at the base of arm I	*Loligo chinensis, 9*
TC	250 mg/l of sw	**Im** – in the solution for 2 h.	*Loliolus noctiluca, 9*
OTC	0.1 ml dw	**Ij** – itm, ventral mantle musculature	*Loligo vulgaris reynaudii, 10*
OTC	0.5 ml / 1.5 ml of sw	**Ig** – squid forced-fed the solution	*Illex illecebrosus, 11, 12*
OTC	25 e 50 mg/kg of bw	**Ij** – itm between lateral line-dorsal fin	*Paralichthys dentatus*, 13*
OTC	100 mg/ml of sw	**Ij** itm. ventrolateral proximal region (mantle)	*Alloteuthis subulata, 14*
OTC HCl	75 mg	**F** – shrimp stuffed	*Illex illecebrosus, 12, 13*
OTC HCl	250 mg/l of sw	**Im** – in the solution for 2 h	*Onychoteuthis borealijaponica, 14*
TC HCl	75 mg	**F** – cooked shrimp stuffed	*Illex illecebrosus, 16*
TC HCl	20 g/l of dw	**F** – fed twice fillets (im. in TC overnight)	*Todarodes pacificus, 17*
TC HCl	500 mg/l of asw	**Im** – in the solution for 2 h	*Tautogolabrus adspersus*, 18*
CTC	10 mg/2 ml of dw	**Ij** – itm. ventrolateral proximal region (mantle)	*Alloteuthis subulata, 14*
Ca + TC	100 mg/l and 250 mg/l (of sw)	**Im** – 1st bath of CA 1.5h; 11 days later 2nd bath of TC 2h.	*Sepioteuthis lessoniana, 19*
CA	100–200 mg/l of sw	**Im** – in the solution for 2–4 h.	*Leiostomus xanthurus* Cynoscion nebulosus*, 20*
CA	25–50 mg/kg of bw	**Ij** – itm. between lateral line – dorsal fin	*Paralichthys dentatus, 13*
AC	2 g/l of dw	**F** – fed twice fillets (im. in AC)	*Todarodes pacificus, 17*
Strontium Chloride	1.2 g/ml of dw	**F** – shrimp cooked and soaked for 24h in the solution	*Illex illecebrosus, 9, 11, 16, 21*

Notes: **AC** (alizarine complexone); **asw** (artificial seawater); **bw** (body weight); **CA** (calceine); **CTC** (clortetracycline); **dw** (distilled water); **F** (food); **Ig** (ingestion); **Ij** (injection); **Im** (immersion); **itm** (intramuscular); **OTC** (oxytetracycline); **OTC HCl**(oxytetracycline hydrochloride); **sw** seawater; **TC** (tetracycline); **TC HCl** (tetracycline hydrochloride); * (fish species).

The band classification was established qualitatively in four categories based on the increasing visibility of the fluorescent bands: 0— weak (bands hardly visible and only in restricted areas of the statolith), 1—sufficient/weak (fluorescent bands smudged, difficulties in determining their width), 2—sufficient (fluorescent bands well defined but irregular at places), 3—good (bands well defined in the entire statolith circumference).

The best fluorescent bands and higher survival were produced by OTC and AC at the lowest concentrations. The fluorescent rings were well outlined around the entire edge of the statolith, allowing a good visibility but not bright. The quality of the bands was similar when using these compounds, for concentrations varying between 0.1 and 0.5 $mg.ml^{-1}$ (Table 2). For concentrations of OTC higher than 1.0 $mg.ml^{-1}$, the bands produced were too thick and too bright, which resulted in unreadable statoliths (Table 2). The statoliths stained with CA showed quite narrow bands with insufficient fluorescence, irrespective of concentrations used (Table 2).

The results obtained in this study also show that slight variations in statolith preparation for analysis can determine the visibility and quality of the fluorescent bands. In fact, some fluorescence can spread both through the *rostrum* and the resin, also resulting in unreadable statoliths. Special care must be taken to avoid this.

The survival increased from 4–5 days, in the first experiment, up to 18 days in the second (Table 2). The survival period increased with the decreasing compound concentration (Table 2). In fact, a significant correlation has been calculated between these two variables ($r = -0.8006$, $P < 0.0001$). However, a non significant correlation has been calculated between survival period and DML ($r = 0.1852$, $P > 0.5$). The low survival of animals could also have been due to a variety of other factors relating to capture, transport, tagging, collision of swimming

Table 2. Results of staining statoliths of *Loligo forbesi*.

