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A first survey on the biochemical composition of egg yolk and lysozyme-like activity of egg envelopment in the cuttlefish *Sepia officinalis* from the Northern Adriatic Sea (Italy)

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ABSTRACT

The cuttlefish *Sepia officinalis* is an important fishery resource in the Northern Adriatic Sea (Italy). During reproduction, fertilised eggs are released by adult females in coastal waters and embryo development can take over two months. During this period, embryos rely on nutrients and other substances, such as immune factors, provided by the female in egg yolk. In cephalopods in general, and specifically in the common cuttlefish, little information is available on yolk biochemical composition and substances included in egg envelopment. In the present study, the main biochemical components of egg yolk and the presence of antimicrobial substances in egg envelopment of *S. officinalis* were determined for the first time. Statistically significant differences in total egg weight and egg yolk weight were observed among batches from different females. Egg and yolk weights were positively correlated, with yolk representing the 13% (±5%) of the total egg weight. Total proteins were the main biochemical component (46%) of egg yolk, followed by total carbohydrates plus glycogen (39%) and lipids (15%). Statistically significant differences among batches were recorded in egg yolk total protein amounts, lipids, carbohydrates and glycogen, but no correlations were found between egg yolk weight and the biochemical components. The Petri dish and the quantitative spectrophotometric assays revealed the presence of lysozyme-like activity in egg gelatinous envelopment.

1. Introduction

Cephalopods are important components of marine coastal ecosystems, from both an ecological and commercial point of view [1–4]. In the Northern Adriatic Sea (Italy), the cuttlefish *Sepia officinalis* is an important halieutic resource, representing one of the main demersal resources [5]. Regarding reproductive strategies, *S. officinalis* is considered to be an intermittent terminal spawner: egg laying occurs in separate batches throughout the spawning period, after which cuttlefish die [6]. The spawning period occurs from early spring to summer but may shift depending on variations in environmental conditions, such as milder or cooler winters [7]. Usually, it involves two groups of reproducers of different ages that spawn in consecutive periods during the same reproductive season. Spawner somatic growth does not occur between spawning events and females show group-synchronous ovulation with at least two groups of oocytes at different stages of development [6]. The maturation of previtellogenic oocytes is likely to continue throughout the spawning period and allows females to spawn about 3000 eggs in captivity [8–10].

Eggs of *S. officinalis* consist of three main components that are easily distinguishable, from the inside to the outside: (1) the mature oocyte, surrounded by the chorion, which is embedded in (2) gelatinous mucosubstances released by the oviducal gland and finally encapsulated by (3) several membranes secreted by the nidamental gland and stained with ink [11]. The outer capsule protects the embryo from the surrounding environment [12] and hardens during the first days following deposition. The dehydration of the gelatinous capsule is responsible for an initial decrease in egg size after deposition, whereas a subsequent increase in size is due to the water diffusion into the hypertonic perivitelline space [13–16]. Eggs, and in particular yolk, provide the main constituents for embryonic development, such as proteins, lipids and carbohydrates which support the formation of cell membranes and

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extracellular structures and further operate as energy storages [17]. According to Bouchaud and Galois [17], maternal nutritional history is likely to affect the survival of early life stages through the quantity and quality of yolk allocated per egg, and nutrient allocation can vary according to local variations of environmental parameters, that influence food availability to mature females. Environmental parameters can therefore affect female energy reserves, maintenance demands and their overall physiological status. While the size of eggs is generally species-specific, it may vary in response to different environmental conditions [18,19] and an increase in egg size with a decrease in water temperature was described for different aquatic species [19].

In lecitotrophs, egg size is often positively related to the size of embryos or larvae and may influence the performance of juveniles [20,21]. In cephalopods, high flexibility in the reproductive strategy occurs and some egg characteristics, such as size, biochemical composition or energy content, can vary markedly according to environmental conditions [12,22]. However, a small number of studies have investigated the biochemical composition of cephalopod eggs during the embryonic development [23], and few surveys have focused on the biochemical composition of egg yolk in cuttlefish species [16,17]. In addition to nutrients, mothers can provide eggs with substances acting as antimicrobials and therefore enhancing hatching success [15,24,25]. Such antimicrobial substances can be secreted around the mature oocyte providing immunosurveillance during embryo development [24,26].

The aim of this study was to obtain first information about the nutritional content available to the early life stages of *S. officinalis*. The main biochemical components (total protein, lipid and carbohydrate) of egg yolk were measured, and correlations with egg yolk weight were evaluated. Moreover, we investigated the presence of antimicrobial activity (lysozyme-like activity) in egg gelatinous envelopment.

