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An unusual finding of *Sepietta oweniana* (Cephalopoda: Sepiolidae) egg clutch

DANILA CUCCU [1], MARCO MEREU [1], RITA CANNAS [1], SANDRO MARCIAS [1], ANGELO CAU [1] and PATRIZIA JEREB [2]

[1] Dipartimento di Biologia Animale ed Ecologia, Università di Cagliari, Tommaso Fiorelli 1, 09126 Cagliari, Italy.
E-mail: cuccu@unica.it
[2] ISPRA ex-ICRAM, via di Casalotti 300, 00166 Roma, Italy.

SUMMARY: An unusual finding of *Sepietta oweniana* egg clutch at 544 m depth is described within an updating study of the reproductive aspects of the species in Sardinian waters. Egg species identity was determined by genetic analysis and morphological and biometrical data of the eggs, embryos and one newly hatched specimen are reported and discussed. Illustrations of the eggs, spermatophores and spermatangia by photographic material are also included. Data on bathymetric distribution and sexual maturity are reported and compared with data already available for the species in other geographical areas of distribution.

Keywords: *Sepietta oweniana*, egg clutch, embryos, reproduction, bathymetric data, Mediterranean Sea.

RESUMEN: Descubrimiento inusual de una puesta de *Sepietta oweniana* (Cephalopoda: Sepiolidae). – Se describe un descubrimiento inusual de una puesta de *Sepietta oweniana* a 544 m de profundidad, en el ámbito de un estudio de aspectos reproductivos de la especie en aguas de Cerdeña. Los huevos se identificaron por medio de análisis genéticos. En este trabajo se presentan y discuten datos morfológicos y biométricos de los huevos, embriones y de un especimen recién nacido. Se incluye material fotográfico de los huevos, espermatóforos y espermatangia. Se presentan datos de distribución batimétrica y madurez sexual y se comparan con datos existentes de la especie en otras áreas geográficas de distribución.

Palabras clave: *Sepietta oweniana*, puesta, embriones, reproducción, distribución batimétrica, Mediterráneo.

INTRODUCTION

The bobtail squid *Sepietta oweniana* (Orbigny, 1839-1842) is widely distributed in the eastern Atlantic and in the Mediterranean Sea, where it is the most abundant sepiolid species and an important by-catch of local trawl fisheries (Reid and Jereb, 2005). Consequently, many observations on the geographic and bathymetric distribution of *S. oweniana* in the eastern Atlantic (see Mangold-Wirz, 1963; Collins *et al.*, 2001) and throughout the Mediterranean Sea are available (e.g. Mangold-Wirz, 1963; Orsi Relini and Bertuletti, 1989; Villanueva, 1995; Jereb *et al.*, 1997; Lefkaditou *et al.*, 2003; Giordano *et al.*, 2009).

The reproductive behaviour has also been investigated, among others, by Salman (1998) and Bello and Deickert (2003), who suggested a continuous spawning strategy for the species. Deickert and Bello (2005) also described in detail egg clutches collected in the Catalan Sea; their tentative identification of the eggs was based on morphological examination and was subsequently confirmed by the examination of newly hatched specimen in aquaria.

In fact, cephalopod eggs and egg masses collected in the field are difficult to identify and are often neglected due to this difficulty (Boletzky, 1998). The use of molecular analysis allowed us to identify all material collected in the present case (i.e. egg clutch as well as the newly hatched and adult specimens) as *S. oweniana*; this, in turn, allowed us to make a confident analysis of various biological and reproductive aspects of the species in the area investigated. Results are compared with the information already available in the literature.

MATERIALS AND METHODS

Samples were collected by bottom trawl surveys carried out in Sardinian waters in summer 2005 and the winters of 2006 and 2007; hauls were performed by daylight and a bottom otter trawl with a 20 mm codend stretched mesh size was used.

