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Egg clutch, sperm reservoirs and fecundity of *Neorossia caroli* (Cephalopoda: Sepiolidae) from the southern Sardinian sea (western Mediterranean)

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First observations on reproduction of *Neorossia caroli* from the southern Sardinian sea are reported, based on the analyses of an egg clutch and specimens collected by bottom trawl on sandy and muddy grounds. The egg clutch, consisting of 13 white eggs of 9 mm length with embryos at an advanced developmental stage, was found at 1200 m depth, attached to a piece of hard substrate. Adult specimens were collected in a depth range between 400 and 1600 metres. DNA sequences indicated that embryos, spermatangia and adults belonged to the same species, the carol bobtail squid, *N. caroli*. Fecundity analysis made on 40 females showed a variable number of eggs (20–611) in the ovaries, with the combined presence of advanced and developing oocytes, and few smooth eggs ready to be spawned in the oviducts of mature females. Several sperm reservoirs (spermatangia) were embedded in the mantle of some of the females, in the anterior ventral area overlying the oviduct.

INTRODUCTION

Reproduction is a key phase in the life history of all living organisms and understanding the different reproductive strategies encoded in the genome of each species is a key to shed light on their entire life cycle. This is definitely true for many cephalopods, given their fast growth and relatively short life span if compared to those of the vertebrates that share the habitat and compete with them.

Fecundity, mating and spawning, egg clutch number and size, all are basic elements in trying to define cephalopod reproduction patterns (e.g. Rocha et al., 2001), but some of them are not easy to get information on, especially for species other than those living in coastal shallow waters and/or on the shelf, and much of the currently available knowledge still comes from observations in captivity (Boletzky & Boletzky, 1973; Boletzky et al., 1973, Bello & Deickert, 2003; Deickert & Bello, 2005). Samples from the field are often difficult to obtain and in some cases also difficult to use in a proper way, due to problems of species identification. This is the case, in particular, for eggs and egg masses/clutches (see Boletzky, 1998 for a review) that may become available through fishery operations or occasional, non professional, individual records and/or accidental transportation by sea currents. If comparison with direct observations in aquarium did turn out useful, the application of molecular analysis and recent advanced technologies can be a remarkable aid for the specific identification of all the many species for which studies in captivity are not available.

The carol bobtail squid *Neorossia caroli* (Joubin, 1902) is distributed in the Mediterranean Sea and in the eastern Atlantic Ocean from south-western Iceland and Ireland southward to the Gulf of Guinea and the Namibian coast of southern Africa; doubtful and sporadic records exist for the western Atlantic, from the southern slope of the Great Newfoundland Bank, the slope of Nova Scotia and the Gulf of Mexico (Reid & Jereb, 2005).

The deepest living sepiolid, collected down to 1744 m in the western Mediterranean basin (Villanueva, 1992a), the carol bobtail squid lives preferentially on deep muddy bottoms (often characterized by *Isidella elongata* populations), usually overlapping with *Rossia macrosoma* in the lower half of the distributional range of the latter. In spite of its wide distribution and the rather common presence in the Mediterranean bottom trawl catches (Reid & Jereb, 2005), the main information on the species' life cycle and reproductive biology is still due to the studies carried out in the early sixties in the Catalan Sea (Mangold Wirz, 1963a,b), with some additional information available in a few other papers (Jereb et al., 1998; Reid, 1991).

The recent need to re-describe the species (Reid, 1991) and the even more recent tentative attribution of *Neorossia* specimens recorded from the Falkland Islands waters to another subspecies, *N. caroli jeannae* (Nesis et al., 2001), clearly emphasize the importance of improving our knowledge on *Neorossia* genus and species.

The aim of this paper is to add information to our knowledge of the reproductive biology of *N. caroli*, by the analysis of the southern Sardinian sea population.

