New protocols to improve the deposition and hatching of Sepia officinalis*' eggs*

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Artificial surfaces,
Controlled induction,
Hatching protocols,
Management resource,
Sepia officinalis,
Spawning.

Summary
The objective of this study was the development of hatching protocols in controlled conditions to obtain juveniles, in order to restock and increase the resource of *Sepia officinalis*. The study was divided into the following phases: development and application of artificial surfaces at specific sites of the Molise coast in Italy; induction of eggs hatching and juveniles maintenance under controlled condition; juveniles introduction into specific sites and assessment their increment; experimental data elaboration. The obtained results concerned both the effectiveness of the artificial surfaces tasted during the study and the importance of the recovery of the eggs laid on artificial surfaces (artefacts and fishing gear) for preservation and the management of the *Sepia officinalis* resource. The induction tests conducted on eggs hatching under controlled conditions confirmed what described in the extant literature. Water salinity was detected as the only limiting factor, with values ≤ 20% related to the absence of hatching. The described practices for harvesting and induction of hatching for the production of juvenile cuttlefish may be endorsed by the operators at relatively low cost and throughout the year, with obvious economic benefits.

Nuove procedure per migliorare la deposizione e schiusa di uova di Sepia officinalis

Parole chiave
Deposizione uova,
Gestione della risorsa,
Induzione controllata,
Manufatti artificiali,
Protocolli di schiusa,
Sepia officinalis.

Riassunto
L'obiettivo del presente studio è stato la messa a punto di procedure di schiusa di uova di *Sepia officinalis* in condizioni controllate ai fini dell'ottenimento precoce di giovanili da utilizzare per l'incremento della risorsa di *Sepia officinalis*. Lo studio è stato articolato nelle seguenti fasi: messa a punto ed applicazione di manufatti artificiali in siti specifici della costa molisana; induzione della schiusa delle uova e mantenimento di una parte di giovanili in condizioni controllate; immissioni nei siti specifici di giovanili di seppia e valutazione dell'incremento degli stessi; elaborazione dei dati ottenuti nella fase sperimentale. I risultati hanno riguardato l'efficacia dei diversi substrati impiegati e l'importanza del recupero delle uova deposte sui substrati artificiali (manufatti ed attrezzi di pesca) ai fini della salvaguardia e della gestione della risorsa *Sepia officinalis*. Le prove di induzione della schiusa delle uova in condizioni controllate hanno confermato quanto descritto in letteratura: la salinità si è rilevata unico fattore limitante, infatti a valori ≤20% non si registra alcuna schiusa. Le pratiche di raccolta e di induzione della schiusa descritte potrebbero essere adottate dagli operatori a costi relativamente bassi per la produzione di giovanili di seppia, in periodi dell'anno diversi da quelli del ciclo naturale, con evidente vantaggio economico.

Introduction

The progressive and increasingly worrying depletion of marine fishery resources occurred during the 20th century has resulted in a serious crisis for the entire fishing industry and the connected activities. The Molise small-scale fishery activity is mainly carried out within 3 miles from the coast. The maritime compartment of Termoli (Molise, Italy) involves 20 gross tonnage boats of less than 10 tonnes and is characterized by small, often family-run, businesses which employs about 130 people considering both those engaged in fishing and those participating in the induced activities (23). The small-scale fishery represents about a third of the entire commercial fleet of Molise.

Sepia officinalis (Linnaeus, 1758) is a common cuttlefish and is of particular importance among the species commercialized and caught within 3 miles from the coast. Using static gears (pots, fyke nets, trammel nets), the small-scale fishery is assiduously dedicated to the capture of this species, especially during the spawning period, which coincides with the warmer seasons (spring and autumn) with a peak in spring, in conjunction with the arrival in the coastal areas of the adult specimens from deeper water, and a peak in the autumn before the wintery migration of these molluscs to open sea (2, 16).