Specimens were identified according to Bello (1995). On 166 females and 188 males dorsal mantle length (ML; to the nearest 0.01 mm) and total weight (TW; to the nearest 0.01 g) were recorded after preservation in 4% formalin. Sexual maturity was determined according to Mangold-Wirz (1963) and Salman and Onsoy (2004), i.e. females with ripe oocytes in the oviduct and/or empty follicles in the ovary were considered mature. Mated females were identified by the presence of spermatangia remainings in the bursa copulatrix. Oocytes, spermatophores and spermatangia were counted and measured (to the nearest 0.01 mm) in all mature specimens, and the presence of empty follicles in the ovaries also was recorded. Potential fecundity (PF) was computed as the sum of all oocytes in the ovary and in the oviduct, excluding empty follicles.

The egg mass and one newly hatched specimen located on an empty case were photographed and kept alive in a shipboard tank with well-oxygenated sea water on the way back to the laboratory. The following day, the eggs, the embryos removed from their cases and the newly hatched specimen were photographed again and measured to the nearest 0.01 mm. Whole embryos and small pieces of mantle tissue of 5 adult females were stored in absolute ethanol at -20°C for the genetic analyses.

Genomic DNA was extracted according to a salting-out method and analyzed through PCR amplification and direct DNA sequencing. Polymerase chain reaction (PCR) was used to selectively amplify the 16S ribosomal mitochondrial gene (16S rDNA) and the cytochrome oxydase subunit I (COI) with universal primers and thermocycling conditions as described in Cuccu *et al.* (2009). Amplification products were purified by magnetic beads (ChargeSwitch® PCR Clean-Up Kit, Invitrogen) and directly sequenced using the same PCR primers. Sequences were aligned with the CLUSTAL-W program implemented in the MEGA4 package (Tamura *et al.*, 2007), and adjusted by eye.

RESULTS

Molecular analysis

A total of 456 bp of nucleotide sequence of 16S rDNA were determined for embryos and females. The 16S sequences were identical to the sequences previously obtained for males of *S. oweniana* (Gen Bank submission number EU203145) (Cuccu *et al.*, 2009). As for the COI gene, embryos shared the same sequence found in males and in females (EU203144); two additional haplotypes (FJ765230-31), differing from the previously cited sequence in only one transition out of 639 bp of total length, were found in females.

