Decapod eggs membranes: powerful barriers or regulatory structures?

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Summary statement: Decapod crustacean´s eggs are not completely isolated by their membranes, which may selectively transport ions to an intra-membrane space. We found evidence of osmoregulation and propose an active role of membranes.

Abstract

Osmoregulatory abilities and mechanisms of adults and larvae of decapod crustaceans have been extensively investigated. However, how embryos carried by their mothers can deal with changing or extreme salinities is less understood. The egg membranes are believed to isolate embryos from a challenging environment, although osmoregulatory ability has been demonstrated in early developing embryos (naupliar stage) of two crabs. To establish whether embryos are isolated by their membranes and/or are able to osmoregulate, we measured the survival and volume change over 48 h of oocytes and embryos in different stages of three carideans (*Betaeus lilianae*, *Palaemon macrodactylus* and *P. argentinus*) and the brachyuran *Neohelice granulata*, subjected to different salinities. In addition, we recorded osmolality changes in homogenates of the same stages in *P. argentinus* and *N. granulata* after 2 h of exposure and mapped the presence of putative sites of ions exchange in the membrane of all species.

High mortality, when existed, was associated to low salinity and their variation with the stage of development depended on the species. All species precipitated silver salts in or under the egg envelope, with a different pattern between carideans and brachyuran. Changes in osmolality and egg volume after hypo/hyper osmotic salinity challenges indicate that eggs are not fully isolated by their membranes, and that some osmoregulatory mechanisms are in

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play to maintain developmental homeostasis. We suggest that egg membranes can participate in osmoregulation by selectively transporting ions to an intramembrane space, with differences between carideans and brachyurans.

Introduction

Females of decapod crustaceans from the suborder Pleocyemata carry their eggs until the larvae or juveniles hatch. Requirements and physiological capacity of early developmental stages often differ from those of their parents. For instance, adults can often live in brackish water or on land, but their larvae need marine conditions to develop. From an extensive revision of the ontogeny of the osmoregulation in crustaceans (Charmantier, 1998; Charmantier and Charmantier Daures, 2001), it appears that much is known about adult osmoregulation, less about juvenile and larval phases and very little about embryos. As natural selection acts on each one of a species' developmental stages, to fully understand the osmoregulatory strategy of a given species it is necessary to know the diverse responses from the reproductive cells of adults to embryos, larvae, and juveniles, which can be strikingly different (Charmantier and Wolcott, 2001).

Charmantier (1988) considered three patterns of postembryonic ontogeny of osmoregulation: in the first one, all phases of the life cycle osmoregulate weakly or not at all, typically involving marine stenohaline species. In the other two, adults are strong osmoregulators, frequently estuarine or freshwater species, and the adult type of osmoregulation is established at the beginning of the larval phase or in the juvenile phase. The least clear point in this scheme is whether the embryos are able or not to osmoregulate, especially for those species following the second and third pattern. In the crustaceans that incubate their embryos in closed chambers (as some Cladocera, Isopoda and specially Amphipoda), the osmolality of the fluids surrounding the embryos is in different ways regulated by the mother. Within the peracarida, different regulatory capacities of the embryos themselves have been observed; while in the isopod *Sphaeroma serratum* embryos have little osmoregulatory ability (Charmantier and Charmantier-Daures, 1994), in the amphipod *Orchestia gammarellus* all embryo stages efficiently hyper-hypo osmoregulate from a few days after the start of development and until hatching, with hatchlings being

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weak regulators (Morritt and Spicer, 1996). The ability to osmoregulate, in this case, seem to be present very early during embryonic development in relation to a system formed by a well-developed embryonic dorsal organ and the vitelline membrane (Meschenmoser, 1989; Morritt and Spicer, 1996). Similar studies are almost inexistent for decapod crustaceans. Pleocyemata embryos develop in an open incubation chamber, and face the same osmotic regimes as their mothers, with little chance of female intervention. The main mechanism proposed so far that would allow these embryos to live in conditions of varying salinity or in fresh water is the osmoprotection provided by the outer membranes of the egg (Charmantier and Charmantier Daures, 2001), while the appearance of the ability to osmoregulate would be associate with the development of the larval/adult osmoregulatory structures and the Na^{+}/K^{+} -ATPase activity at the end of development. Nevertheless, as noted by the authors, to accomplish its role of osmoprotection, egg envelopes need to be highly impermeable to water and ions, involving serious incompatibilities with other processes, for example gas exchange. Taylor and Seneviratna (2005) demonstrated for the first time that embryos of two species of grapsid crabs were able to hyper osmoregulation in low salinity as early as the naupliar stage (called stage 2 or gastrula stage by the authors). No other studies have shown that osmoregulation occurs in other decapod embryos.

The egg membranes as described for different pleocyemata decapods has a complex structure, formed by several layers, and to some extent, comparable among different species. The first two egg's layers that constitute the vitelline membrane, are formed while oocytes are in the ovary, and follicular cells seem to cooperate in their formation (Goudeau and Lachaise, 1980; Talbot and Goudeau, 1988). The vitelline membrane turns into the fertilization membrane in brachyurans after the cortical reaction (Goudeau and Becker, 1982). This process has two steps, one immediate liberation of fine granular material to the perivitelline space during the first 15 minutes after fertilization, and a second massive extrusion of a huge quantity of ring-shaped structures, stored in the cytoplasm; rings coalesce and free their content forming a new layer between the vitelline membrane and the plasmalemma. This second part of the process takes 7 to 8 hours to be complete (Goudeau and Becker, 1982; Talbot and Goudeau, 1988). At the end of this process, the fertilization

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membrane is fully formed. In shrimps and lobsters, all the process is triggered by the massive influx of Mg^{+2} independently of fertilization, (Goudeau et al., 1991). Additional layers are added when the developing embryo reaches successively the naupliar and metanaupliar stages. (Goudeau and Lachaise, 1983).

Nowadays, whether the external egg membranes have a passive (osmoprotective function) or an active role in developing embryos of decapod crustaceans is still uncertain, as for several other groups of invertebrates and vertebrates (Charmantier and Charmantier Daures, 2001).