The reproductive period of *Sepia officinalis* lasts about 7 months (4, 21). The freshly laid eggs have a diameter between 5 and 9 mm and are fixed by the female to any type of natural (rocks, macroalgae, seagrass) and artificial (ropes, fishing gear, pieces of iron, etc.) substrate.

Development is direct, incubation lasts in average 1 month at 20 °C, however it can spam from 30 to 60 days, depending on the temperature. At the time of hatching the young cuttlefish, which measure about 10 mm (length of the mantle), are already able to hunt independently and assume quickly a benthic lifestyle. The cuttlefish present along the Atlantic coast reach a maximum size of 45 cm and weight of 4 kg, while in the Mediterranean basin they reach a maximum length of 35 cm (usually from 15 to 25 cm). The lifetime is generally between 18 and 24 months although some males can live longer (up to 36 months) (3).

For small fisheries, *Sepia officinalis* is of commercial importance and, consequently, it is necessary to put in place proper management procedures devoted to protect this resource and to increase presence of this species along the shores. Considering the 2004-2007 national catches, the common cuttlefish is 1 of the 9 species with the highest landed amount. During 2007, 7,500 tonnes of cuttlefish were landed in Italy, corresponding to 17% of small fishery catches (19). During the last 5 years, however the landed of *Sepia officinalis* decreased markedly. For example, in 2012 a decrease of 10% in terms of both quantity and price was registered [4,172 tonnes caught with a turnover of 35.94 million euro was detected with respect to 2011 (24)].

Several studies have been conducted (7, 10, 25, 27, 29) focusing on the distribution, biology, physiology and ethology of this species and on techniques for innovative fishing and protection (1, 6). Of particular interest are those studies related to the development of the resource such as the use of new artificial substrates for eggs collection (1). It has been previously reported (3) that the choice of deposition surfaces depends on female preferences: usually cuttlefishes lay eggs on long surfaces with a diameter lower than 10 mm. In this respect, the research analysing the conditions for embryos development and growth both in captivity and in nature is particularly worthwhile (12, 14, 15, 17, 18, 20, 22, 28).

In the present study, we have developed and tested new artificial substrates for egg collection and hatching controlled induction in order to obtain organisms for resource restocking.

Materials and methods

Identification of sites

The seabed typology along the coast of Molise was evaluated through diving floor survey. The survey showed that the seabed is sandy, muddy or rocky-sandy. Cuttlefish most populated areas were chosen to place the artificial substrates: 2 areas were in the Northern part of Termoli city ('Skyscraper' and 'Starfish') and 2 in the Southern part ('Rio vivo' and 'Campomarino Lido').

Development and positioning of artificial substrates

On the basis of previous research (27), two easy handling and long lasting artificial substrates were produced to collect eggs and reduce fisheries losses (due to nets, ropes, etc.): modified cuttle-traps and modified grids, armed with ropes of various sizes (diameter of 8 mm, 10 mm and 12 mm) and different colours (red and white with black dots), with and without floatation devices (Figures 1, 2 and 3).

In May 2006, we placed the artificial substrates (modified cuttle-traps and modified grids) on the selected areas in the Northern part of Termoli, in order to evaluate their efficiency and, if necessary, endurance. Until November 2006, we weekly controlled the anchorage and the status of substrates and counted the laid eggs. In March 2007, we positioned 5 modified cuttle-traps at the 'Starfish' site for 6 months.

