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Ionic movements across the chorion in newly shed salmon eggs (*Salmo salar* L.)

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Summary. Sodium and chloride exchange and trans-chorion potentials were investigated in newly shed eggs from Atlantic salmon. Exposure of eggs to pH 3.5 caused the non-labile sodium fraction at pH 7.0 to become labile and lost from the egg. Chloride fluxes appear unaffected by the pH of the external medium. Trans-chorion potential was inside negative (about −100 mV) in dilute media (10^{-5} mol·l^{-1} NaCl or KCl) and immediately decreased as external cation concentration was increased, by about 46 mV/decade change in cation concentration, reaching about 0 mV in 10^{-2} mol·l^{-1} cation. Return to dilute cation solutions resulted in a slow increase in potential (repolarisation) and the time course of these potential changes was paralleled by the rate of sodium efflux, although chloride efflux was very rapid. After exposure to acid conditions repolarisation of the egg on return to dilute cation concentrations was preceeded by a phase when the pvp became inside positive. The results are discussed in terms of chorion structure, anionic charge on the perivitelline molecules and unstirred layers within the chorion.

Key words: Perivitelline potential – Salmon eggs – Donnan effect

Introduction

While bathed in the ovarian fluids salmon eggs are flaccid but upon shedding and contact with water they swell as a result of water absorption and become turgid. During water hardening the vitelline membrane surrounding the yolk becomes increasingly impermeable to water (Potts and Rudy 1969), and egg weight increases by around 25%, associated with the formation of the perivitelline fluid which exerts an outward pressure on the chorion (shell or zona radiata). The mechanism of water absorption is associated with release from the yolk of Golgi-derived secretory vesicles (cortical granules or alveoli) whose contents are discharged by exocytosis to the perivitelline space. These substances are initially of high molecular weight (200 kDa) but within a few minutes depolymerise to smaller units of 9 kDa, containing a high proportion of sialic acids attached to a tridecapeptide (Inoue and Inoue 1986, Kitajime et al. 1986). The perivitelline colloids, which are too large to penetrate the outer chorion pores, apparently possess a net negative charge in media of high pH resulting in an ability to concentrate cations in the perivitelline fluid from the external medium (Rudy and Potts 1969, Eddy and Talbot 1983, 1985) and the development of a potential difference across the chorion (Peterson 1984, Peterson and Martin-Robichaud 1986). The regulation of ionic balance in the developing salmon ovum presents a number of challenging physiological problems, two of which have been addressed in this study. Firstly, how do extra-embryonic compartments of the egg respond to changes in the cation concentration of the external environment, and secondly what is the influence of acidification of the environment on the egg?

Abbreviations: pvp perivitelline potential

Materials and methods

Freshly shed River Tay salmon eggs were obtained from the Department of Agriculture and Fisheries hatchery at Almondbank, Perthshire and immediately fertilised.

Sodium and chloride fluxes. Batches of 50–100 water hardened eggs were placed in 25 ml 8.3 mmol·l^{-1} sodium chloride solu-

tion to which 37 kBq of 22-Na or 74 kBq of 36-Cl had been added. Allowing 1–2 h for equilibration the required number of eggs were removed on a plastic mesh, briefly rinsed with distilled water to remove surface activity and transferred to 200 ml of 0.1 mmol·l^{-1} NaCl at pH 6.5 or a similar solution acidified to pH 3.5 with sulphuric acid. Eggs were removed at intervals and gamma activity determined using a Panax Reigate iodide crystal detector and counter, or 36-Cl beta activity by scintillation counting. Eggs which had been exposed to acid for 40 min (Fig. 1) were removed, rinsed with distilled water, returned to the loading medium for 1 min and rinsed as previously described prior to counting. A known weight of eggs with a minimal volume of water were finely ground in a glass homogeniser and the resulting suspension spun in a bench centrifuge for a few minutes to form a solid pellet principally composed of yolk, and a clear supernatant used for beta activity by scintillation counting. The scintillant contained 2:1 toluene, Triton X100, and 4 g PPO in one litre. The counter was a Packard 3600. Eggs dissolved in a tissue solvent (Soluene 350, Packard) produced identical counting characteristics. The egg supernatant was analysed for sodium content using an EEL 100 flame photometer.