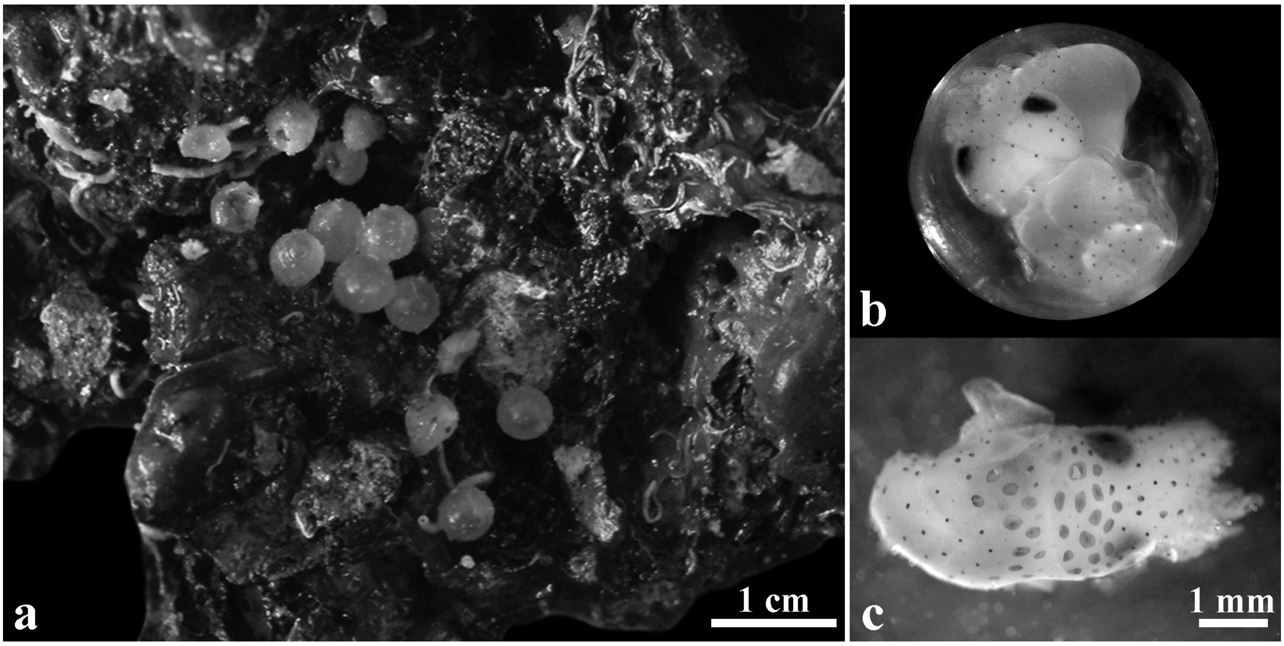

Fig. 1. – *Sepietta oweniana* from the Sardinian Channel: egg clutch attached to a piece of hard substrate (a); embryo inside the egg (b); newly hatched specimen (c).

Table 1. – *Sepietta oweniana* from the Sardinian waters: measurements of six embryos (in bracket mean values ± SD) and one newly hatched specimen described in this paper.

	Embryos	Newly Hatched
Dorsal mantle length (mm)	2.18-2.21 [2.20 ± 0.02]	2.68
Ventral mantle length (mm)	1.76-1.82 [1.79 ± 0.03]	2.61
Mantle width (mm)	1.98-2.01 [2.00 ± 0.02]	2.10
Head length (mm)	1.35-1.39 [1.37 ± 0.02]	1.50
Head width (mm)	1.35-1.39 [1.38 ± 0.02]	1.60
Eyes diameter (mm)	0.49-0.51 [0.50 ± 0.01]	0.76

Egg clutch and embryos

The egg clutch was found on 22 February 2007 in the Sardinian Channel (geographic position: 38°44’840N 9°31’650E - 38°42’590N 9°29’320E) at a mean depth of 544 metres. Six eggs and six empty egg cases were attached to a piece of rocky substrate covered by a few small calcareous serpulids; one newly hatched specimen was resting on an empty case (Fig. 1a). The eggs were approximately spherical to lemon-shaped and greyish-white in colour. They measured 5.70x4.80 mm on average and were covered by a hard shell. After shell removal, the advanced developmental stage of the embryos was clearly visible; it corresponded to Naef stage XIX (Naef, 1928) (Fig. 1b). Embryos differed from the newly hatched specimen (Fig. 1c) in their smaller size (Table 1), the presence of embryonic organs and mantle transparency.

Sampled specimens

A total of 166 females (ML: 12.90-30.80 mm; TW: 1.29-8.30 g) and 188 males (ML: 13.40-29.10 mm; TW: 1.45-6.33 g) of *S. oweniana* were found between 53 and 598 m depth, but the bulk of the sample came from 300-500 m in both summer and winter. Mature and maturing specimens were present in both seasons. Of the total, 116 females were mature (Fig. 2a); they ranged in size between 18.50 and 30.80 mm ML and size at 50% of maturity was 24.00 mm.