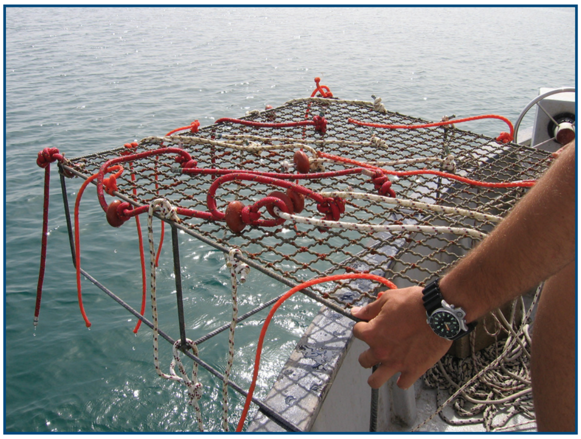

Figure 1. *Metallic grid with different ropes.*

Figure 3. *Cuttletraps with 8 mm ropes.*

In May 2008, 5 modified cuttle-traps were placed in the 'Campomarino Lido' site. The substrates were kept there until July 2008 when, due to scanty deposition, they were moved to the 'Starfish' site and left there until November 2008. In October 2008, 8 modified cuttle-traps were placed in the 'Skyscraper' and 'Rio Vivo' sites. The activities of these traps lasted until November the 10th 2008, when all substrates were removed and taken to laboratory for storage and winter maintenance.

In February 2009, 5 modified cuttle-traps were placed in the 'Starfish' site until May 2009.

Surveys of *Sepia officinalis* catches

The assessment of the amount of cuttlefish along Termoli coastline was determined by daily catches operated by small commercial fishing. The amount of cuttlefish was assessed according to the area and distribution. Despite the fact that the resource is available throughout the year, investigations concerned only spawning periods (March-October 2007 and February-May 2008).

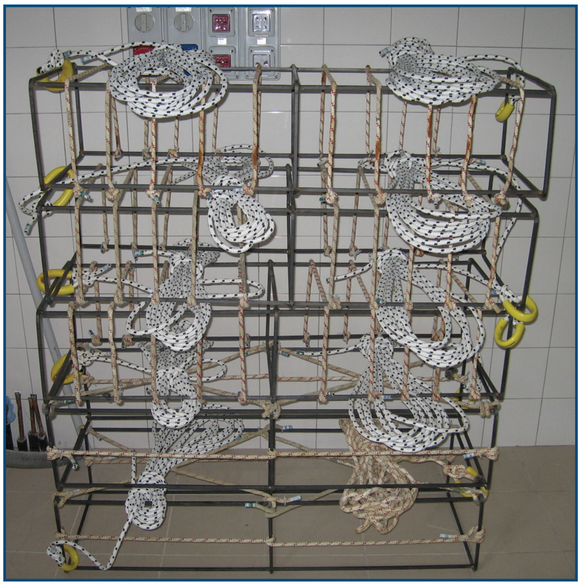

Figure 2. *Cuttletraps.*

Induction of egg hatching

Hatching test in aquaculture plant

In June 2006, we collected and transported about 2000 cuttlefish eggs to the local aquaculture plant named Ittica Molisana (Campomarino, Molise, Italy). The ropes with the egg clusters were placed vertically in special racks and were planted in a circular fiberglass pool of 20 m^3 capacity in open circuit. During the test the water salinity, due to problems related to plant water intake, decreased significantly (<18‰). Therefore, it was deemed useful to transfer the experiments in laboratory to follow the hatching in small volumes: a batch of about 100 eggs was collected, transferred to the laboratory and planted in 2 racks placed in 2 tanks of 60 l.

Hatching test in hatchery

In the period January-March 2007, the hatcheries for incubation of large volumes of eggs were set up and prepared, under controlled conditions of temperature and salinity at Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale', Centro Sperimentale Regionale per la Pesca ed Acquacoltura of Termoli. The hatchery (horizontal trough) is composed of 2 rectangular tanks with bottom flat and 220 l of capacity, each containing 7 racks with a maximum capacity of 1,500/2,000 eggs corresponding to about 1.5 kg of weight.

The eggs retrieval concerned 5 artefacts placed on the site 'Starfish' and was conducted with the cooperation of Termoli small fishery operators. Eggs placed in the tanks were taken between March the 27th and 9th April the 19th 2007, at a temperature of 12-15°C and transported to the laboratory.