DML (mm)	Sex / Maturity	Compound – Concentr. (mg/ml)	Survival (days)	Bands Classification	Bands Quality
1ST Experiment					
284	M / V	OTC – 0.5	4–5	3	Well defined
306	F / V	OTC – 1.0	"	2	
364	M / V	OTC – 1.0	"	2	Bright
345	F / V	OTC – 2.0 " 2			
430	M / V	OTC – 5.0	2–4	1	
270	F / V	OTC – 5.0	"	1	Too
455	M / V	OTC – 10.0	"	1	bright
610	M / V	OTC – 10.0	"	1	
2ND Experiment					
304	F / V	OTC – 0.5	1	3	Well
310	F / V	OTC – 0.25	8	3	defined
330	F / V	OTC – 0.1	8	3	
530	M / V	AC – 0.25	18	3	Well
290	F / ?	Ac – 0.1	16	3	defined
557	M / V	Ca – 0.5	11	0	Very
460	M / III	CA – 0.25	13	0	narrow
360	M / III	CA – 0.1	17	0	

Notes: **Ac** – alizarine complexone; **CA** – Calceine; **DML** – Dorsal mantle length; **F** – female; **M** – male; **OTC** – Oxytetracycline; **III** – maturing stage; **V** – mature stage; **(?)** – maturity stage unknown due to cannibalism during captivity; **3** – Sufficient/good; **2** – Sufficient; **1** – Sufficient/weak; **0** – weak.

squid with cage walls and agonistic interaction between squid.

In statoliths of individuals surviving for longer (from 16 to 18 days in captivity) it would be expected to find growth rings beyond the fluorescent band, indicating that some growth occurred after the administration of the marking compounds. However, this was not observed. This fact can be explained either by an improper method of preparation or the slower growth rate of the individuals. Thus, the rings close to the edge of the *rostrum* would be more difficult to identify.

In future studies experimental validation of daily ring deposition of *L. forbesi* should be made in juveniles and using OTC and AC as fluorescent markers at low concentrations. A less harmful method of administration, like immersion or stained food, should also be tried since the injection causes skin damage.

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Field observations of schooling in the oval squid, *Sepioteuthis lessoniana* (Lesson, 1830)

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Some species of squid school[1] (i.e. swim together in polarised groups[2]). Understanding the details of schooling in squid is important for two reasons: 1) to estimate sustainable catch sizes[2] and 2) to search for examples of complex social behaviour in cephalopods. Cephalopods, such as squid, possess large brains whose organisation differs from that of similarly sized vertebrates such as fish[1]. Are squid brains capable of producing behaviour as complex as that observed in schooling fish (e.g. observational learning,[3] recognition of members of the same school,[3] and intraschool communication[2])?

Many of these questions are difficult to study in the field. Recently Boal and Gonzalez[4] found no evidence of complex social behaviour in *Sepioteuthis lessosniana* in the laboratory. In contrast, field studies on a related squid *Sepioteuthis sepioidea*[5,6,7] suggest that these squid do exhibit complex social behaviour such as the division of labour within the school (e.g. sentinels). Are these and other differences (see Table 1) indicative of: 1) a species difference between *S. sepioidea* and *S. lessoniana*, 2) a lack of rigorous behavioural studies on *S. sepioidea*, or 3) a change in the behaviour of *S. lessoniana* when confined to a tank?

In this study we examined the group behaviour of *S. lessoniana* in the field for 9 consecutive days (Oct. 30 to Nov. 7, 1997). Observations were made while snorkelling in a lagoon in front of the Lizard Island Marine Research Station (Casuarina Beach, Lizard Island, Great Barrier Reef, Queensland, Australia (14°40′S, 145°28′E)). *S. lessoniana* is the only *Sepioteuthis* found at this location.[12,13] During the day, squid could be found under a 7.2 m boat moored in the lagoon. Squid arrived after dawn and departed between 1–4 hours prior to dusk. Each observation session lasted approximately 2 hours, and one or two observation sessions were made each day. After the first 15 min, squid would sometimes drift under us, suggesting that they had habituated to our presence. We recorded our observations on the number of squid in the group, the geometry of the group formation, the direction each squid was facing, and its position within the group (when we could identify animals because of unique scars) on underwater slates. We estimated the mantle length (ML) of the largest and smallest squid by holding a measuring tape as close as possible to them. On day 2–5 and day 8, one observer videotaped the group's behaviour using a Sony Hi 8 mm TR 910 housed in an Ewa-marine videohousing. Videotape was analysed to determine nearest neighbour distance, orientation, and the response of squid to the behaviour of their neighbours.

