

A study of the electrical polarization of *Sepia officinalis* yolk envelope, a role for Na⁺/K⁺-ATPases in osmoregulation?

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The cuttlefish *Sepia officinalis* mate and spawn in the intertidal zone where eggs are exposed during low tide to osmotic stress. Embryonic outer yolk sac is a putative site for osmoregulation of young *S. officinalis* embryos. By using electrophysiological recordings and immunostaining we showed, (i) that the chorion is only a passive barrier for ions, since large molecules could not pass through it, (ii) that a complex transepithelial potential difference occurs through the yolk epithelium, (iii) that ionocyte-like cells and Na⁺/K⁺-ATPases were localized in the yolk epithelium and (iv) that ouabain sensitive Na⁺/K⁺-ATPase activity could participate to this yolk polarization. These data warrant further study on the role of ion transport systems of this epithelium in the osmoregulation processes in *S. officinalis* embryos.

Introduction

Marine organisms exist in osmotic balance with their environment. Seawater salinity is environmental variable that imposes physiological limits for the embryonic development of marine organism and the osmotic stresses acting on species depend upon individual adaptations. The regulation process is controlled by specialized cells called ionocytes (or mitochondria-rich-cells) located in numerous organs essentially gills, kidney and/or tegument. Ion movements are mediated by different enzymes, primarily Na⁺/K⁺-ATPase located in the basolateral or apical membranes of the ionocytes.^{1,2} The Na⁺/K⁺-ATPase is an ubiquitous plasma membrane pump whose enzymatic activities constitute some of the principal means through which all animal cells convert the energy embodied in ATP into electrochemical gradients that can be exploited by all manner of metabolic pathways;¹ its structure is adapted in marine organisms.³

Among cephalopods, the common cuttlefish *Sepia officinalis* mate and spawn in the coastal zone.^{4,5} Eggs are fixed on hard substrata, in the intertidal zone, and are exposed during low tide to osmotic stress for all their embryonic development. Embryos are protected by an eggshell composed of several black envelopes that can prevent physical damage due to desiccation and can limit osmotic problems due to variation in salinity. As the embryo grows, the eggshell becomes thinner and permeable to seawater, which allows the supply of the various ions and respiratory gas required by the embryo.^{6,7}

The influence of salinity stress on embryonic development and hatching success in cephalopods have been evidenced by numerous authors.⁸⁻¹² Increases in salinity can produce smaller embryos with less ability to swim and predate, decreasing the individuals' chances of survival.¹³ Cephalopods are less efficient in osmoregulation than fish: their tolerance to salinity variation is weak and their development is rapidly affected by an increase or a decrease of 10% around their optimal. Nevertheless salinity ranges for embryonic development and hatching success are species specific.^{9-11,14}

In adult fish, the gills are well established as being the predominant site of osmoregulation.^{15,16} Unlike adults, the early stages of fish show alternative sites for ion-regulation among them extra branchial integument that has a major role in the embryo and the post-hatching stages.^{16,17} The integument comprises the head, the trunk and fins and the yolk sac which was shown to be a major site for osmoregulation in euryhaline teleosts by numerous ionocytes located in the epithelium.¹⁸⁻²⁰

In adult cephalopods, including the cuttlefish *S. officinalis*, ionocytes and Na⁺/K⁺-ATPase have been reported in gills essentially but also in excretory organs suggesting a role of these organs in ion and acid-base regulation.²¹⁻²³ A recent study on two species of cephalopods (*Loligo vulgaris* and *Sepia officinalis*) has shown a role of gills and Na⁺/K⁺-ATPase in the embryo before hatching showing the importance of osmoregulation process during the development. Differences between the two