Table I. *Eggs positioning for hatching tests in hatchery during 2007.*

N° rack	Eggs number	Introduction date
1	1,762	27/03/2007
2	790	28/03/2007
3	1,001	29/03/2007
4	2,839	30/03/2007
5	1,309	02/04/2007
6	2,532	05/04/2007
7	1,032	18/04/2007
8	2,381	19/04/2007
9	2,026	19/04/2007
10	899	19/04/2007
11	1,502	19/04/2007

After a few hours of conditioning, eggs were transferred into tanks with water at a temperature of 20 ± 1°C, salinity of 38 ± 1‰ and dissolved oxygen concentration above 8 mg/l. The eggs were counted, measured (length and width) and positioned in the racks (Table I).

Hatching test in acquarium

Four hundred eggs (divided in 8 lots consisting of 50 eggs each) were taken from the traps placed in the 'Starfish' site on September the 12th and on October the 10th 2008. The collected eggs were transported to the laboratory to carry out 2 hatching tests under controlled conditions. In both tests, eggs were placed in 4 small racks suspended in 4 aquaria with a capacity of 80 l. In the first test of hatching, the following parameters were set: temperature of 20 ± 1°C and salinity of 20‰, 25‰, 30‰ and 35‰. The second test was performed at 2 values of salinity (25‰ and 30‰) and at 2 temperatures (20 ± 1°C and 25 ± 1°C). In May 2009, a test was repeated under the same experimental conditions of the first hatching test conducted in 2008.

Maintaining of juveniles under controlled conditions

In the maintenance phase, juveniles were daily fed *ad libitum* with live food consisting of adult *Artemia salina*. They were produced by incubation of cysts under controlled conditions (salinity: 26.5‰, temperature: 27 ± 1°C, pH: > 8, surface brightness: 1,800/2,000 lux; density of incubation: 1.5 g of cysts/litre) with a final hatching percentage close to 70% after 36 hrs. The *nauplii* were then transferred into cylinder-conical tanks of 250 l capacity equipped with aeration (salinity: 37‰; temperature: 25 ± 1°C, pH: > 8; breeding density: 8000 nauplii/l, concentration dissolved oxygen: > 7 mg/l). In the growth phase, *Artemia salina* was fed on phiytoplankton coltures (*Chaetoceros calcitrans, Phaeodactylum tricornutum* and *Tetraselmis suecica, Isochrysis galbana, Dunaliella tertiolecta*) and yeast (*Saccharomyces cerevisiae*), after 16 days adults of *Artemia salina* were obtained to be used as food. This feeding mode has been selected on the basis of previous studies (5, 8, 9, 11, 13).

Releasing of post-hatching juvaniles

The cattlefish juveniles obtained in the laboratory hatching trial performed in 2007 were released in the 'Starfish' site near the bottom sea at 4 different sowing times 72-96 hrs after the hatching event. The site was selected as a poorly productive site for *Sepia officinalis* according to local the fishery operators.

In July and September 2007, 4 sea samplings were performed in the restocking area to evaluate the efficacy of the releasing. The fishing was carried out with gillnet fine mesh of 22 mm and 400 m in length sailed after 48 hrs. For each sampling, all captured *Sepia officinalis* juveniles were counted and measured.

Statistics

In order to assess the effect of the different types of artificial substrate on the amount of laid eggs, analysis of variance (ANOVA one-way, type of artificial substrate) was performed on the data concerning both the eggs deposited on the racks and on the modified pots at the 'Starfish' site in 2006. Furthermore, in order to evaluate the effect of the different size of the ropes on the amount of laid eggs, an analysis with a single source of variability (diameter of the ropes) was performed on dataset relating to depositions number on modified cuttle-traps.

Data of hatching tests with a salinity of 20‰, 25‰, 30‰ and 35‰ conducted in the aquaria during 2008 and 2009 were analysed (ANOVA one-way, salinity) in order to evaluate the effect of this parameter on the number of hatched eggs. When differences were significant, a post hoc Tukey's test was conducted.

The analysis was conducted on transformed data (square root) using the SPSS 22.0 software.