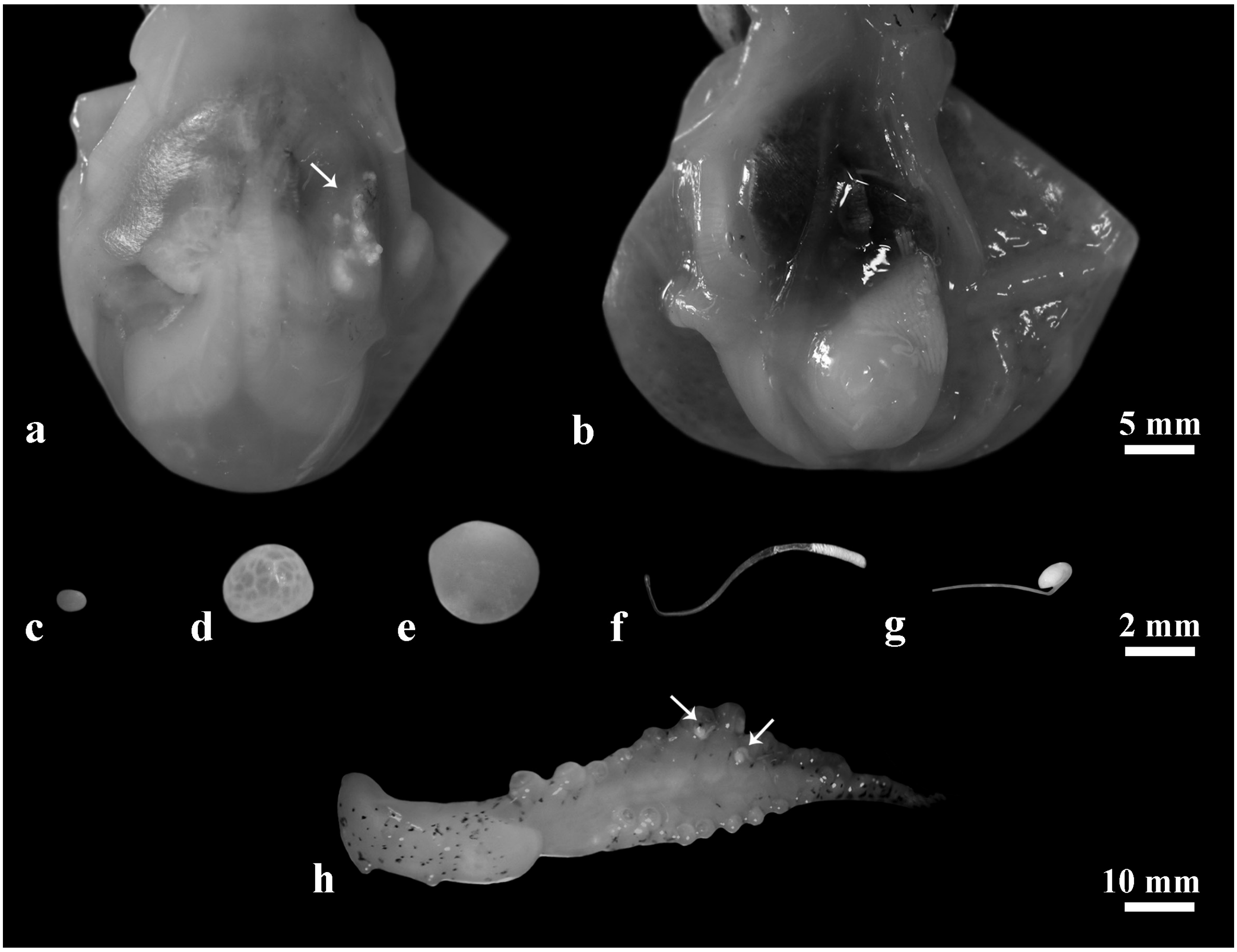

Fig. 2. – *Sepietta oweniana* from Sardinian waters: female (a) and male (b) reproductive system, fresh material. Small (c), reticular (d) and ripe (e) oocytes. Spermatophores (f), spermatangium (g) and hectocotylus (h). The arrow in (a) shows spermatangia inside the copulatrix bursa and the arrows in (h) indicate spermatangia imbedded in the hectocotylus.

TABLE 2. – *Sepietta oweniana* from the Sardinian waters: number, size and % of the oocytes observed in the ovaries of two groups of mature females. In brackets mean values ± SD.

	89 females (82% mated) 18.50-30.80 mm ML			27 females (100% mated) 18.60-30.40 mm ML		
	Number	Size (mm)	%	Number	Size (mm)	%
Small Oocytes	11-179 [70±32]	0.10-1.77 [0.90±0.26]	51	24-128 [70±30]	0.46-1.48 [0.91±0.28]	58
Reticulated Oocytes	6-99 [51±17]	1.80-3.35 [2.60±0.30]	38	21-93 [47±17]	1.80-3.33 [2.40±0.40]	42
Ripe Oocytes	1-94 [20±18]	2.00-4.37 [3.24±0.50]	14			
Potential Fecundity	45-263 [141±41]			61-185 [117±33]		

Potential Fecundity computed for the 89 females with ripe oocytes was 141±41, and included oocytes at various stages of maturation (see Table 2, Fig. 2 c,d,e). Up to 94 ripe oocytes were counted in the oviduct: they were smooth in surface and brownish in colour. Potential Fecundity of females with no smooth oocytes ranged between 61 and 185 oocytes (Table 2). The bursa copulatrix measured 13.00x7.60 mm in mature females, and in those already mated it contained from 19 to 231 (mean= 130±58) spermatangia (Fig. 2g). Spermatangia total length ranged between 3.02 and 4.11 mm (mean= 3.75±0.26 mm).