2. Materials and methods

2.1. Cuttlefish sampling and egg yolk collection

Specimens of *S. officinalis* were sampled during the reproductive period - from April to June 2013 - by means of traps along the west coast of the Northern Adriatic Sea (near Chioggia, Italy). Both females and males were transported to the laboratory in containers with aerated seawater and kept in outdoor plastic tanks (55 × 38 × 30 cm) containing about 50 L of aerated seawater. A flow-through system was used and each tank was equipped with PVC curved panels to allow cuttlefish to refuge and a rope for egg deposition. Tanks were gently cleaned daily without removing animals, thus minimising possible stress. Each specimen was fed with one crab (*Carcinus aestuarii*) per day and eggs were removed daily. The eggs used for the biochemical analyses were deposited between 2 and 4 weeks after the capture of adult cuttlefish and only eggs attached to the suspended ropes or to the aerator tube were used, whereas eggs left on the bottom of the tanks or with anomalous capsules were discarded.

In this study, 12 different cuttlefish females (length size range: 114–173 mm) were employed and 15 eggs were collected randomly from each of 25 batches differing in average egg size (average egg size range: 5.11–10.58 mm, expressed as egg width measured from one to the other side of the egg and perpendicularly to the major axis). Before egg yolk collection, total wet weight (including yolk, capsule and the perivitelline liquid) of individual eggs was measured with an analytical balance (Mettler Toledo XS 105 DualRange). In order to determine the biochemical components, three pools of egg yolks (from five eggs each, for each batch) were used. Egg yolk was obtained by removing carefully gelatinous envelopes that constitute the egg capsule (see Fig. 1 and the video provided as supplementary file). Egg yolk wet weight was immediately measured. Egg yolks were then placed on ice and pooled in 5 mL of distilled water. Pooled egg yolks were then homogenised by an UltraTurrax T10 (Ika) and 4 aliquots (1 mL for protein assay, 1 mL for carbohydrate assay, 1 mL for glycogen assay and 1,2 mL for lipid assay) were prepared, frozen in liquid nitrogen and stored at −20 °C until the analyses.

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2.2. Biochemical analyses

Total lipid levels were extracted [27] and then quantified according to the method of Pande et al. [28]. Absorbance was measured at 590 nm and a standard curve of stearic acid was prepared.

Total protein content was measured according to the colorimetric method of Lowry et al. [29], the absorbance was measured at 590 nm and a standard curve of bovine serum albumin (BSA) was prepared.

Carbohydrate and glycogen levels were determined by the colorimetric method of DuBois et al. [30]. Absorbance was measured at 490 nm and a standard curve of glucose was prepared.

For each egg yolk pool, the measurement of the biochemical component was repeated three times. Results are expressed as μg/mg egg yolk wet weight.

2.3. Determination of lysozyme-like activity in egg gelatinous envelopment

Lysozyme-like activity was determined in egg gelatinous envelopment samples by means of two assays, the first qualitative (Petri dish assay) and the second quantitative (turbidimetric assay). Three pools of gelatinous envelopment samples (from 5 eggs each) for each female (5 females in total) were used. Samples were thawed on ice and homogenised in five volumes of 0.1 M Tris–HCl buffer, pH 7.5, containing 0.15 M KCl, 0.5 M sucrose, 1 mM EDTA, 1 mM dithiothreitol (DTT; Sigma) and 40 μg/mL aprotinin (Sigma). Samples were then sonicated for 30 s at 0 °C with a Braun Labsonic U sonifier at 50% duty cycles and centrifuged at 10,000 × g for 30 min at 4 °C. Supernatants (SN) were collected for assays.

Enzymatic assay on Petri dishes was performed as described in Matozzo et al. [31]. Briefly, 1% of methylene blue 0.13% in distilled water was added to a 0.25% suspension of *Micrococcus lysodeikticus* (Sigma) in 0.1 M phosphate/citrate buffer, pH 5.8, and incubated for 30 min at 25 °C to stain cells. Seven mL of the stained *Micrococcus* suspension were then added to 50 mL of 1.5% agarose, previously heated in 0.1 M phosphate/citrate buffer, pH 5.8. Five mL of this solution were then poured into Petri dishes. When the agarose has become solid, 5 mm wells were filled with 50 μL of SN. Lysis ring diameter was observed after overnight incubation at 37 °C and compared with those obtained from a known solution of lysozyme (1 mg/mL) from chicken egg white (Sigma) in 0.1 M phosphate/citrate buffer, pH 5.8.

In the spectrophotometric assay, 50 μL of SN were added to 950 μL of a 0.15% suspension of *M. lysodeikticus* (Sigma) in 66 mM phosphate buffer, pH 6.2, and the decrease in absorbance (ΔA/min) was continuously recorded at 450 nm for 5 min at 20 °C. Standard solutions containing 1, 2.5, 5 and 10 μg lysozyme per mL of 66 mM phosphate buffer, pH 6.2, were prepared from crystalline chicken egg white lysozyme (Sigma). The average decrease in absorbance per minute was determined for each enzyme solution and a standard curve of enzyme concentration versus ΔA/min was drawn.

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