Results and Discussion

Selection of the substrates

The endurance and efficiency of 'grids' and 'modified cuttle-traps' were compared; grids had the disadvantage of poor handling, high production costs and tendency of silting also with low surges, whereas modified cuttle-traps, showed a reduced tendency of silting and reduced resistance to oxidative processes.

Data of 2006 deposition events on grids and modified cuttle-traps have been compared in order to assess the effect of the different types of substrates on the amount of laid eggs. Considering that a unique deposition event was recorded in the 'Skyscraper' site (150 eggs on July the 25th 2006), statistical analyses were performed only with respect to the deposition data of the 'Starfish' site. Significant differences were recorded in number of eggs laid on different substrates, with significantly higher values obtained on traps compared to grids (one-way ANOVA, $p < 0.001$). Consequently, the 'modified cuttle-trap' a parallelepiped of 30x45x120 cm dimension, iron built with a diameter of 10 mm was selected for the following trials.

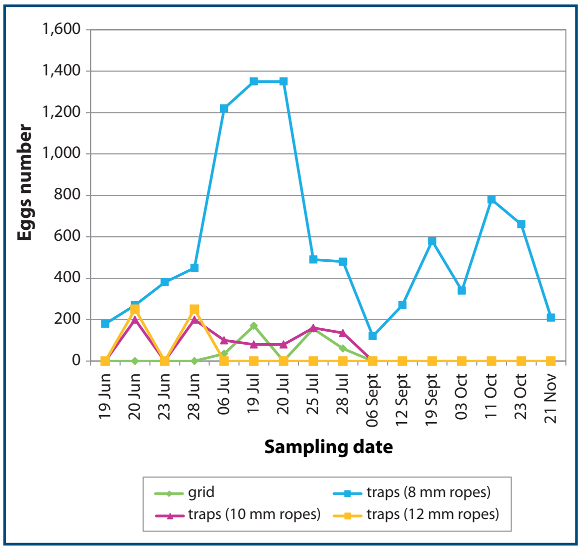

Figure 4. *Temporal variations of eggs hatching on artificial substrates in 'Starfish' site during 2006.*

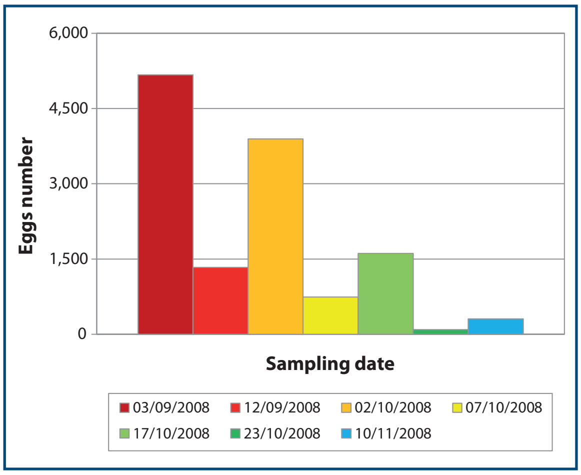

Figure 5. *Eggs laid number on cuttletraps in 'Starfish' site during 2008.*

In addition, statistical analysis showed that egg deposition was significantly greater on 8 mm diameter ropes than on those with a diameter of 10 cm and 12 cm (Tukey test, $p < 0.001$). Whereas, the number of eggs laid on ropes with a 10 cm and 12 cm diameter was not significantly different (Tukey test, $p < 0.001$). Finally, the egg amount on different artificial substrates varied according to the monitoring period (Figure 4).

In 2007, the deposition found on 8 mm diameter rope traps placed on the 'Starfish' site was greater than that reported in the 2006 monitoring (1,871 vs 571 eggs on average).

In 2008, the trials included 'Starfish', 'Campomarino Lido', 'Rio Vivo' and 'Skyscraper' sites (Figures 5, 6, 7, 8). The highest number of eggs was laid on the 'Starfish' site (1,878, 218, 63 and 450 eggs on average, for 'Starfish', 'Campomarino Lido', 'Rio Vivo' and 'Skyscraper, respectively').