In the present study, we gather information on the functional link between egg membrane development, changes in egg volume and embryo survival during early embryonic development from 4 species of pleocyemata decapods challenged by abrupt salinity changes. Selected species occur from marine and estuarine to freshwater environments. *Palaemon macrodactylus* Rathbun, 1902 (Caridea, Palaemonidae) is a strongly hyper/hypo osmoregulator invasive species commonly found between 2 and 34 psu (González-Ortegón et al., 2006), *Palaemon argentinus* (Nobili, 1901) (Caridea, Palaemonidae) is a strongly hyper osmoregulatory freshwater species (Charmantier and Anger, 1999), able to develop up to 25psu (Ituarte et al., 2005). *Betaeus lilianae* Boschi, 1966 (Caridea, Alpheidae) is the only intertidal caridean species other than *P. macrodactylus* found in the coastal area of Argentina (Spivak et al., 2019). Its physiology has not been studied, but since it has never been reported out of the marine condition, it was considered marine stenohaline. *Neohelice granulata* (Dana, 1851) (Brachyura, Varunidae) is a strongly hyper/hypo osmoregulatory semiterrestrial crab (Luquet et al., 1992; Charmantier et al., 2002) that lives in areas where salinity varies widely (Luppi et al., 2013). All species have a typical development with the embryonic phase ending with the hatching of a zoea, and several larval stages before decapodid and juvenile. We evaluated embryonic survival and changes in egg volume in response to acute salinity changes as a proxy for water permeability. We also measured osmolality in early developing embryos of the freshwater *P. argentinus* and the estuarine *N. granulata*. Deposition of silver salts was used to localize possible ion exchange surfaces in eggs of all species. Our general hypothesis is that egg membranes do not completely isolate embryos but also, they have a more

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active role in the mechanisms that allow decapod embryos to develop in salinity challenging environments.

Materials and Methods

Collection and rearing of specimens:

Females and males of *Palaemon macrodactylus* were caught with hand nets at the Mar del Plata harbor, Buenos Aires province, Argentina (38°02'28'' S, 57°32'18'' W), a marine habitat with freshwater supply during the rainy season, along the reproductive period 2016-2017 (October-March) and reared in 15psu.

Females and males of *Palaemon argentinus* were obtained with hand nets from the freshwater La Brava Lake, Buenos Aires province, Argentina (37°51'57'' S, 57°58'55'' W), along the reproductive period (September-March) from 2016 to 2018 and also reared in 15psu.

Betaeus lilianae females and males were caught with hand nets in tidal pools at Las Grutas, Rio Negro province, Argentina (40°48'26'' S, 65°04'44'' W), an intertidal habitat with no freshwater input except for scarce rainfall, in February 2017, and reared in seawater (34psu).

Ovigerous or mature fertilized females of *Neohelice granulata* were captured by hand at Mar Chiquita lagoon, Buenos Aires province, Argentina (37°45' 0.8'' S, 57°25'22'' W), a coastal lagoon with wide and unpredictable variations in salinity because of wind and rainfall, during the reproductive season (September to march) between 2016 and 2019. They were reared in 25psu.

Ethical consideration of species

Experimentation with the species used here do not require approval by an ethics committee, however, protocols were applied to minimize suffering and the number of individuals used.

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Definitions and stages of embryos:

The general term “egg” includes both, oocytes end embryos. We will call them oocytes (O) when referring to unfertilized eggs, and we refer to “embryos” to denote stages occurring between the beginning of development of fertilized eggs and hatching. The first stage of development used in experiments, referred to as morula stage (MS) corresponds to the stage 1 described by Bas and Spivak (2000) for *N. granulata* and by Ituarte et al. (2005) for *P. argentinus.* At this stage, eggs’ appearance varies from a totally uniform, undivided cell, to a typical morula, although it also includes the blastula and gastrula stages which are undistinguishable by direct observation. The second stage used in experiments, referred to as naupliar stage (NS) is the embryonized nauplius stage (Goudeau and Lachaise, 1983). It is fully formed after three days of development at 20°C in *N. granulata* and after five days in *P. argentinus* (stage 3 in Bas and Spivak, 2000 and Ituarte et al., 2005). It is observed as a translucent area occupying about 10% of volume in an egg pole.

Obtention of oocytes and embryos:

Oocytes of caridean species were obtained by leaving females with mature ovaries (visible dorsally through carapace) in aquaria without males. Since fecundation in those species is external and occurs immediately after female molting, the absence of males ensures that eggs extruded to the pleon for incubation are unfertilized oocytes. Brachyuran females instead, store sperm internally, and eggs are fertilized before being extruded. Then, to obtain oocytes of *N. granulata*, non-ovigerous females at the beginning of the reproductive season (October, when most females have ripe ovaries) were killed in cold, and oocytes immediately extracted from the ovary (after corroborating females were fully mature).

Embryos from all Caridean species and stages were obtained by monitoring mature females in aquaria where males were present and following them from the time of egg extrusion and fertilization until the appropriate stage of development was reached. Fertilized eggs from *N. granulata* were obtained from fertilized females kept in laboratory and monitored to detect the time of eggs extrusion, or from ovigerous females captured in the field with eggs at the appropriated or at an earlier developmental stage and conditioned at least 24 h

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in laboratory conditions. Recently hatched zoea I larvae of *N. granulata* and *P. argentinus* were obtained from some females after the complete embryonic development had finished.

Selection of salinity levels for experiments

Salinity levels to evaluate oocytes and embryos' performance were selected to ensure some osmotic challenge within a range (if known) they could survive, for 48 h. Oocytes and embryos from *N. granulata* were faced to 5 and 45 psu, near the limits they can tolerate. The intermediate rearing salinity (25 psu), where development is optimum (Bas and Spivak, 2000), was used as control. The same three salinity levels were used for *B. lilianae* oocytes and embryos, although in this case, the intermediate, 25 psu, differed from the rearing salinity (34 psu). The oocytes and embryos of the freshwater shrimp *P. argentinus* were exposed to1 psu (normal salinity) and 25 psu, their limit for normal development (Ituarte et al., 2005), with the rearing salinity (15 psu) as control. The same salinity range was used for *P. macrodactylus*, although in this case the lowest salinity was the lower limit for the species while the highest and intermediate levels represent frequently occurring conditions.