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Results and Discussion

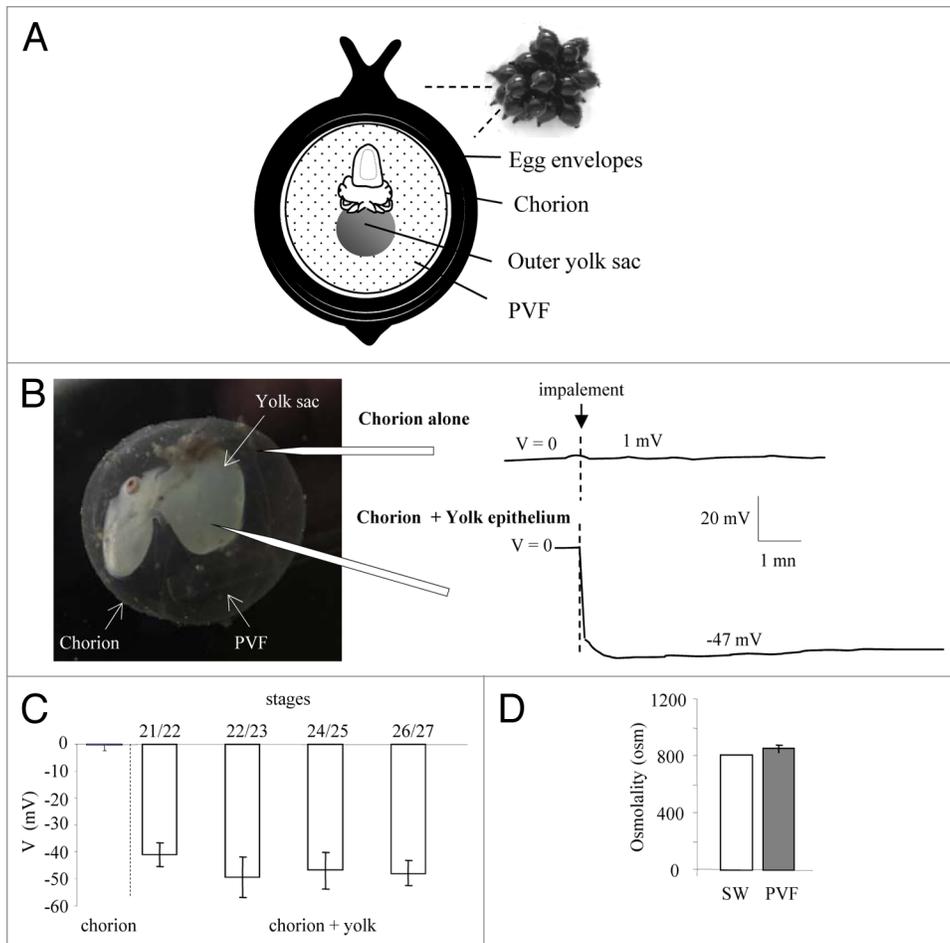


Figure 1. (A) Egg elements surrounding the *Sepia* embryo during its direct development: embryo bathed in the perivitelline fluid (PVF) is protected by chorion and several dark envelopes. (B) *Sepia* embryo inside its chorion. Upper arrow indicates the position of the microelectrode for measuring difference of potential (V) across chorion. Diagram up: Recording of potential difference upon insertion of the microelectrode in the perivitelline space. Diagram down: Recording of potential changes upon insertion of the microelectrode in the outer yolk sac through the chorion. (C) Mean values of potential difference for chorion alone and yolk with chorion between stages 21 and 27. (D) Osmolality of the perivitelline fluid (PVF) and sea water (SW). The data correspond to the means of at least 5 replicates and error bars correspond to standard deviations.

species studied probably in relation to their lifestyle have been evidenced.²⁴ But in cephalopod embryos, protected from the external environment by the egg envelopes and chorion, the importance of the integument, including the outer yolk sac epithelium is poorly understood. Recently, ionic and acid-base regulation through Na^+/H^+ exchangers (NHE3) was described for the yolk sac of cephalopod embryos.^{25,26}

In this context, we decided to explore how these compartments and limits (chorion and perivitelline envelope) intervene in the ion movements, in order to compare the mechanisms involved in fish and cephalopods and between tissues within the same organism. Na^+/K^+ -ATPase is involved in plasma membrane polarization^{27,28} and regarded as an excellent marker for the ion-regulatory capacity of a given tissue.²⁹ We thus undertook an electrophysiological study of the chorion and yolk sac envelope of *Sepia officinalis* eggs and further searched for putative role of Na^+/K^+ -ATPases.