Furthermore, the amount of eggs laid on 'Starfish' site during late winter-early spring of 2009 was much lower than the one collected in same period of 2007

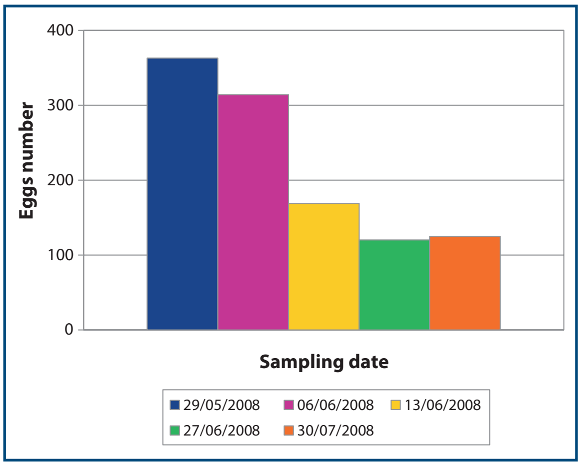

Figure 6. *Eggs laid number on cuttletraps in 'Campomarino Lido' site during 2008.*

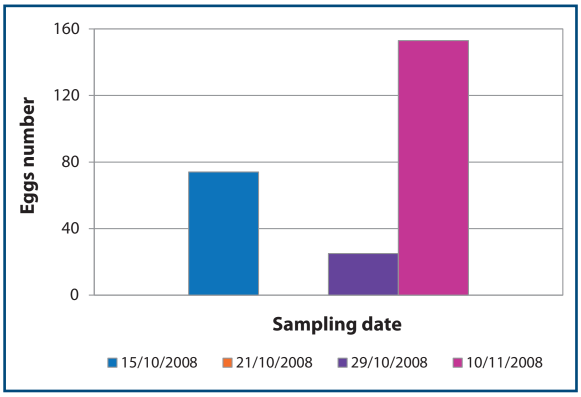

Figure 7. *Eggs laid number on cuttletraps in 'Rio vivo' site during 2008.*

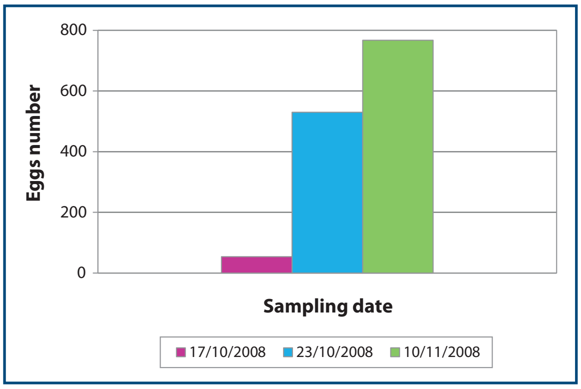

Figure 8. *Eggs laid number on cuttletraps in 'Skyscraper' site during 2008.*

Table II. *Catched specimens during 2007 samplings.*

Catch area	N° catched specimens	Average of specimens in each sampling
Saccione (Southern Coast)	1,900	95
Vallone 2 miles (Southern Coast)	4,000	200
Rio vivo (Southern Coast)	260	13
Cala Saraceni Sud (Northern Coast)	1,120	56
Cala Saraceni Nord (Northern Coast)	1,640	82
Modenese (Northern Coast)	1,800	90

Table III. *Catched specimens during 2008 samplings.*

Catch area	N° catched specimens	Average of specimens in each sampling
Vallone 2 miles (Southern Coast)	3,624	151
Porto di Campomarino (Southern Coast)	4,056	169
Cala Saraceni Nord (Northern Coast)	3,360	140
Torre diroccata (Northern Coast)	3,216	134
Modenese (Northern Coast)	4,224	176

(946 vs 1,878 eggs on average). This was probably due to adverse weather and sea conditions.