Salinities of 15 and 25 psu were prepared by diluting marine water with tap water, and 45psu was obtained by diluting mineral salts to prepare artificial salt water (Aquavitro Salinity, Seachem laboratories, USA) with tap water. Salinity was measured with a hand refractometer.

Volume change and survival:

Survival and volume change were evaluated in the four species, in O, MS and NS embryos. Three replicates of ten eggs (from three different females, except in some cases specified below), were obtained from ovigerous females of all four species at each of the three stages of development. Eggs (except those of *N. granulata* obtained from ovaries) were gently separated from pleopods with fine forceps and put in small petri dishes with filtered and UV sterilized water of one of the three experimental salinity levels selected for each species and reared at 20°C and 12:12 L:D light regime. Water and dishes were changed after 24 h.

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Volume was estimated at time 0, just before starting the experiments in the female rearing water, and then, in the experimental salinities 1, 2, 24 and 48 h after the beginning of the experiments. Major (D) and minor (d) diameter of each egg was measured with a stereomicroscope with a graduated eyepiece. Volume was estimated as $V=\pi. D.d^2/6$.

Mortality was defined by changes in yolk color (compared to normal eggs, different for each species) and/or texture (the yolk typically loses its globular texture when the embryo dies), and/or membrane rupture, at the same times volume was measured. Only those eggs considered alive were measured, except those from *B. lilianae* at 5 psu which all died during the first hour (see below), some of which were nonetheless measured to show volume change.

Osmolality experiments:

The initial osmolality (in rearing salinity) and after two hours in an experimental salinity (the shortest period in which changes in the volume were observed), was measured in O, MS NS embryos and ZI larvae of *P. argentinus* and in MS and NS embryos and ZI larvae of *N. granulata*. The osmolality of the larvae was measured to compare the values obtained from whole larvae (as used here) with the direct measurement of hemolymph available from previous studies. Experimental salinities were the same as in survival experiments. The protocol for measuring osmolality was modified from Taylor and Seneviratna (2005). Masses of eggs were removed from *N. granulata* females and kept in petri dishes at the rearing salinity. From there, different portions were drained by placing them on a mesh on blotting paper and then transferred to a dish with water of one of the experimental salinities. Larvae were directly transferred from the hatching water to the experimental salinity with a similar procedure. After two hours, eggs and larvae were quickly blotted dry and homogenized 10 s in 0.5 ml ice-cooled Eppendorf tubes, using a motor-driven pestle. Washing with distilled water was avoided because *N. granulata* embryos varied their osmolality after a few seconds of washing. Three different broods (females) were used for each developmental stage and salinity, with three replicates per brood.

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Since egg mass volume of *P. argentinus* is small, to reach the necessary volume of homogenate, the complete brood of embryos or larvae from between 4 and 7 females (depending on the brood size) were pooled for each measure. Three replicates of each salinity condition and development stage were made from pools with the same procedure described above. Homogenates (50µl) were measured with a freezing point osmometer (Osmomat 030, Gonotec, Germany). Samples of water of the experimental media were measured by triplicate for each egg sample.

Silver staining of eggs:

Staining of eggs to reveal putative sites of ion transport was made following Seneviratna and Taylor (2006). Eggs were detached from ovigerous females of the same four species and at different developmental stages depending on their availability. O, MS, NS and more advanced stages were used. All samples were rinsed in distilled water for about 30 s and transferred to 5 g·l^{-1} $AgNO_3$ for 5 min, rinsed again in distilled water and exposed to sunlight for 10-15·min in water at the rearing salinity. Treated eggs were then fixed 24 h in 4% formalin in water of the rearing salinity and transferred to 70% alcohol. They were observed in toto under stereomicroscope and microscope. Conventional Hematoxylin-Eosin staining was made after paraffin inclusion of some samples.

One sample of eggs from *N. granulata* in a stage posterior to NS (Stage 4 in Bas and Spivak, 2000), O and MS eggs from *P. argentinus* treated with $AgNO_3$ were immediately fixed for SEM (Jeol JSM-6460LV Scanning Electron Microscope), and the deposits in the membranes analyzed with energy-dispersive X-ray spectroscopy (EDS; EDAX Genesis XM4 Sys-60) to determine their composition. Eggs were fixed in 2% glutaraldehyde in 0.2M buffer phosphate with sucrose to adjust osmolality (700 mOsm kg-1 for *N. granulata* and 450 mOsm kg^{-1} for *P. argentinus*) and OHNa to adjust pH to 7.8, punctured to facilitate the penetration of fixative. Then, the outer egg membranes were partially removed with the tip of a scalpel to expose all layers. Eggs were dehydrated in graded series of ethanol, dried in HMDS and mounted in aluminum stubs with double-sided tape, sputter coated with gold/palladium,

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observed in SEM, and different areas and layers of membranes and eggs surface analyzed with EDS.

Data analysis

All analysis were computed in R (R Core Team 2013). Volume and survival were analyzed using the generalized linear mixed model function glmer from package lme4 (Bates et al. 2015). Volume was ln-transformed to comply with normality and homoscedasticity assumptions. Survival was analyzed as a binomial variable, i.e. alive or dead. Salinity and time were treated as fixed factors and female (clutch origin) was treated as random effect. Only survival at time 48 h was compared, between those salinities in which there were survivors.

For the analyses of volume variation, we used the function dredge from package MuMln (Barton, 2020) to perform information-theoretic model selection using Akaike's Information Criterion (AIC) comparing all-subsets of the global model (complete model including all interactions terms). Pairwise multiple comparisons were performed, when applicable, with the Holm-Sidak method. In cases where most of eggs at any stage died and/or their volume changed disproportionately compared to the other salinities, data were not included in analysis.

Osmolality data were analyzed with two-way ANOVAs using the function lm in R, with stage and salinity as factors. Pairwise multiple comparisons were performed, when applicable, with the Holm- Sidak method.

In all the analysis the selected significance level α was 5%.