A first set of electrophysiological experiments was conducted on embryos surrounded by their chorion. When the electrode tip was located through the chorion in the perivitelline space (Fig. 1A,B), we failed to record any increase in resistance and variation in transmembrane electrical potential (Fig. 1B,C) suggesting that sea water can diffuse freely through the chorion in accordance with the iso-osmolality recorded for the perivitelline fluid and the sea water (Fig. 1D). These results show that there is no direct regulation by the chorion and dark envelopes. These are passive barriers for ionic movements. When the tip of the electrode just began to penetrate the yolk we sometime record instable voltage values around -60 mV (data not shown). On the other hand we recorded an abrupt shift of voltage when the electrode penetrated into the yolk. In this configuration, we recorded an increase in resistance and a clear negative electrical gradient across the yolk epithelia (Fig. 1B,C). The outer yolk sac envelope consists of an epidermal layer, cell layers delimitating the blood sinus/blood lacunae that lines the yolk syncytium. The inner epithelium is in direct contact with the yolk whereas the outer epithelium is in direct contact with the perivitelline fluid.³⁰ Thus, the

voltage we recorded probably corresponds to a complex potential difference across several epithelia (TEsPD). The TEsPD recorded were closed to -45 mV and constant for the embryos between stages 21 to 27 (Fig. 1C) suggesting that the size reduction of the yolk implying modifications of the epithelia during the development has no consequence on the TEsPD. The second set of experiments conducted after removal of the chorion (Fig. 2A) displays TEsPD of the same order of magnitude than the one recorded with the chorion (Fig. 2A,B). These results confirm that the chorion consists in a passive barrier for ionic movements and that the yolk epithelia are involved in ionic exchanges. We further examine if the TEsPD observed vary when embryos were submitted to variable salinity conditions. Whatever the hypo— or hypersalinity tested embryos at stage 21/22 and 25 preserved a TEsPD around -45 mV (Fig. 2C). However, the yolk polarization and the stable values of TEsPD

upon variable salinity conditions strongly suggest the existence of mechanisms for ionic regulation. In fish, several studies have evidenced a role of Na^+/K^+ -ATPase in cells of the yolk epithelium.¹⁸⁻²⁰ The Na^+/K^+ -ATPase should be preferentially located in membranes of specialized cells called ionocytes and known to be involved in ionic regulation processes.^{24,31} In *S. officinalis*, such cells and their crucial role in osmoregulation have been evidenced.^{24,26} We therefore search for presence of putative ionocytes on *Sepia* embryo yolk. Light microscopic observations of the yolk surrounding structure show a continuous cellular layer on all the surface of the yolk without any heterogeneity in the density or size of the cells as observed with DAPI staining (Fig. 3A). The silver staining procedure of the yolk epithelium revealed brownish patch indicating large amounts of proteins on a restricted surface (Fig. 3B,C) which could correspond to ionocyte-like cells.²⁶ The patch is located on the ventral side of the yolk near the junction between the external yolk sac and the internal one. Positive immunostaining of patch of cells on yolk epithelium (Fig. 4A,B), using antibody H300 evidences the presence of Na^+/K^+ -ATPases. The NaK-ATPase α subunit monoclonal antibody ($\alpha 5$ antibody available through the Development Studies Hybridoma Bank, Iowa City, IA) gave the same results (data not shown). We thus checked if Na^+/K^+ -ATPase could be functional on the yolk epithelium of *S. officinalis*. Na^+/K^+ -ATPase activity is effectively known to be involved in TEsPD of most of moist glands, ducts and vessels of all animal.³² By using ouabain at 100 μM , a well known Na^+/K^+ -ATPase inhibitor,^{28,33,34} we recorded when the yolk is impaled, a rapid depolarization of $+16 \pm 4$ mV ($n = 5$) of the yolk (Fig. 5A,C) in the embryo without chorion. It is to be noted that this depolarization was not recorded when the yolk was impaled in presence of the chorion (Fig. 5B,C), suggesting that ouabain did not reach the Na^+/K^+ -ATPases and that the chorion is not permeable to such molecules. These envelopes are known to play a role as a physical barrier for some molecules, with an evolution of the selectivity as the same time the membranes thickened.^{35,36} These observations are also consistent with previous reports indicating that metals are bound to the chorion ensuring a protection of fish egg embryos³⁷⁻⁴⁰ or *Daphnia magna* eggs.⁴¹