Surveys of *Sepia officinalis* catches

Considering the surveys conducted in 2007, the Northern part of Termoli fishing areas were less profitable than those located in the Southern part of the city. *Sepia officinalis* showed a not uniform distribution in the inshore areas (Table II). In contrast, in 2008, a more uniform spatial distribution was observed (Table III). Considering sample average value for each survey, the amount of cuttlefish caught in the Northern Termoli areas was higher than the one recorded in the previous year and almost similar to that captured in the Southern areas.

Induction of egg hatching

Test of hatching induction conducted in 2006 at the Ittica Molisana aquaculture farm highlighted that, in order to have an optimal hatching rate, the chemical-physical parameters such as water sea temperature and salinity should be within specific ranges (18-25°C for temperature and 30-35‰ of salinity). In fact, when water salinity decreased significantly (< 18‰) due to water intake problems, eggs regressed in size and eggs' maturation stopped. Consequently, the test was stopped and 100 eggs were transferred to laboratory, where complete hatching occurred in 10 days. Juveniles were fed on adult Artemia salina after 24 hrs for about 3 days and were released in the 'Starfish' site 4 days after hatching.

With reference to the induction test conducted in the hatchery during March 2007, the first hatching events occurred after about 30 days from incubation and continued for 19 days. Hatching rate was 35% for a total of 6,351 live organisms (Figure 9).

This study also demonstrated that salinity affects the hatching rate. In fact, the amount of hatched eggs significantly changed according to different salinity levels (Tukey test, $p < 0.05$). Minimum values of hatching (0%) was recorded at a salinity of 20‰, while maximum (> 90%) at 30‰ (Figures 10 and 11).

In tests conducted in aquarium at 2 salinities levels (25‰ and 30‰) and 2 temperatures (21°C and 25°C), hatching percentages ranged from 84% (for the batch incubated at 21°C and 25‰ of salinity) and 98% (for the batch incubated at 21°C and 30‰ of salinity) (Figure 12).

Furthermore, comparing tests at 25‰ of salinity, egg hatching started after 17 and 11 days, respectively at temperatures of 21 and 25°C, while in tests at salinity of 30‰, it started after 11 and 7 days respectively at 21 and 25°C. These data suggest that temperature affects the length of incubation period, as reported in previous studies (15).

Finally, evaluating different percentages of hatching obtained in tests conducted in 2007, 2008 and 2009 at similar conditions (20°C, 35-38‰), it is noteworthy that hatching values obtained in hatchery (35%) were notably lower than those in aquarium (80% in 2008 and 84% in 2009).