Results

Mortality

Oocytes showed the highest rates of mortality relative to the other stages in all species. Overall, mortality was higher in low vs. intermediate or high salinity. Due to the small number of individuals available, only one sample of *B. lilianae* oocytes was obtained. All died within 1h at low salinity while the survival was higher and comparable between the other two salinities ($p= 1$; Fig.1A). Oocytes from *P. macrodactylus* survived a longer period in low salinity but were all dead

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after 48 h; survival in the upper salinity level was high, and higher than at the intermediate level (p = 0.02) (Fig.1B). All oocytes from *P. argentinus* were alive after 24 h and nearly 80% remained still alive after 48 h, with no differences between salinities (all p> 0.1, Fig.1C). Oocytes from *N. granulata,* as those of *P. macrodactylus*, died after 48 h in low salinity, while nearly 50% remained alive in the other salinities (p= 0.09; Fig. 1D).

Early eggs in the stage of morula showed a higher survival than unfertilized oocytes at least in some salinities. MS eggs from *B. lilianae* died in low salinity, as oocytes, in the first hour of exposition, but near 90% remained alive after 48 h in the intermediate and high salinity (p= 0.25; Fig. 1E). MS eggs from *P. macrodactylus* showed high and comparable survival in all salinities (all p> 0.05; Fig. 1F). MS embryos from *P. argentinus* were almost all alive at the end of experiment and there were no differences between salinities (all p> 0.05; Fig. 1G). Although MS embryos from *N. granulata* increased their survival in all salinities, mortality after 48 h in low salinity was still high and different from the others (both p< 0.001; Fig. 1H).

The more advanced (NS) *B.lilianae* embryos showed a slightly higher survival in low salinity as compared to the previous stage, but all died after 24 h. At the intermediate salinity instead, survival was lower than in the previous stage and different from that at high salinity, where no mortality occurred (p< 0.001; Fig. 1I). NS embryos of *P. macrodactylus* showed survival values like the previous stage, high and with no differences between salinities (all p> 0.05; Fig. 1J). All the NS embryos of *P. argentinus* and almost all *N. granulata* survived 48 h at all three salinities (Fig. 1K, L).

Volume change:

There was an interaction between salinity and exposure time in all the species and stages analyzed (all p< 0.05). Volume increases at low salinity were more noticeable and tended to appear earlier than volume reductions at high salinity in all cases where significant differences were recorded.

Betaeus lilianae oocytes increased their volume during the first hour at low salinity before dying. An increase in volume was also recorded in 25 psu, after 24 and 48 h. In 45 psu oocytes tended to decrease volume after two hours but at the end of experiments they did not differ from time 0 (Fig. 2A). The

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oocytes from *P. macrodactylus* increased their volume rapidly in low salinity and do it also in 15 and 25 psu after 24 and 48 h. (Fig. 2B). In the freshwater species *P. argentinus*, oocytes increased their volume slightly but significantly in low salinity only after 48 h of exposition (Fig. 2C). The volume of oocytes from *N. granulata* were not statistically contrasted because overfit was recorded (the random effects structure was too complex to be supported by the data). Volume in low salinity increased markedly from the first hour of exposure, reaching more than 130% of the initial volume after two hours. In the other salinities volumes showed large variations within each condition for the first two hours and then stabilized. The average volume was higher in 25 psu than in 45 psu after 24 h (Fig. 2D).

Fertilized eggs from all species also varied their volume with regard to salinity and time. MS and NS eggs from *B. lilianae* increased in volume at 5 and 25psu even during the first hour, while at 45 psu the volume decreased significantly after 24 h (Fig. 2E, I).

Embryos of *P. macrodactylus* at the beginning of development (MS) only showed volume changes after 48 h, when eggs in 1 and 15 psu were larger than those in 25psu, which remained unchanged (Fig. 2F). The more advanced (NS) eggs of this species increased their volume after 48 h, with the largest and smallest eggs at 1 and 25 psu, respectively; and those kept at 15 psu with intermediate volume. (Fig. 2J).

The only volume variation observed in embryos from *P. argentinus* (MS and NS) was an increase in 1psu compared to the other salinities after 48 h (Fig. 2G, K).

MS embryos of *N. granulata* increased markedly in volume from the first hour of exposure at 5 psu, and eggs in 45 psu decreased their volume after 48 h (Fig. 2H). More advanced, NS embryos instead, did not show changes in volume at any salinity during the first 24h, and only after 48 h embryos at 5 and 25 psu were larger than those at 45 psu, which did not change from the start of the experiment (Fig. 2L).

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Osmolality:

The osmolality of the homogenates of both species depended on the interaction between the external salinity and the stage of development (both $p< 0.001$), and the osmotic response of each stage differed between species.

P. argentinus:

At the rearing salinity of 15 psu oocytes and MS embryos were isosmotic with the medium, while NS embryos were 134 ± 23 mOsm kg^{-1} above the isosmotic line. When transferred two hours to 1 psu, the usual salinity for the species, all stages differed to each other, oocytes reached the lowest osmolality, 198 ± 12 mOsm kg^{-1} above the external medium, MS embryos were 268 ± 40 mOsm kg^{-1} above the external medium, and NS embryos continued having the highest relative value (443 ± 16 mOsm kg^{-1} above the external medium). Surprisingly, when transferred two hours to 25 psu, oocytes and MS embryos did not change their internal osmolality, while NS embryos became near isosmotic (30 ± 10 mOsm kg^{-1} below the line; Fig. 5A)

Zoea I larvae of *P. argentinus* were measured only in 1 psu, 2 hours after transferring them from the rearing salinity of 15 psu. They reached 400.3 ±11.5 mOsm kg^{-1} (Fig. 5A).