The group contained between 10 and 186 squid and the number varied during the day (Fig. 1). Most squid faced the same direction (within 15° of the mean orientation;[8] median 83%, 72% (1st quartile)—94% (3rd quartile)), $n = 5$ randomly chosen sequences from 5 observation sessions). Squid usually faced the same direction as their nearest neighbours, forming clusters within the school (Fig. 2). Animals that were not facing the same direction as the school were usually facing in the opposite direction (180°). The squid moved and/or turned as a group, with all squid completing a turn less than a second after the first squid began to turn ($n = 10$ sequences, 5 videotaped observation periods). When jetting away, the squid became pale and the inter-squid distance decreased (from nearest neighbour distance, median—1.8 mantle lengths (1.0 mantle length (1st quartile)—3.7 mantle lengths (3rd quartile)) to median—0.5 mantle lengths, (0.5 mantle lengths (1st quartile)—1.5 mantle lengths (3rd quartile)), $n = 6$ sequences; Wilcoxon matched pairs, $p < 0.05$).Schooling fish also decrease the distance

Table 1. Differences among the group behaviour of *S. lessoniana* in the field, *S. lessoniana* in the laboratory[4] and *S. sepiodea* in the field[5].

	S. lessoniana (Field)	*S. lessoniana* (Lab.)	*S. sepioidea* (Field)
Group size	10–186	4–18	20–40 (range 2–200)
Group shape	Linear (with clusters)	Roughly spherical	Typically linear (but others observed, e.g. roughly spherical)
Intersquid distance	1.8 mantle lengths	2.7 mantle lengths	varies
% squid oriented in same direction	83%	50%	caries
Size segregation	smallest squid at one end of school	smallest at periphery	smallest sometimes at one end of school
Group fidelity	Low	Low	Low
Cannibalism	None	None reported	None
Less synchronised swimming by squid at periphery	No	Yes	Not recorded
Sentinels probably present	No—But some large squid stayed a few mantle lengths in front of the rest of school	No	Yes

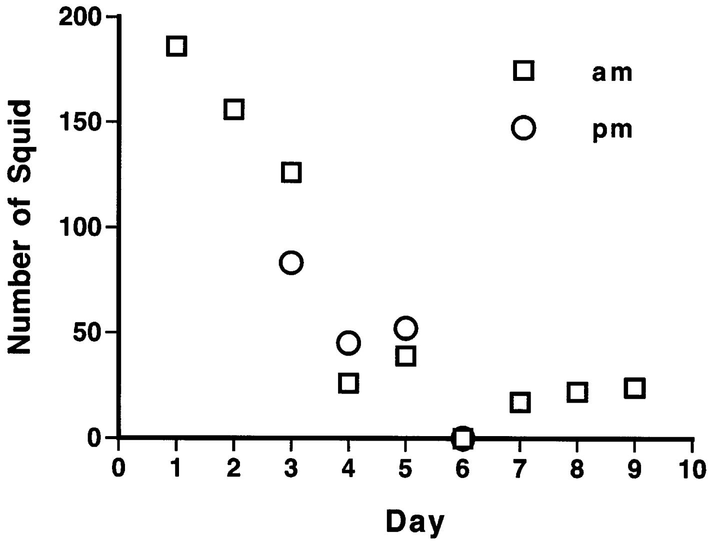

Figure 1. Number of squid in the school during each day of observation. Some days had only one observation period.

between schoolmates during rapid swimming.[2] Because of the squid's synchronisation and swimming polarity, the group meets the criteria[2] for a school. Similar synchronisation has been observed in captive groups of two other species of squid (*Loligo opalescens*[8] and *Illex illecebrosus*[9]).