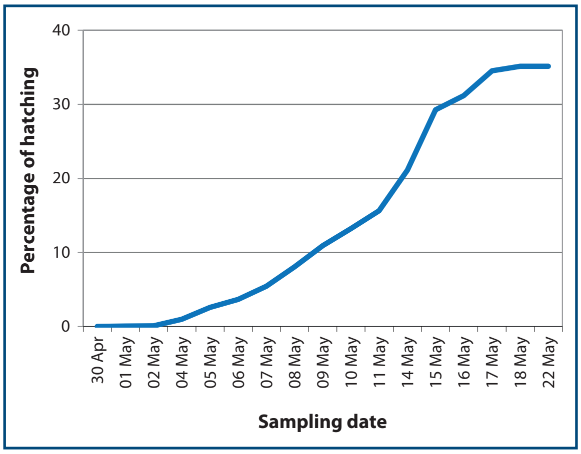

Figure 9. *Hatching trend during 2007 experimental test.*

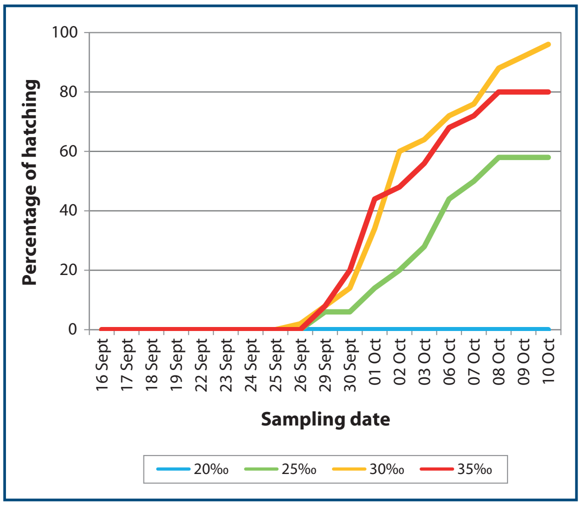

Figure 10. *Hatching trends during 2008 experimental tests at 20‰, 25‰, 30‰ and 35‰ of salinity.*

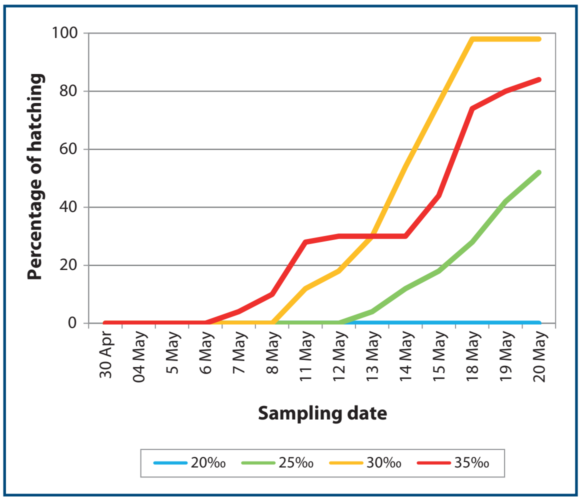

Figure 11. *Hatching trends during 2009 experimental tests at 20‰, 25‰, 30‰ and 35‰ of salinity.*

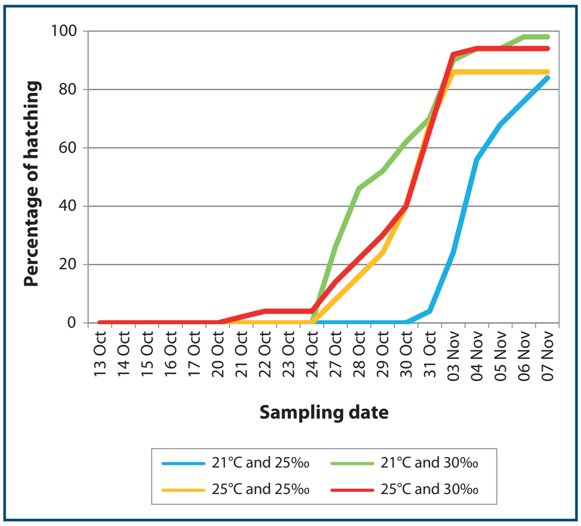

Figure 12. *Hatching trends during 2008 experimental tests at temperature of 21°C, 25°C and salinity of 25‰, 30‰.*

Introduction of post-hatching juveniles

During samplings carried out 2 and 4 months after introduction, about 600 specimens of *Sepia officinalis* with size compatible to the 6,321 individuals previously introduced were captured. Considering that the restocking site had been previously reported by fishing operators as a non-productive area, specimens could be presumably attributed to the juveniles entered.

Conclusions

The results of this study provided information on effectiveness of different substrates and importance of recovery of eggs laid on artificial substrates for preservation and management of *Sepia officinalis* resource. The tests of hatching induction, carried out in this study under controlled conditions, confirmed what has already been reported in the extant literature: the water salinity was the unique limiting factor, in fact hatching events were not recorded at salinity lower than 20‰.

The techniques of collecting and hatching induction described in this study could be adopted by operators in order to lower the costs for the production of juveniles throughout the year independently from the natural cycle, with obvious economic benefits.

Finally, it would be useful to develop protocols for the growth and marking of specimens, in order to provide more accurate quantitative estimates of the increasing of resource following restocking.

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