This depolarization was observed for crab gills epithelia treated with ouabain and the authors suggested Na^+/K^+ -ATPases as the main driving force for ion extrusion during salinity stress response.⁴² Similarly, although the TEsPD reflect the functioning of a complex structure, the efficiency of ouabain from the external face of this epithelium suggests an insertion of this transporter in the external membrane of the yolk epithelium, and thus a role for Na^+/K^+ -ATPases in the excretion of salt from yolk. Actually, the TEsPD decrease recorded upon ouabain addition on *S. officinalis*

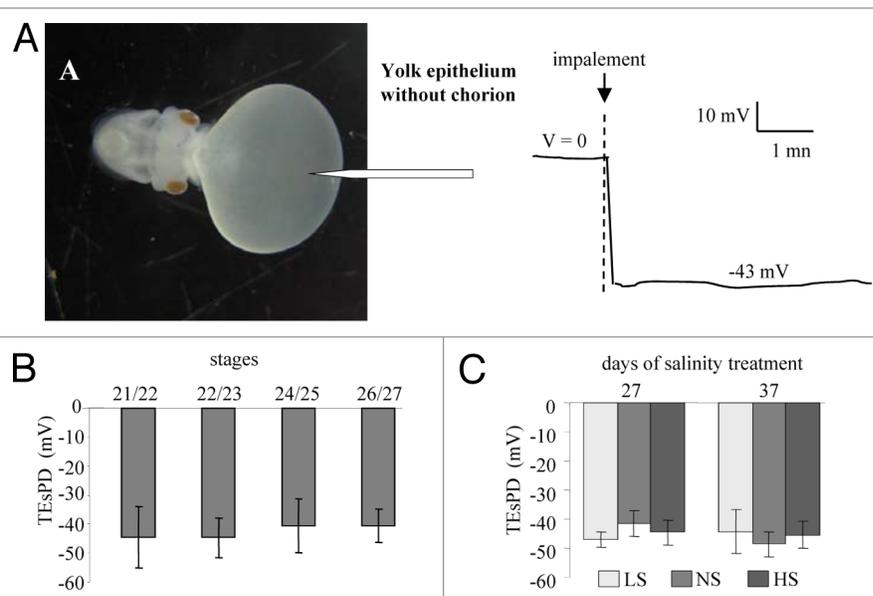


Figure 2. (A) *Sepia* embryo without chorion. The arrow indicates the position of the microelectrode for measuring difference of potential (V) across yolk epithelium alone. Diagram: Recording of potential changes upon insertion of the microelectrode in the yolk. (B) Mean values of difference of potential for yolk without chorion between stages 21 and 27. (C) Mean values of difference of potential for embryos bathed from the beginning of cleavage in low salinity, LS (26 ppm), normal salinity, NS (30 ppm) and high salinity, HS (34 ppm). Measures were taken after 27 and 37 d of treatment. The data correspond to the means of at least 5 replicates and error bars correspond to standard deviations.

yolk was rapidly reversed even in presence of ouabain (Fig. 5A), indicating that other ion transporters, putatively NHE3,²⁶ actively participate to yolk TEsPD. However, further studies are needed to analyze the ionic composition of *S. officinalis* yolk and create a model for this complex system to explain the yolk polarization and the involvement of Na^+/K^+ -ATPase in the TEsPD we recorded.