The school contained squid of different sizes (ML range—25 mm–160 mm) and different stages of sexual development (squid with a mantle length of less than 50 mm were probably not sexually mature[14]). Different class sizes tended to cluster together, with smaller squid at one end (Fig. 2). However small squid (ML < 50 mm) were also found interspersed among larger squid (ML > 100 mm). The school typically had the appearance of a ragged line (Fig. 2). Clusters within the school were not stable from day to day, and squid were observed to change position within the school. Two–five large (ML > 120 mm) squid maintained positions a few mantle lengths in front of the rest of the school (Fig. 2).

Within the school, all squid usually displayed the same body pattern; however, the darkness of the

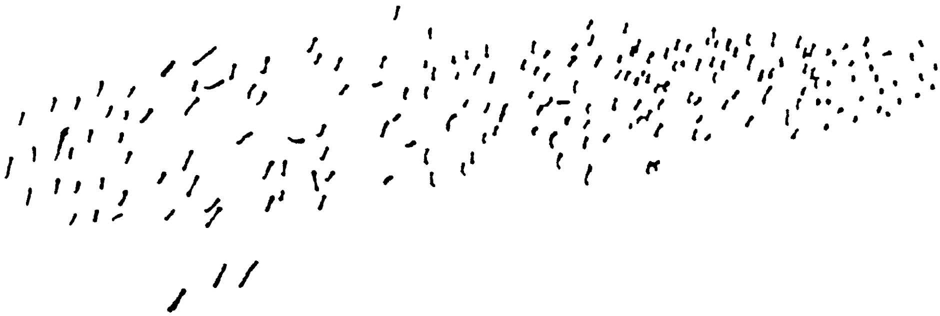

Figure 2. Schematic drawing of a typical school of *S. lessoniana*. Length of line approximates relative squid mantle length. Figure was drawn from video images of the school.

markings could vary between squid. The squid usually exhibited one of four basic chronic body patterns (i.e. patterns of skin colour, skin texture, posture and locomotion that were maintained for prolonged periods of time[10]). The most common body pattern was a uniform dark brown on both arms and mantle (All dark[10]) with the arms held in a cone shape horizontally or at an angle (< 30°). The second most common body pattern was identical to the previous one, except that the mantle was mottled (spotted) and there was a dark band between the eyes (Shaded eye[10]) and at the tip of the arms (Dark arms[10]). In a third body pattern, the mantle was pale with a dark colouring in a rough V-shape on the mantle near the head. When disturbed and/or swimming rapidly, the squid adopted a fourth body pattern. They turned pale, becoming almost translucent (Clear[10]). The animals were difficult to see while showing this body pattern.

Squid sometimes left the school to hunt fish. The hunters turned pale as they left the school. The hunting squid did not always return to the same position in the school. We noticed no change in behaviour or body pattern in the squid that were joined by their 'new' neighbour. We also observed squid attacking smaller squid in the school. The attacks did not result in injury; the smaller squid jetted to another part of the school.

S. lessoniana schools resembled those of the Caribbean squid *S. sepioidea* (Table 1). However, we did not see 'sentinels' as described by Hanlon and Forsythe[5] (i.e. squid facing different directions that appear to be watching for predators). For example, the large squid that stayed at the front of the school all faced the same direction. We do not know if these squid played a special role within the school.

It is unclear why the squid congregated under the boat. it is possible that by staying under the boat they could hide from predators such as birds. However, the ends of the school typically extended beyond the margins of the boat. Therefore, it seems odd that animals not under the boat would adopt a body pattern that made them more visible from the air (i.e. the dark coloration). Why not maintain the pale coloration that makes them virtually invisible?

Two squid could be recognised by unique scars. One of these squid reappeared on three different days (days 1, 2, and 5). We observed the second squid on two different days (days 1 and 2). This suggests that at least some squid return to the same area to school. It also suggests that school composition is fluid, and different animals may participate in different schools on different days.

One possible explanation for the difference between our results and those of Boal and Gonzalez[4] (Table 1) is that we were not studying the same species. There is evidence that *S. lessoniana* encompasses more than 1 species.[11,12] It is also possible that our presence induced changes in the school structure. For example, perhaps there was less synchrony of orientation within the school when we were absent.