A few spermatangia similar in shape and in size to those of the bursa were found embedded in the hectocotyli of some mature males (Fig. 2h). Mature males (Fig. 2b) ranged in size between 14.30 and 29.10 mm ML; size at 50% of maturity was 20.00 mm. Spermatophore number varied between 12 and 558 (mean= 202±116) (Fig. 2f); they ranged in size between 3.40 and 8.30 mm (mean= 6.00±1.00 mm).

DISCUSSION

In recent years, molecular analysis has played a key role in taxonomic and phylogenetic studies of cephalopod species (e.g. Piertney *et al.*, 2003; Strugnell, 2007). It has also already proved to be very useful to the authors of the present paper for the identification of species characterized by morphologic variability (Cuccu *et al.*, 2009) and samples belonging to early developmental stages, like egg clutches and spermatophores (Cuccu *et al.*, 2007). Within this study, the indisputable genetic identification of adult specimens and the egg clutch found in bathyal waters as *S. oweniana* represented the starting point for a confident analysis.

Shape and size of the egg clutch matched the description available from the literature (Mangold-Wirz, 1963; Bergstrom and Summers, 1983; Deickert and Bello, 2005); however, the egg shell was hard, not flexible as described for the eggs found in shallow waters in the Catalan Sea (e.g. "leur enveloppe est mince et souple...", Mangold-Wirz, 1963, p. 135). Also, the depth of the finding (544 m) was unusual when compared with the previous records: *S. oweniana* egg masses, in fact, were recorded at depths ranging between 8 and 130 m in the Atlantic Ocean (Bergstrom and Summers, 1983) and between 90 and 120 m in the Catalan Sea (Deickert and Bello, 2005), while Naef reported an egg clutch found at 200 m in the Gulf of Naples (in Mangold-Wirz, 1963). Mangold-Wirz (1963) does not give depth records of the egg masses collected in the waters off Rosas and Port Vendres (Catalan Sea), but stresses the preference of the species for spawning onto the ascidian *Microcosmus sabatieri*, a typical inhabitant of shallow waters. Therefore, this is the deepest record of identified egg clutches reported so far for the species.

No littoral or sub-littoral algae were found on the rocky substrate where the egg clutch of the present record was attached. This leads us to exclude the possibility that the eggs were laid in shallower waters and washed down to deeper bottoms later on. Furthermore, the present finding does not contrast with what is known about the bathymetric preference of the species. Typically widely eurybathic (8-1000 m, Reid and Jereb, 2005), *S. oweniana* has been shown to prefer a depth range of 200-500 m in several areas of its distribution within the Mediterranean Sea (e.g. Sartor and Belcari, 1995; Villanueva, 1995; Jereb *et al.*, 1997; Lefkaditou *et al.*, 2003; present results). Therefore, the possibility of spawning events in deep waters is not so unlikely, in principle. Observations from deep bathyal waters are less common than those on the shelf and are most often carried out during experimental operations, far less frequent than fisheries activities; these, in turn, avoid bottoms with hard substrates. The scarcity of egg findings in deep waters may therefore be more related to the scarcity of observations than to the actual scarcity of material. Obviously it is also possible that similar findings occurred in the past but the eggs were neglected, discarded and/or not identified.

A list of size-at-maturity data for both sexes as reported in the literature is given in Table 3. As can be observed, while a certain degree of variability does exist in the minimum size as well as in the size at 50% maturity in both sexes, sizes are definitively comparable for males of the Mediterranean and the Atlantic populations.