MATERIALS AND METHODS

Samples were collected during the daytime by bottom trawl net on sandy and muddy bottoms in the south

Figure 1. Western Mediterranean Sea. The grey network south of Sardinia indicates the investigated area.

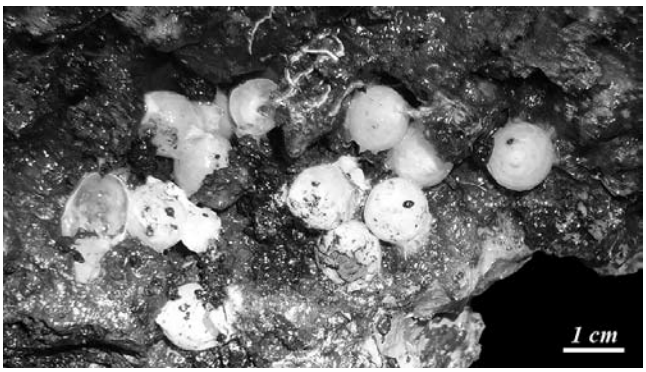

Figure 2. Egg clutch of *Neorossia caroli* attached to a piece of hard substrate.

Sardinian sea (western Mediterranean) (Figure 1). Fishing efforts concentrated at depths from 400 to 600 m, with disproportionately lower effort being deployed at depths from 700 to 1600 m.

The egg clutch was found in May 2006 at a depth of 1200 metres, the adult specimens (106 females and 94 males) were collected during the same year from January to December, between 400 and 1600 metres depth. The egg clutch was photographed and the eggs were counted, photographed and individually measured to the nearest 0.1 mm.

Specimens were classified as *Neorossia caroli* according to the most recent systematic keys (Reid & Jereb, 2005). Dorsal mantle length (ML) and total body weight (TW) of all specimens were measured to the nearest 1 mm and 0.01 g, respectively.

Molecular analysis was applied to test the species identification. Whole embryos, spermatangia and small pieces of adult mantle tissue were sampled for the genetic analyses, and stored in absolute ethanol at −20°C. Genomic DNA was extracted according to a salting-out method (Miller et al., 1988). All samples were analysed through PCR amplification and direct DNA sequencing. The polymerase chain reaction was used to selectively amplify the 16S ribosomal gene (16S rDNA), using the primers described in Palumbi et al. (1991). Amplification products were purified by magnetic beads (ChargeSwitch® PCR CleanUp Kit, Invitrogen) and directly sequenced using the same PCR primers. Sequences were aligned with the CLUSTAL-W program implemented in the MEGA3 package (Kumar et al., 2004), and adjusted by eye.

Embryos where photographed and preserved for future analysis.

Sexual maturity was assessed based on the three stages maturity scale used within the International Research Project MEDITS (Bertrand et al., 2000), where the presence of smooth eggs in the ovaries/oviducts and spermatophores inside Needham's sac indicate mature females and males respectively.

The occurrence of spermatangia in the females also was recorded.

Forty ovaries (30 mature and 10 immature) were randomly sampled and fixed in 4% formalin for fecundity analysis. All the oocytes of mature females ovaries were counted, measured (nearest 0.1 mm), and assigned to three stages of development depending on the size: small (0.1–3.0 mm), medium (3.1–5.5 mm) and large (5.6–7.5 mm). In immature specimens only the oocytes larger than 0.1 mm were considered for the analysis. Potential Fecundity (PF) of mature females was computed as the sum of the oocytes in the ovary plus the eggs in the oviducts.

Spermatangia where taken and preserved for future observations.

Stomachs were removed and assigned to a subjective fullness index category (FUI; Rasero et al., 1996), where 0 = empty; 1 = containing very scarce remains; 2 = from significant remains to full repletion.