To our knowledge it is the first report dealing with polarization of the yolk of cephalopods and the probable presence of Na^+/K^+ -ATPase activity on ionocytes in yolk epithelium of *S. officinalis*. The characterization of the ionocytes-like cells in relation to the types and localizations of ion channels and/or pumps in this epithelium must be a challenge. These data will allow drawing a complete scheme of the tissues/organs and the regulation systems involved in the osmoregulation processes in *Sepia* embryos.

Materials and Methods

Animals

All animal procedures were in compliance with the guidelines of the European Union (directive 86/609) and the French law (decree 87/848) regulating animal experimentation. All efforts were made to minimize animal suffering and to reduce the number of animals used. *Sepia officinalis* embryos used in this study were gathered from just-fertilized egg batches that were collected from the marine station of Luc-sur-mer (University of Caen-France) between April to June. Eggs at the beginning of gastrulation (stage 10 according to Lemaire's system for *S. officinalis*,⁴³) were kept at 20°C in oxygenated sea water (30ppm). Eggs are surrounded by a chorion and

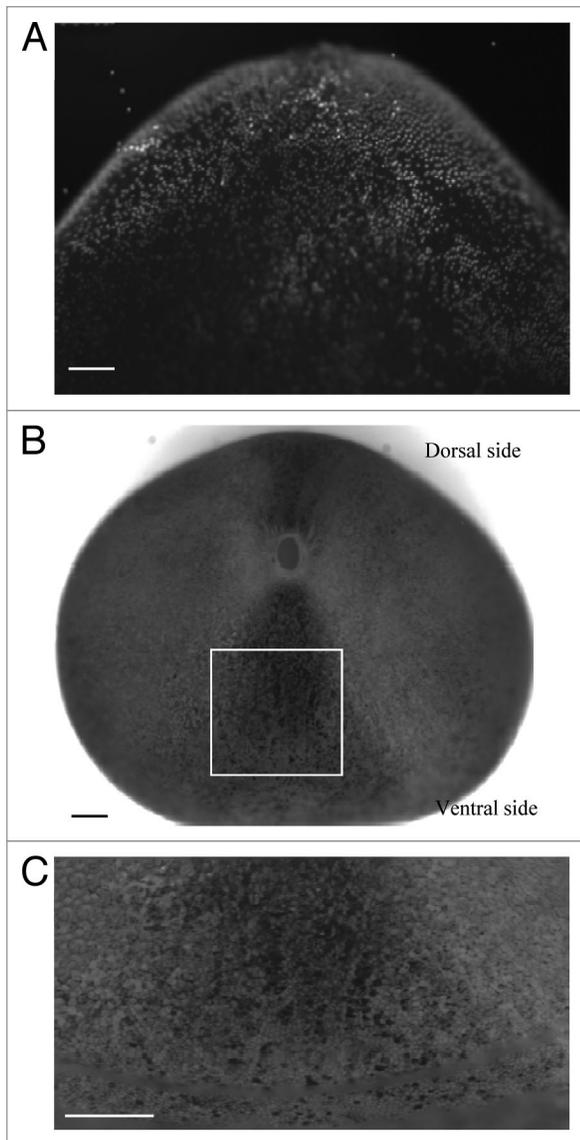


Figure 3. (A) DAPI staining of the yolk; (B) Silver staining of the yolk perivitelline epithelium of *Sepia officinalis* embryo. The aperture is the constriction limit between the external and the inner yolk sac. The dark patch indicates a zone with high ionocyte-like cells density. (C) Magnification of the dark area showing ionocyte-like cells. Scale bars: 500 μm