Despite the differences between the schooling behaviour of *S. lessoniana* in the field and in the laboratory (Table 1), the major conclusions of the laboratory[4] study, i.e. size based segregation and the absence of preferential associations or group fidelity, were supported. We found more polarisation within the school, a linear as opposed to spherical schooling geometry, the tendency for large squid to stay a few mantle lengths in front of the rest of the school and less strict size segregation than in the laboratory study.[4] More research is necessary to determine whether *S. lessoniana* exhibits complex social behaviour. This study suggests that both field and laboratory studies will be needed.

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Histioteuthis bonnellii (Férussac, 1835) (Cephalopoda) in the Eastern Mediterranean: new record and biological considerations

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Information on the cephalopod molluscs of the Eastern Mediterranean prior to the 1990s has been sparse and consisted mainly of faunistic recording.[1,2,3,4,5,6] Since the early 1990s, however, the distribution and abundance of cephalopods at least in the north-eastern part of that sea, (Aegean and Ionian seas), have been regularly monitored and cephalopod samples collected from the continental shelf region to the bathyal slope.[7,8,9] In August 1997 a specimen of an uncommon species of squid, caught in Korinthiakos Gulf (Western Greece) by a local fisherman, was sent to the Institute of Marine Biology of Crete (IMBC). The rather good condition of the animal allowed its safe identification as *Histioteuthis bonnellii* (Férussac, 1835).

Histioteuthis bonnellii, the umbrella squid, is one of the three species of the family Histioteuthidae reported so far from the Mediterranean Sea.[10,11] This species is distributed in the Mediterranean Sea as well as in the Atlantic and Indian oceans.[12]Records of the species in the Mediterranean (Fig. 1) have been reported mainly for the Western (Catalonian coasts;[13] Gulf of Lions, Nice;[14] Corsica, Sardinia;[15] Genoa;[16] Tyrrhenian Sea[17,18,19]; Gulf of Naples[20]) and Central Mediterranean (Straits of Messina;[21] Taranto Gulf;[22,23,24] Adriatic Sea[25]). The occurrence of the species in the Eastern Mediterranean was only recently reported in Aegean Sea[8,26] but in both cases neither the location of the sampled specimens nor any biological information on the specimens are given. The present finding of *H. bonnellii* represents a new record of the species in the Eastern Mediterranean confirming its presence and completing its distribution in that part of the Mediterranean. Despite the records of the species in the Mediterranean referred to above, biological knowledge of this squid is rather limited and its abundance is still unknown in that sea.[24]

The squid (Fig. 2A) was caught at Korinthiakos Gulf with gill nets on August 18th, 1997 from a mesobathyal bottom at a depth of 320 m (38°16′ N, 22°34′ E). Unfortunately the specimen was cut by the fisherman and internal organs (digestive and genitals) had been removed in order to be used as bait for fishing. After communication with the IMBC the remaining animal was fixed in buffered formalin and sent to the Institute where it was preserved in 70% ethanol.

All the characteristic features of *H. bonnellii*, i.e. the six-membered buccal membrane, the deep inner web connecting the arms, the number of photophores around the right eyelid, the single greatly enlarged elongate photophores borne terminally on arms I, II and III, were recognized in the newly reported specimen. These features clearly differentiate this species not only from *H. elongata* (Voss & Voss, 1962) and *H. reversa* (Verrill, 1880) the other two species of the family Histioteuthidae in the Mediterranean, but also from the other species in the family distributed in other areas of the world.[27,28]

The specimen in our collection has a dorsal mantle length of 135 mm and could be considered as one of the largest specimens reported so far.[13,16,20,27]. Accord-

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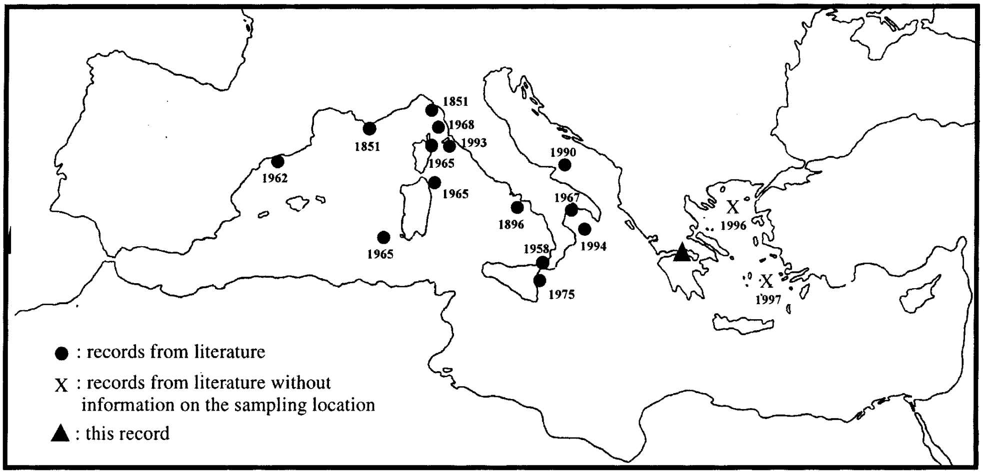