Table 1. *Distribution of female and male of* Neorossia caroli *at different stages of sexual maturity from the southern Sardinian sea.*

		Immature (Stage 1)		In maturing (Stage 2)		Mature (Stage 3)	
		Females	Males	Females	Males	Females	Males
400–500 m	No. specimens	9	3	2	1	6	8
	Range ML (mm)	22–46	27–35	38–52	29	51–70	41–47
	Mean ±SD	35 ±9	30 ±4	45 ±10		58 ±7	44 ±2
501–700 m	No. specimens	37	9	10	14	37	54
	Range ML (mm)	18–52	22–31	28–50	20–45	36–70	25–50
	Mean ±SD	33 ±8	26 ±3	40 ±7	34 ±6	55 ±9	39 ±6
701–1600 m	No. specimens	2	0	2	0	1	5
	Range ML (mm)	38–45		33–44		69	19–43
	Mean ±SD	42 ±5		39 ±8			31 ±9

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Table 2. *Parameters of 30 mature females of* Neorossia caroli.

				Occurrence of egg at different length and average length in brackets [ ]				
ML (mm)	TW (g)	FUI	SP	Small eggs	Medium eggs	Large eggs	Novid	PF
38	42.56	0		95% [1.2]	4% [4.1]	1% [6.1]	6	364
44	31.27	1	*	33% [1.6]	38% [4.7]	29% [6.5]	7	24
45	45.19	2	*	90% [1.3]	4% [4.1]	6% [6.1]	8	468
47	52.77	2		90% [1.4]	8% [4.3]	2% [6.0]	8	611
48	41.64	1	*	78% [1.0]	13% [4.3]	8% [6.5]	18	372
50	46.99	1		49% [1.1]	23% [3.8]	28% [6.5]	7	47
51	51.39	2		80% [1.1]	11% [4.1]	9% [6.2]	11	279
52	37.82	1		90% [1.1]	6% [4.0]	4% [6.5]	14	361
52	54.63	1	*	82% [1.2]	12% [4.1]	6% [6.1]	20	368
54	52.70	2		53% [1.5]	23% [4.0]	22% [6.5]	11	63
54	59.20	2		74% [1.3]	18% [4.1]	8% [6.5]	19	301
55	42.30	2	*	69% [1.3]	17% [3.8]	13% [6.2]	28	221
55	51.50	0		68% [1.0]	12% [4.1]	19% [6.5]	18	222
55	63.20	0	*	68% [1.4]	18% [4.1]	12% [6.4]	16	158
56	50.10	2		77% [1.2]	12% [4.3]	11% [6.5]	24	293
56	48.10	2		76% [1.3]	14% [4.2]	10% [6.2]	16	177
56	48.20	2	*	70% [1.1]	15% [4.3]	14% [6.5]	20	281
57	52.20	2	*	73% [1.1]	16% [4.2]	11% [6.5]	24	304
58	51.70	2		81% [1.4]	12% [3.5]	7% [6.3]	16	259
58	63.87	2	*	70% [1.3]	20% [4.1]	10% [6.4]	26	379
58	50.04	2		84% [1.0]	12% [4.5]	4% [6.4]	5	169
59	63.33	2	*	94% [1.4]	5% [4.1]	1% [6.1]	2	604
60	48.42	1	*	66% [1.4]	32% [4.2]	1% [6.1]	16	357
60	61.37	1		80% [1.4]	16% [4.1]	4% [6.5]	14	508
62	66.60	1	*	76% [1.6]	15% [4.1]	9% [6.2]	16	376
63	83.34	0	*	83% [1.4]	12% [4.3]	6% [6.1]	15	440
64	61.68	1		74% [1.2]	16% [4.2]	10% [6.3]	18	243
69	84.80	1	*	77% [1.8]	17% [4.2]	6% [6.1]	19	400
70	61.98	0	*	80% [1.4]	14% [4.3]	6% [6.5]	19	399
70	73.40	1	*	71% [1.1]	16% [4.1]	11% [6.2]	16	472

ML, Mantel Length; TW, Total Weight; FUI, Fullness index category; *, occurrence of spermatangia (SP); Novid, ripe eggs number inside the oviduct; PF, Potential Fecundity.