N. granulata:

In the rearing salinity of 25 psu the internal osmolality of the three tested stages, MS, NS and ZI did not differ each other with values of 770 ±40 mOsm kg^{-1}. When transferred two hours to 5 psu, MS embryos and ZI dropped about 500 mOsm kg^{-1} and kept only 84 ± 17 mOsm kg^{-1} above the surrounding medium. NS embryos, instead, did not change significantly. Finally, when transferred to 45 psu, ZI and MS embryos were near isosmotic with the external medium, although MS embryos showed a broader variation between replicates from different females. NS embryos instead, were able to keep 229 ± 4 mOsm kg^{-1} below the medium (Fig. 5B)

Silver staining of eggs

All caridean eggs (O, MS, NS or more advanced embryos of the three species) stained uniformly with the solution of $AgNO_3$, leaving an opaque, dark cover that made difficult to see any detail inside the egg case (Fig. 4A-C). In

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oocytes and MS stage embryos it was not clear from the observation with optical microscopy whether the deposits occurred between membrane layers or below the membrane, on the cell surface or the ectoderm (Figs. 4D, E). In more advanced NS eggs, silver deposits appeared clearly inside the egg membrane, forming a layer between external and internal envelopes (Fig. 4F).

By contrast, the oocytes of *N. granulata* seemed not to stain with $AgNO_3$ solution, and after washing them with distilled water only some granules remained adhered, but the surface was not darkened (Fig. 5A). Nevertheless, when histological sections were observed under optical microscope, a thin line presumably of silver granules could be seen directly on or within the cytoplasm (Fig. 5B). MS embryos of *N. granulata* darkened only slightly, and black spots appeared scattered inside the membrane, (Fig. 5B, E). When more advanced embryos (NS or later) were immersed in the silver solution, a very definite area was revealed, as a skullcap over the egg zone containing the yolk (Fig. 5C). In this case also, the silver deposits appeared inside the layers of the fertilization membrane, but only in the patch area (Fig. 5F).

The analysis with energy dispersive X ray spectrosocopy of treated eggs and embryos confirmed the presence of a high proportion of Cl^- and Ag^+. In MS embryos of *P. argentinus* peaks of these ions only appeared when intermediate layers of the membrane, exposed by rupture of the upper layers, were analyzed. In oocytes, on the other hand, the deposits appear to be inside the egg, since the peaks were recorded when analyzing the surface of an oocyte that had had its envelope completely removed. In NS embryos of *N. granulata*, Cl^- and Ag^+ peaks were recorded only inside the dark calotte, in an intermediate layer of the membrane, in an area where the outermost layer has been removed.

Discussion

All decapod species studied here showed changes in volume and/or survival when exposed to salinities above and below the rearing intermediate salinity. Even in the freshwater species, successful embryo development is not associated with an impermeable embryonic membrane isolating them from the external environment.

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In all species the oocytes showed the greatest mortality levels and/or changes in volume, while a significant increase in survival and volume stability appeared in fertilized eggs with developing embryos, suggesting that it is the complete fertilization membrane which is fully functional.

On the other hand, survival and volume stability in low salinity were markedly improved between the morula and nauplius stages in *N. granulata*. In a previous study of this and another species of grapsoid crabs, Bas and Spivak (2000) found that embryos died at salinities below 12 psu if exposed before the nauplius stage but were able to develop normally if exposure occurred thereafter. Based on the presumed role of isolation assigned to embryonic membranes, it was suggested that the appearance of the naupliar membrane was responsible for a more effective isolation, and thus greater survival. Taylor and Seneviratna (2005) found a very similar survival pattern in other Varunidae crabs, *Hemigrapsus sexdentatus and H. crenuatus* They showed that membrane permeability did not change substantially between pre- and postnaupliar stages, and associated instead the capacity of osmoregulation that occurred from the naupliar stage onwards to the appearance of a dorsal organ, which allowed the embryos to maintain their internal osmolality balanced through an active mechanism of water and ion filtration. An embryonic dorsal organ has been described in Malacostraca crustaceans as a thickening of the extraembryonic ectoderm in the dorsal midline of embryos (Anderson, 1973). This structure is functional only during the embryonic period and is then reabsorbed before hatching. Although its assumed general function was primarily the histolysis and reabsorption of the extraembryonic ectoderm and yolk degradation (Anderson, 1973), it was described in some peracarida as clearly osmoregulatory, with typical ionocytes associated to the external membrane (Stromberg, 1972; Meschenmoser, 1989), and correlated to the ability of some species to osmoregulate inside the female brood chamber (e.g. Morrit and Spicer, 1996).The function and structure of the embryonic dorsal organ in decapods are not clear; and it is even possible that there is not a single type, having appeared independently on multiple occasions in different groups of decapods (Robertson, 2013). More research on this topic is obviously needed.

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The presumed position of the dorsal organ in embryos of Hemigrapsus species was revealed with silver salts by Seneviratna and Taylor (2006) and appeared essentially the same as that found here in *N. granulata*. Silver salts have long been used to detect ion exchange sites and was the first technique allowing functional discrimination of the branchial tissues in Brachyura (osmoregulatory vs respiratory gills; Ewer and Hattingh, 1952). Although the involved process was not always clear, it is effective to reveal sites of chloride flux through some surfaces, which reduces silver ions, forming black deposits of ClAg (Copeland, 1967; Seneviratna and Taylor, 2006). In this study, this simple technique has proven to be useful to compare the areas of ion exchange between species.

We have shown here that *N. granulata* embryos from the nauplius stage are able to maintain an osmotic difference with the medium, when exposed to low salinity, definitely indicative of an active mechanism of osmotic regulation. This suggests that an osmoregulatory capacity, possibly associated with a dorsal organ, may be a common trait in intertidal and/or estuarine brachyurans species usually exposed to low salinities during embryonic development.

In the carideans studied here, the deposition of silver salts is uniform throughout the egg membrane and occurs as early as oocytes in all species. At the same time there is no clear improvement in survival and volume stability between morula and nauplius stages exposed to low salinities as recorded in *N. granulata* alongside with the appearance of the silver salt patch.