Figure 1. Distribution of *Histioteuthis bonnellii* in the Mediterranean Sea. Records derived: (1962)[13]; (1851)[16]; (1965)[15,17]; (1968)[18]; (1993)[19]; (1896)[20]; (1975)[21]; (1958)[14]; (1994)[24]; (1967)[22]; (1990)[25]; (1996)[8]; (1997)[26]

ingly, it should be considered as an adult specimen and, despite the fact that genitals were missing, it probably represents a female in mature condition as the head is not wider than the mantle width normally found in this species,[27] a fact possible caused by the large mass of eggs inside the mantle cavity. This argument has also been presented for a specimen collected in West Greenland[29] and is strengthened by the fisherman's statements about its enlarged internal organs which were used for bait. The measurements of the morphomeric characters of the specimen are presented in Table 1, while radula is shown in Fig. 2B.

Korinthiakos Gulf is a late quaternary fault-controlled basin with an average width of approximately 30 km. The deep water basin (> 900 m) is connected with the Patraikos Gulf (and open Ionian Sea) through straits incorporating a shallow sill (65 m) at its western end; there is an 8 m deep artificially-dredged channel (Corinth Canal) at the southeastern limit connecting the gulf with the Saronikos Gulf (western Aegean Sea). Such conditions create a bathymetrically-restricted 'fjord-like' marine embayment with water exchange with the open Ionian Sea across the Rion-Antirion sill.[30] This water exchange controls to a certain degree the downwards extension of the surface water masses to deeper depths in the gulf. Accordingly the presence of *H. bonnellii* in the Korinthiakos Gulf should be related to the flow of the water originating from the Ionian Sea. The oxygen content of the Gulf maintains high values (i.e. dissolved oxygen > 2 ml/l) at water depths of about 700 m, indicating the absence of any anoxic conditions in the deeper basin waters.[28] However, to what extent these conditions allow the recognition of an *H. bonnellii* population in Korinthiakos Gulf awaits future intensive scientific surveys.

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Figure 2. (A) Collected specimen of *Histioteuthis bonnellii* from Korinthiakos Gulf. **(B)** Radula × 400 (SEM).

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Table 1. Measurements of morphometric characters in *Histioteuthis bonnellii* from Eastern Mediterranean after fixation: BW = Body weight; TL = Total length; DML = Dorsal mantle length; MW = Mantle width; HL = Head length; HW = Head width; FL = Fin length; FW = Fin width; AL = Arm length; IWL = Inner web length; ED = Eye aperture diameter; U = Upper beak; L = Lower beak; CL = Crest length; HL = Hood length; RGL = Rostral gap length; WL = Wing length

BW	DML	MW	HL	HW	FL	FW	AL	AL	AL	AL	AL	AL	AL	AL
g	mm	mm	mm		mm	mm	RI	RII	RIII	RIV	LI	LII	LIII	LIV
							mm	mm	mm	mm	mm	mm	mm	mm
975	135.0	99.3	94.5	45.4	80.7	100.2	252.8	330.3	282.6	222.7	222.9	291.8	295.5	266.2
IWL	IWL	IEL	IWL	IWL	ED	ED	UCL	UHL	URGL	UWL	LCL	LHL	LRGL	LWL
(A)	(B)	(C)	(D)	(E)	(R)	(L)	mm	mm	mm	mm	mm	mm	mm	mm
mm	mm	mm	mm	mm	mm	mm								
93.8	148.3	184.8	163.8	172.3	31.6	15.6	38.4	29.2	8.1	12.1	20.1	15.5	8.6	16.4

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