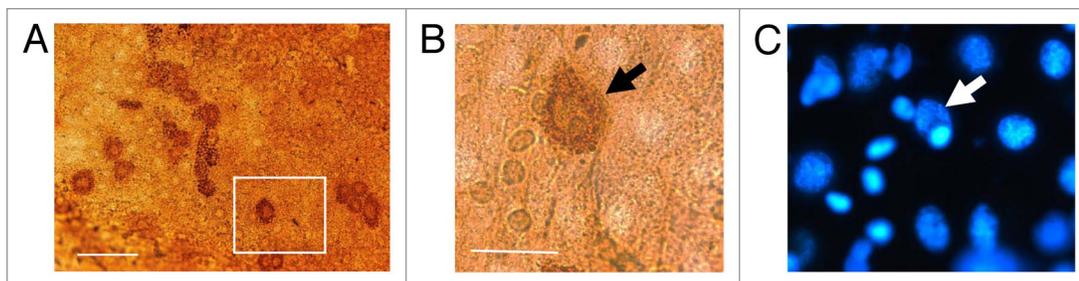


Figure 4. Na^+K^+ -ATPases immunostaining on *S. officinalis* yolk from embryo at stage 27. (A) Staining of the perivitelline envelope showing the patchy staining. (B) Magnification on a stained cell. (C) DAPI staining for the same magnification. Scale bar: A: 100 μm ; B,C: 30 μm .

several dark envelopes, constituting an eggshell (Fig. 1A). From stages 22 to 27, a few of them were opened daily by removing all the black envelopes. In this late embryonic development stage, the perivitelline fluid is increasing as a consequence of seawater entry^{7,44} and all the main structures and organs are developed.

The eggshell constitutes an interface, a physical barrier between the external surrounding water and the perivitelline fluid which creates the embryonic microenvironment.⁴⁵ The external black envelopes were removed in seawater to enable easier insertions of microelectrode into the perivitelline space through the chorion or into the outer yolk sac through the perivitelline envelope (Fig. 1A,B). The samples were bathed in 5 ml of sea water during the whole experiments.

Measurements of osmolarity

The osmolarities of the seawater and of perivitelline fluid samples were determined. The chorion and black envelopes were rapidly removed and the PVF collected directly in a microtube. Measurements were made on 100 μl by peltier cooling effect using an osmometer (Roebbling microosmometer).

DAPI and silver staining of yolk

Embryos with yolk were separated from the eggshell. Yolk sacs were bathed for 15 min in a solution of 200 $\text{ng}\cdot\text{ml}^{-1}$ of DAPI/PBS, and then observed using a Leica M16 2F binocular stereomicroscope. Other embryos with yolk were rinsed rapidly in distilled water. They were then transferred to 10 $\cdot\text{gl}^{-1}$ AgNO_3 for 5-min, again rinsed in distilled water and exposed to sunlight for 10–15-min. Observations were done with a Leica M16 2F binocular stereomicroscope.

Immunohistochemistry

The eggshell was removed in sea water and embryos were fixed for 1h in 3.7% paraformaldehyde (PFA) in phosphate-buffered saline (PBS 1X) at room temperature. After stage determination by binoculars, the yolk sac was separated from the embryo and washed 3 times for 15 min. in PBS, and then stored at -20°C in 30% glycerol. Fixed yolk sacs were washed in PBS and permeabilized overnight at room temperature in 10% dimethylsulfoxide (DMSO) in PBS to enhance antibody penetration. To reduce non-specific binding by antibodies, sites were blocked by incubating the yolk sac in blocking buffer [PBS / 0.05% Saponin / 1% bovine serum albumin (BSA)] overnight at 4°C and rinsed in PBSS [PBS/ 0.05% saponin] 4 times for 1 h.

Yolk sacs were then incubated overnight at 4°C with a NaK-ATPase $\alpha 1$ antibody of human origin [5 $\mu\text{g}/\text{ml}$ working dilution; NKA α (H-300), Santa Cruz Biotechnology, Santa Cruz, CA), which specifically recognizes the α subunit of the cephalopod NaKATPase²⁴ and then rinsed 3 times with PBSS (1 h each). A second incubation in horse

biotinylated anti-rabbit IgG antibody was performed overnight at 4°C in blocking buffer (dilution 1:100).