RESULTS

Egg clutch

The egg clutch consisted of 13 spherical eggs attached to a piece of hard substrate (Figure 2); the eggs were covered by a hard white coat and ranged from 9.0 to 9.3 mm in diameter. They were at the same stage of development, as seen after removing the external cover and extracting the embryos. In all embryos an orange pigmentation of the retina was observed (Figure 3), indicating that the IX Naef's (1928) development stage had been reached.

Specimen distribution

Males (19–50 mm ML) and females (18–70 mm ML) occurred mostly together within a depth range between 400 and 1600 m (Table 1). The bulk of the samples came from the depth range between 501 and 700 m. The combined presence of mature and immature females was observed in the whole bathymetric range investigated, while mature

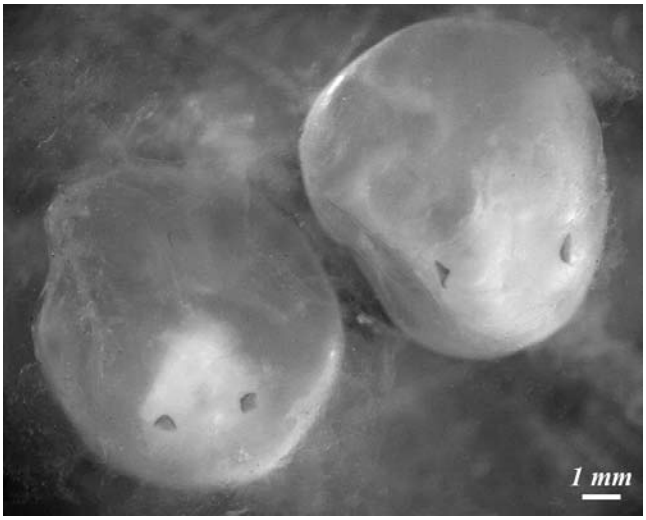

Figure 3. Pigmentation of the retina in two embryos of *Neorossia caroli*. (The egg shell has been removed).

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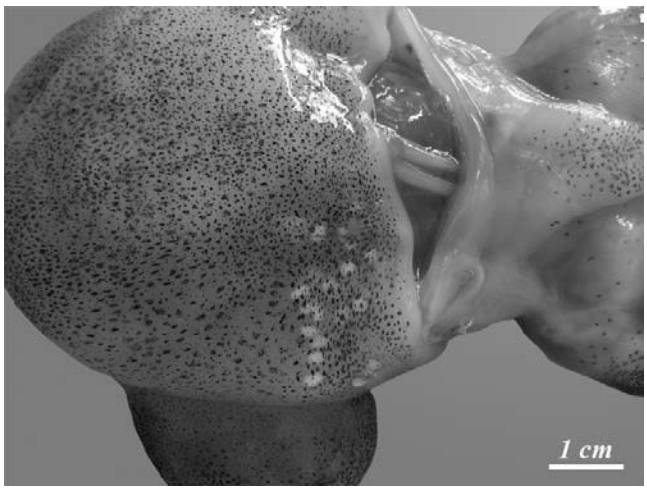

Figure 4. Spermatangia (white spots) implanted in the inner ventral mantle tissue of a mature female of *Neorossia caroli*.

males were more abundant >500 m depth. The smallest mature male (19 mm ML) was caught at 1600 metres.

Molecular analysis

A total of 470 bp of nucleotide sequence of 16S rDNA were determined from adults (both males and females), embryos and spermatangia. All sequences were identical (GenBank submission number EF057734), indicating that the whole sample belongs to the same species, *Neorossia caroli*.

Female sexual maturity

Mature as well as immature animals were present year-round.