Regarding the osmolality of the different stages (oocyte, morula and nauplius) of the caridean *P. argentinus*, it varied markedly and differently after two hours from salinity transfer. The maintenance of an osmolality much higher than that of fresh water (between 200 and about 500 mOsmol kg^{-1} depending on the stage) after a rapid drop of between 100 and 200 mOsmol.kg^{-1}, in a non-isolated internal medium, can only been explained by the existence of an active osmoregulatory mechanism. It is also interesting to note the difference observed between stages in the osmotic performance at a salinity higher than the isosmotic point. Nauplius stage embryos were unable to hypo osmoregulate and rapidly become isosmotic with the external medium at 25 psu, whereas oocytes and morula stage embryos were able to maintain their osmotic

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concentration as in 15 psu after two hours. This suggests that the mechanisms operating in the different stages are different.

The osmotic capacity of ZI of *P. argentinus* obtained from our measurements in homogenates (370 ± 11.5 mOsm kg^{-1}) is slightly higher to that obtained by Charmantier and Anger (1999) from the ZI hemolymph reared 24 h in 1 psu (340 mOsm kg^{-1}). The results obtained here for ZI of *N. granulata* were very close to those reported by Charmantier et al. (2002) after 24 h of exposition to 5.3, 25.5 and 44.3 psu. Hence, our results highlight that the approximation of measuring whole embryos and larvae homogenates is useful to measure the osmotic capacity of early embryos which has been one of the main problems in this kind of studies. Also, it is necessary to develop methods to obtain good histological sections for both, optic or electronic microscopy, which are hampered by problems in the fixation of the eggs of many species, (Hubble and Kirby, 2007). In the species studied here, results were poor, despite having attempted different protocols.

In addition to the hypothesis of the impermeability of the membranes as the mechanism of embryo protection (Charmantier and Aiken, 1987; Susanto and Charmantier, 2001), a limitation of water influx by the tensile strength of bounding membranes has also been proposed (Charmantier and Charmantier Daures, 2001). Nevertheless, not only water but ions have been shown to flow through eggs membranes (Seneviratna and Taylor, 2006; this study) and this ends up causing swelling of the embryos, osmotic imbalance, and death when there are no regulatory mechanisms as noted in the initial embryos of *N. granulata*.

All species tested here are at least partially permeable to water and ions. Then, external membranes are not simply barriers isolating embryos from the external media, although it is also likely that they could change their permeability through embryonic development. Alternatively, if membranes were to function as regulatory organs (or were part of one) as proposed by Morritt and Spicer (1996) for the isopod *Orchestia gammarellus,* it would be possible to explain most of the results both previous and reported here. According to this hypothesis, although the membranes (possibly associated with ionocytes from a certain point in development) would not totally prevent the flow of water and ions in a medium with an osmolality differing from the internal one, they would

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at least have partial capacity to actively regulate the flow of some ions to rebalance the internal medium. A membrane with these characteristics was described in some adult crustaceans. The cuticle covering the gills of adult *A. leptodactylus* has a high directional selectivity to ions as Cl^- and OH^+ (Avenet and Lignot, 1985). Such selectivity is likely located in the epicuticular layer, being specific of the gill lamina of the crayfish (no other parts of the cuticle showed this property). Avenet and Lignot, (1985) suggest that the cuticle could favor Cl^--OH^- exchange and Na^+-Cl^- co-transport through the gills. In addition, Barra et al. (1983) observed in gills of the crab *Eriocheir sinensis* acclimated to freshwater and treated with silver salts, that silver chloride deposits occurred uniformly inside the thin cuticle of the osmoregulatory posterior gills. In the adjacent respiratory gills, instead, deposits appeared only as small clusters inside the membrane. Egg membranes studied here and by Seneviratna and Taylor, (2006) allowed the passage of Ag^+ ions from the outside and Cl^- ions from the inside into an intermediate space. We have no evidence of a structural or composition similarity between the gill epicuticle of *A. leptodactylus* or *E. sinensis* and the envelopes of decapods eggs. However, if an acellular protein cover present in decapods, presumably the epicuticle, can play an active role in ion exchange through the gills, it is worthy to explore such a possibility in the egg's envelope as well.

Although selective membranes are not expected to function as a complete mechanism of regulation, it could have been a necessary prior-condition for the development of efficient cellular mechanisms. In our study, the Alpheid shrimp *B. lilianae* showed low tolerance to salinity changes, but its eggs envelopes still formed silver salts deposits like the other, highly tolerant palaemonid species.

In summary, since decapod embryos are not totally isolated from the environment by membranes with some degree of selectivity and may in some cases osmoregulate from very early stages, we consider that further studies of egg membranes and the underlying extraembryonic ectodermal (where a dorsal organ could develop), are necessary to fully understand the ability of embryos to survive and develop in anisosmotic media.

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The authors declare no competing or financial interests

Author contributions:

Design and experimentation, animals collect and culture: C.B. and R.I.; statistical analysis M.K.; writing original draft: C.B.; writing review: R.I. and M.K. Fundings administration: C.B.

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Figures:

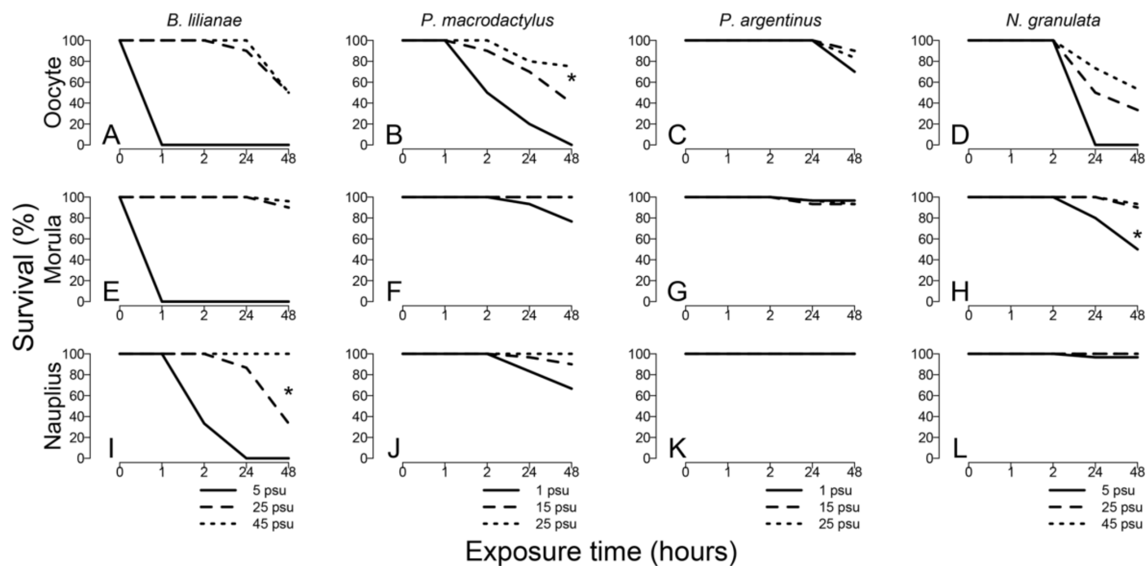

Figure 1: Survival of oocytes and embryos of *Betaeus lilianae*, *Palaemon macrodactylus*, *P. argentinus* and *Neohelice granulata*. Survival was estimated at the rearing salinity (time 0) and along the experimental period (1 to 48 h), in three salinities. Oocyte: Unfertilized eggs; Morula: morula stage embryos; Nauplius: nauplius stage embryos. Asterisks indicate significant differences in the comparisons of survival after 48 h. Initial number of eggs at each stage and salinity was n= 30 (10 eggs from three different broods) except for A (n=10, one brood) and B (n=20, two broods).

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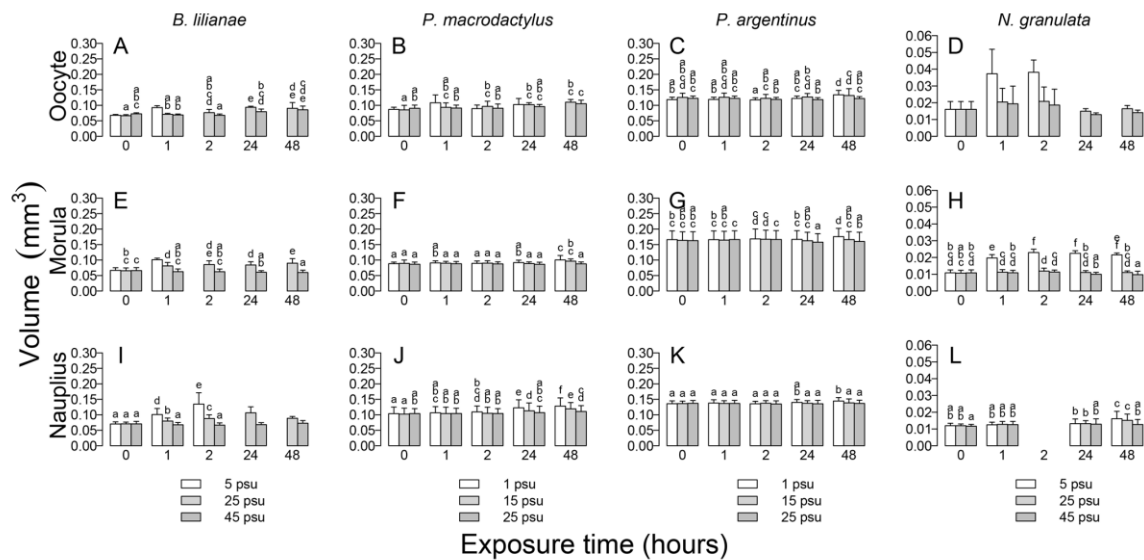

Figure 2: Volume of oocytes and embryos of *Betaeus lilianae*, *Palaemon macrodactylus*, *P. argentinus* and *Neohelice granulata*. Volume was estimated in all individuals considered alive at each time at the rearing salinity (time 0) and along the experimental period (1 to 48 h), in three salinities. Oocyte: Unfertilized eggs; Morula: morula stage embryos; Nauplius: nauplius stage embryos. Volumes are mean + s.d. Lower case letters above bars indicate the results of multiple comparison between all pairs. Different letters denote differences in volume. Bars without letters were not included in comparisons (see text for details). Initial number of eggs at each stage and salinity was n= 30 (10 eggs from three different broods) except for A (n= 10, one brood) and B (n= 20, two broods).

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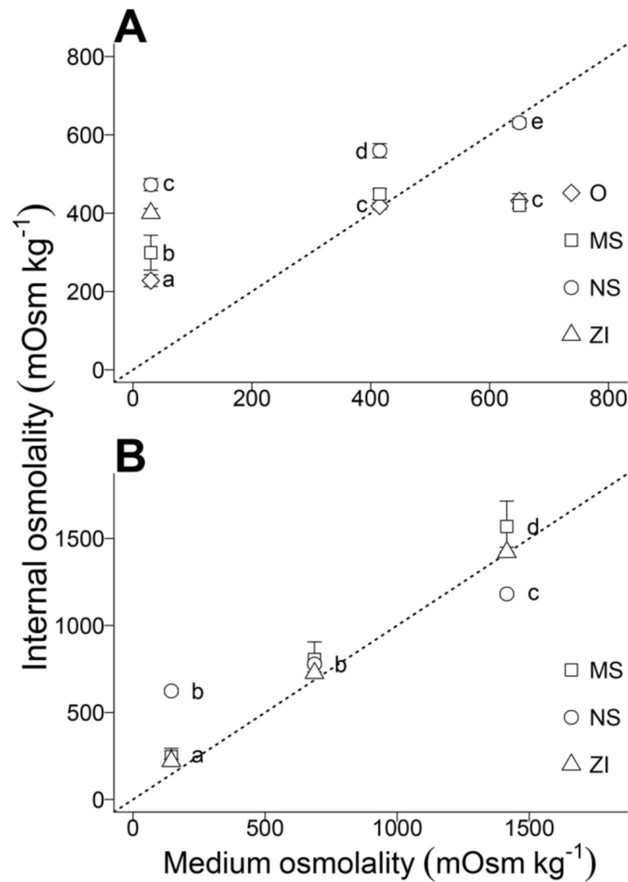