After additional washes (3 times, 1 h in PBSS), staining was performed using the Vectastain ABC Kit (Vector Laboratories) and 3,3-diaminobenzidine (DAB-nickel Kit, SK-4100, Vector Laboratories); a dark brown colored substrate of peroxydase. After 10 min., the coloration was stopped in PBSS and embryos were fixed with 3.7% PFA in PBSS. The yolk sac was dissected in order to remove the yolk and the perivitelline envelope was mounted on glass slide in 3:1 glycerol to PBS for viewing on a Leica DMLB microscope, and photographed with a cool-snap color camera. A solution of 200 ng.ml⁻¹ of DAPI/PBS was applied on slides.

Electrophysiology

Potential differences were measured with glass microelectrodes pulled on a Narishige PA81 electrode puller (CLARK GC 150F with a tip diameter thinnest than 1 µm). Micropipettes were filled with 600 mM KCl (resistance of about 80 M) and were connected by a reversible Ag/AgCl half-cell to the measuring set-up consisting of an electrometer (F223A WPI) and a pen-recorder (Kipp and Zonen BD8). *Sepia* eggs were bathed from stage 10 in three different salinities (low salinity, LS: 26 ppm; normal salinity, NS: 30 ppm and high salinity, HS: 34 ppm). After 27 d, embryo is free in the PVF and reached stage 21/22 in which the yolk is separated in an internal sac inside the embryo and an external sac visible outside. After 37 d they reached stage 25 at which all the organs are set up. Yolk embryos at stage 21/22 and at stage 25 were impaled using a three axis hydraulic micromanipulator Narishige WR91 clamped to the binocular loupe. Experiments were performed on a vibration free table enclosed in a faraday cage, in which all the apparatus were earthed. Experiments were performed at 22 ± 2°C. The Na⁺-K⁺ ATPase inhibitor ouabain (100 µM final concentration) was added directly to the sea water.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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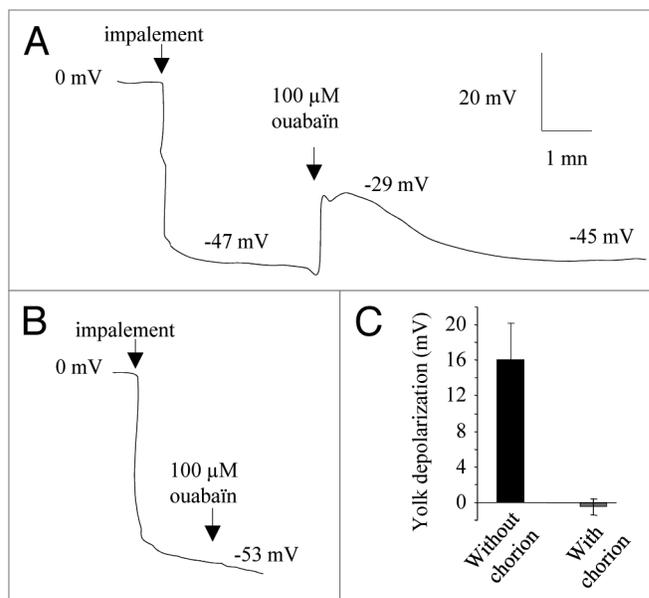


Figure 5. Recording of the transepithelial difference potential (V) of *Sepia* embryo yolk without (A) or with (B) chorion, and variation of the potential upon addition of the Na⁺K⁺-ATPase inhibitor ouabain at 100 µM. C) Mean values of potential variation for the different configurations. The data correspond to the means of 5 replicates and error bars correspond to standard deviations.

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Author Contributions

LB and FB designed the experiments. LB, FB, DF, LV performed the experiments. LB and FB analyzed the data and wrote the paper.

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