Forty-four females (42%) were mature, their size ranging between 36 and 70 mm (ML). The mantle cavity was mostly filled with the ovaries, covered by the white, creamy nidamental glands, and ripe eggs were found in the oviducts. In 38 (86%) of them the presence of spermatangia (from 5 to 24) was recorded, indicating that mating had already occurred. The spermatangia were implanted in the inner left side of the ventral mantle surface, the area overlying the oviduct (Figure 4).

From 24 to 611 oocytes of different sizes were counted inside the 30 mature ovaries analysed, indicating asynchronous ovulation; their relative number for each developmental stage is reported in Table 2. The highest percentage was represented by small, immature oocytes of 1.3 ±0.3 mm average size, followed by medium size oocytes of 4.1 ±0.2 mm average; larger oocytes, average size 6.3 ±0.3 mm, were far less numerous, and some of these (from a minimum of 5 to a maximum of 28) were smooth and located inside the oviducts.

The fullness index indicated recent feeding in 83% of the mature specimens analysed.

Also, ten immature females were analysed, their mantle length ranging between 33 and 46 mm. The ovaries contained from 115 to 210 small oocytes smaller than 3.0 mm (0.8 ±0.3 mm) along with numerous oocytes too small to be measured. The fullness index indicated a regular feeding activity.

DISCUSSION AND CONCLUSION

Our findings confirm the presence of *Neorossia caroli* well below 1000 m depth also in the southern Sardinian sea, as reported for the areas where bathyal depths have being investigated, e.g. the western Mediterranean and the eastern North Atlantic (Reid & Jereb, 2005). Bathyal hauls (i.e. deeper than 700 m in the present case) are too few to allow quantitative comparisons with the other investigated bathymetric ranges, but results indicate that *N. caroli* of the Sardinian sea is more abundant at depths below 500 m.

Although data available do not allow us to make any final statement, the observed mature males abundance variation with depth is interesting; such is the fact that mature as well as maturing females are well represented >500 m depth. Recent data on *Rosssia macrosoma* of the Portuguese waters (Rosa et al., 2006) indicated that larger animals are significantly more abundant in deeper waters (i.e. between 300 and 600 m). If future data and analysis should confirm that in *N. caroli* the preference for deeper waters becomes stronger the closer to spawning the animals are, this would explain, at least partially, the scarcity of egg clutch findings reported in the literature.

To the best of the authors' knowledge, in fact, these are essentially limited to the observations by Mangold Wirz (1963a,b) and Villanueva (1992a). In both cases, however, due to the apparent similarity of *N. caroli* and *R. macrosoma* mature smooth eggs (i.e. Mangold Wirz, 1963 a,b), it seems that the attribution of the egg masses found in the wild to either species was done mainly on the basis of indirect considerations, like depth records and the presence of the hard case. This is particularly evident for the eggs found in the Catalan Sea in December 1958 (Mangold Wirz 1963a, p. 124), only tentatively attributed to *R. macrosoma*, and was confirmed to the authors for the egg clutch found in 1992 by Villanueva in the western Mediterranean, eventually attributed to *N. caroli* (R. Villanueva, personal communication).

DNA sequencing proved to be very useful in identifying species samples at any developmental stage, in particular eggs, spermatophores, larvae, juveniles and supposed cryptic adults, that is to say whenever the available morphological characters are not adequate to allow discrimination to the specific level and/or not adequately developed yet. The use of molecular analysis in the present study proved to be of paramount importance, since it allowed the indisputable attribution of the egg clutch found to the bobtail carol squid, *N. caroli*. Also, the 16S sequence obtained represents the first nucleotide sequence known for the species.