As for females, leaving aside the data from the Catalan Sea (Mangold-Wirz, 1963), a difference in size between the Mediterranean and Atlantic populations at 50% maturity is indeed confirmed, i.e. females reach maturity at larger sizes in Atlantic waters; on the other hand, sizes at maturity are comparable among Mediterranean populations, i.e. they show the same degree of variability observed for male sizes. Our observations of mated females lacking smooth oocytes but carrying empty follicles in the ovaries suggest that

Table 3. – *Sepietta oweniana*: data on bathymetric distribution, sexual maturity for both sexes and female potential fecundity as available from the literature. ML_m, mantle length of the smallest mature specimens; ML_{50}, mantle length at 50% sexual maturity.

References and geographical region	Range of capture (m) [Preferential range]	Range of ML (mm)	Sex	ML_m (mm)	ML_{50} (mm)	Potential fecundity (ripe oocytes)
Mangold-Wirz (1963)	80-700	20.0-40.0	Females	30	>35	150-200
Mediterranean (Catalan Sea)	[-]	20.0-35.0	Males		20*	(30-40)
Bergstrom and Summers (1983)	60-300	-	Females	-	33	max: 130
Atlantic (Gullmar Fjord)	[-]	-	Males	-	23	(–)
Guescini and Manfrin (1986)	10-200	-	Females	24	-	–
Mediterranean (Adriatic Sea)	[-]	-	Males	17	-	
Orsi Relini and Bertuletti (1989)	50-700	10.0-35.0	Females	20	-	–
Mediterranean (Ligurian Sea)	[400-500]	14.0-30.0	Males	18	-	
Jereb *et al.* (1997)	27-708	14.5-36.8	Females	18.5	-	–
Mediterranean (Strait of Sicily)	[200-400]	14.0-34.0	Males	14	-	
Salman (1998)	26-500	14.0-36.0	Females	22	28	58-236
Mediterranean (Aegean Sea)	[-]	18.0-35.0	Males	21	24	(–)
Sartor *et al.* (1998)	60-680	13.0-40.0	Females	19	26	–
Mediterranean (Tyrrhenian Sea)	[350-550]	14.0-35.0	Males	15	21	
Bello and Deickert (2003)	93-98	16.5-38.5	Females	24.5	-	719-1613
Mediterranean (Adriatic Sea)	[-]					(27.2)
Giordano *et al.* (2009)	100-200	18.0-34.0	Females	24	24	60-106
Mediterranean (Tyrrhenian Sea)	[62-405]	15.0-29.0	Males	18	18	(–)
Present results	53-598	12.9-30.8	Females	18.5	24	45-263
Mediterranean (Sardinian seas)	[300-500]	13.4-29.1	Males	14.3	20	(20)

* size corresponding at 75% of the mature specimens

these females had already spawned shortly before being caught. Similarly, it is possible that females from the Catalan Sea carrying large oocytes had already spawned their smooth oocytes before being captured. If this is the case, size at maturity of females from the Catalan Sea would be comparable with data from other Mediterranean areas.

The mean number of ripe oocytes found in mature ovaries (mated and not mated) is in agreement with data from the Catalan Sea and the Adriatic Sea (Mangold-Wirz, 1963; Bello and Deickert, 2003) (see Table 3). Potential fecundity values are also similar to those reported for the southern Tyrrhenian (Giordano *et al.*, 2009), the Aegean Sea (Salman, 1998) and females of the Catalan Sea (Mangold, 1989), all markedly lower than those reported by Bello and Deickert (2003) for females of the same area (see Table 3).

The number of spermatangia found in the *bursa copulatrix* indicates that females store a suitable number of sperm reservoirs to fecund all the eggs laid during the protracted spawning process. Male fecundity was lower than that reported for the eastern Mediterranean population (Salman, 1998) but higher than that reported for the Catalan Sea population (Mangold-Wirz, 1963). The large number of spermatophores present in the Needham's sac, much larger than that found inside the *bursa copulatrix*, confirms that a single male can fecund several females.

The present results, i.e. a spawning season extended throughout the year, the asynchronous ovulation and the presence of a low number of ripe oocytes in the oviduct, confirm a continuous spawning strategy for this species, as proposed by Bello and Deickert (2003), and as adopted by other species within the family Sepiolidae (Gabel-Deickert, 1995; Cuccu *et al*., 2007). Our observations underline the importance of carrying out bathyal investigations to add new and important information on the biology of even well studied species such as *S. oweniana*.

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