Figure 3: Variation in the osmolality of homogenates of oocytes, eggs and Zoea I larvae from *Palaemon argentinus* (A) and *Neohelice granulata* (B). The intermediate value of osmolality corresponds in both species to the rearing salinity (415 mOsm kg^{-1}, 15 psu in *P. argentinus*, 687 mOsm kg^{-1}, 25 psu in *N. granulata*). The lowest and highest values were measured in homogenates of each stage after two hours of an abrupt change to the experimental salinity. Zoea I of *P. argentinus* were tested only after transferring to freshwater. Larvae were not included in statistical comparisons. O: oocytes; MS: morula stage embryos; NS: nauplius stage embryos; ZI: zoea I larvae. Lowercase letters next to the symbols indicate the result of multiple comparisons between all pairs. Different letters denote differences in homogenates osmolality. Points in A are means for three independent replicates obtained from homogenates of 7 to 10 entire broods. Points in B are means from three different broods, three replicates each. s.d. are small and fall within the limits of symbols except for MS embryos of *N. granulata.*

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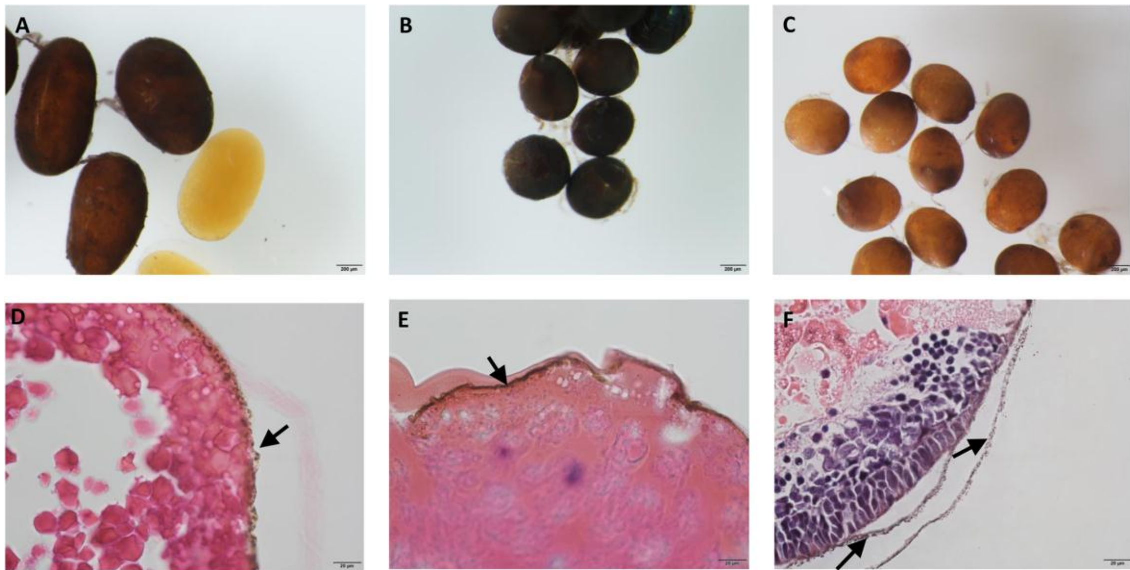

Figure 4: Oocytes and embryos of caridean species treated with silver nitrate and exposed to sunlight. Upper row: whole eggs under stereomicroscope; lower row: sections of similar eggs stained with H/E, under microscope. A,D: Oocytes of *P. argentinus*; B,E: Morula stage embryos of *B. lilianae*; C: Nauplius and F: Metanauplius stage embryos of *P. macrodactylus.* In A one not treated oocyte is on the right to show the natural appearance of untreated material. Arrows indicate the silver salts deposits. The outermost layer is lost in F.

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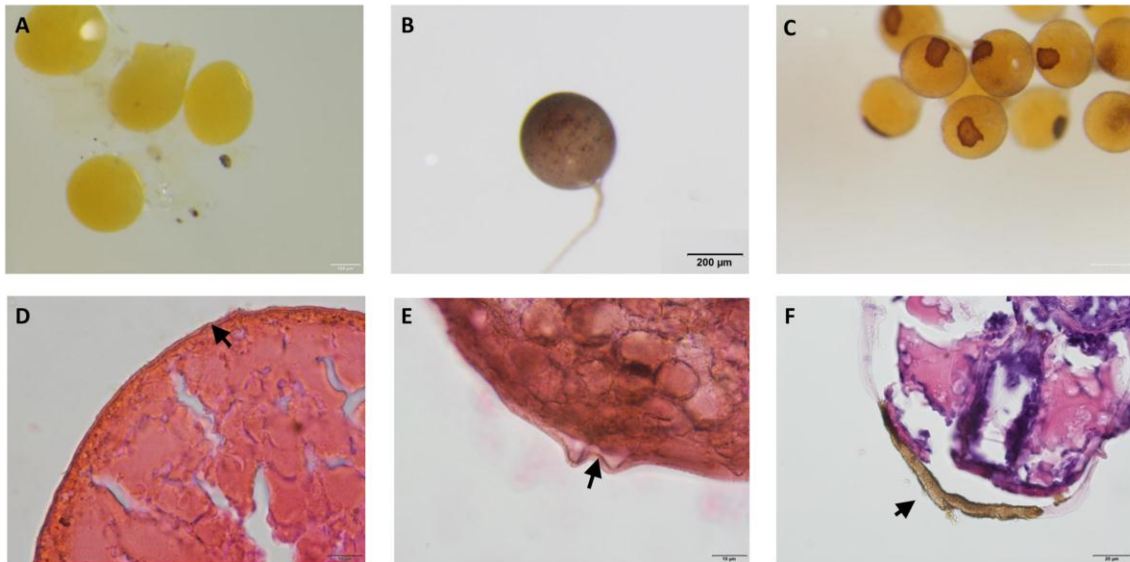

Figure 5: Oocytes and embryos of the brachyuran *Neohelice granulata* treated with silver nitrate and exposed to sunlight. Upper row, whole eggs under stereomicroscope; lower row, sections of similar eggs stained with H/E, under microscope. A, D: Oocytes; B, E: Morula stage embryos; C: Nauplius stage embryos, F: Postnaupliar embryo. Arrows indicate the silver salts deposits.

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