Egg size, arrangement and general structure conform to what is known for eggs spawned by the species belonging to the subfamily Rossiinae (e.g. Boletzky, 1998); in particular, the characteristic thick, hard and highly protective case, so unique for decapod cephalopod eggs, was unmistakable. It was observed that eggs of *Rossia* 'do not decay for several months after hatching of the young animals, nor are they colonized by sessile organisms' (Boletzky & Boletzky, 1973); therefore, they surely offer a remarkable protection to the embryos. Since the only other known hard case formation in coleoids is that of the cirromorph octopod suborder Cirrata, which lay large, individually encapsulated eggs, generally

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at depths over 1000 m (e.g. Boletzky, 1998), the peculiar hard thick covering may be an adaptation to deep water embryonic development (Boletzky, 1994).

The egg white colour observed in the present case does not conform to the 'violet très soutenu, plus foncée que celle de *R. macrosoma'* described by Mangold Wirz (1963a).

However, white eggs are common in the subfamily (e.g. Aldrich & Lu, 1968; Anderson & Shimek, 1994) and it is known that, occasionally, egg case colour may be different from the usual one (e.g. Boletzky et al., 2006). The above mentioned records of *R. macrosoma* in waters clearly deeper than those once considered as its bathymetric upper limit (Rosa et al., 2006), confirm a wider bathymetric distributional range also for the stout bobtail squid; therefore, and albeit unlikely, it is not possible to discount the hypothesis that the egg clutch reported by Mangold Wirz as belonging to *N. caroli*, was in fact a *R. macrosoma* egg clutch.

As for the number and dimensions of mature eggs in the ovaries, our observations agree with what was reported for the Catalan sea animals (Mangold Wirz, 1963a). Unfortunately no comparison with other *N. caroli* populations is possible at the moment.

The high variability of the observed PF, the combined presence of oocytes at different developmental stages and the low number of ripe eggs inside the oviducts, are elements in favour of a 'multiple' spawning pattern, with the production of various egg batches during an extended period of time. Within this frame, the occurrence of mature females with the lowest number of eggs and spermatangia inside the mantle, would testify that these animals had already spawned more than once. Such a 'continuous spawning' strategy, following the classification proposed by Rocha et al. (2001), is typical of the bathyal cirroctopods and was recently described for the common bobtail squid *Sepietta oweniana* (Bello & Deickert, 2003). According to Rocha et al. (2001) this strategy could be considered an adaptive response to 'stable' and relatively 'safe' environments, where predation pressure and competition for resources may be very low. In such environments, i.e. deep, bathyal waters, spawning can be slow and continuous with a limited number of eggs per clutch. The high occurrence of feeding activity in *N. caroli* mature females suggests that somatic growth is not inhibited by the maturation and spawning processes and may continue throughout the whole spawning phase.

As for the spermatangia found embedded in the inner side of the left ventral mantle tissue, this is the first record of the kind for the species. However, the phenomenon itself is rather widely spread among cephalopods (Nesis, 1995), and it affects mostly species known or supposed-to mate and spawn in deep waters. Observations made by several authors (see Nesis, 1995 for details) suggest that the outside attached spermatophores somehow melt the mantle tissue to locate themselves onto the inner mantle surface, close to the oviduct openings or on the way of the eggs from these openings to the outside of the mantle cavity.

In any case and independently from the implantation way this kind of sperm reservoir storage would prove to be among the most effective ones, especially in a situation where mating itself is a fast process and/or can occur quite a long time before spawning.

Even though only further observations will allow to better understand the phenomenon, it can be suggested that the embedded spermatangia found in the present studies represent the most effective way to allow sperm storage, due to the lack of a functional bursa copulatrix in the species and to the mating process itself. This, in fact, is a fast and rather violent event (see also Mangold, 1987), where males, which are smaller than females, have to grasp them from below and transfer as many spermatophores as possible to the mantle cavity, in the shortest possible time-range.

Further studies will help to better frame present results in order to improve our knowledge on the biology of this species, which is still poorly known. Recent concerns (Boyle et al., 1998) about our lack of knowledge on deep-water and bathyal cephalopods, due to the increasing exploitation of the deep sea—the largest single habitat of the planet—underline the importance of this goal.

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