Potential measurements. Water hardened eggs were stored in distilled water, and then transferred into 10^{-5} mol·l^{-1} cation as the control perfusion fluid. Eggs were attached to a small perspex slide using Parafilm softened in liquid paraffin. The slide was placed in a small bath through which the appropriate bathing solution was continually perfused at up to 10 ml·min^{-1}. The egg was covered with approximately 0.8 ml solution, and so very rapid changes in the ionic composition of the bathing fluid could be achieved by switching reservoirs to expose the egg to a different concentration of NaCl or KCl. Acidification was by addition of HCl. Experiments were carried out at room temperature (19–20 °C). Perivitelline potentials (pvp) were measured between the inside and outside of the egg using glass micropipettes of <0.5 µm tip diameter. When filled with 3 mol·l^{-1} KCl or 2 mol·l^{-1} NaCl, electrodes had a tip resistance of $10–20 \cdot 10^6$ ohm. The indifferent electrode was an agar-KCl bridge connected to an Ag/AgCl electrode. Penetration of the tough chorion with the electrodes sometimes reduced the electrode resistance to less than $2 \cdot 10^6$, but the measured pvps were similar and the eggs responded to solution changes in a quantitatively similar way whether or not the tips had broken. On balance it was felt than electrolyte leakage into the relatively large volume of perivitelline fluid was sufficiently small even from enlarged tips to make the use of fine-tipped electrodes preferable to the very large diameter agar-electrolyte pipettes previously used (Peterson 1984). The choice of filling electrolyte in our experiments seemed less important than reported by Peterson and Martin-Robichaud (1986) which may simply reflect the different electrode configurations or differences in media in which the eggs were kept. KCl was the preferred electrolyte in our experiments in order that electrode tip potentials were kept small. Insignificant changes in junction potential were noted when the perfusion fluid was changed with the electrode tip just outside the egg. Eggs which had been subjected to these electrode impalements continued to develop and hatched normally subsequent to the experiments. All values are reported as means ± standard error of the mean.

Results

Ionic exchange studies

Salmon eggs which had been equilibrated in 8.3 mmol·l^{-1} NaCl rapidly lost sodium when placed in dilute media (Fig. 1) with about 40% of the total labeled sodium released in ten minutes. This presumably represents the bulk of the freely exchangeable sodium, the remainder being less labile and released by acid treatment within 30 min. Return of acid exposed eggs to the loading medium (8.3 mmol·l^{-1} NaCl) for 1 min resulted in a gain in activity to 62% of the initial activity (Fig. 1). The mean sodium content of an egg equilibrated in the loading medium was 18.7 ± 0.37 µmol which decreased to 13.3 ± 0.35 µmol after 30 min in acid and immediately rose to 17.8 ± 0.6 µmol on return to the loading medium. The equilibrium for chlo-

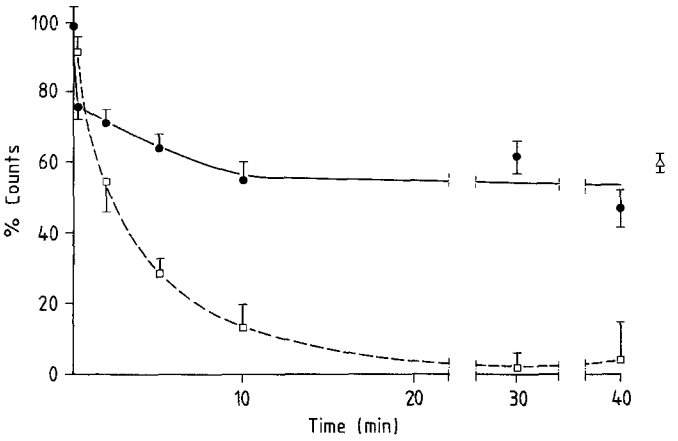

Fig. 1. Sodium efflux of salmon eggs loaded in 8.3 mmol·l^{-1} NaCl then rinsed and placed in dilute media or acid media. Solid circles, eggs to 0.01 mmol·l^{-1} NaCl. Open squares, eggs to acid water pH 3.5 (sulphuric acid). Open triangle, counts reaccumulated when eggs were returned from acid to the loading medium for 1 min. Values are the means ± standard error

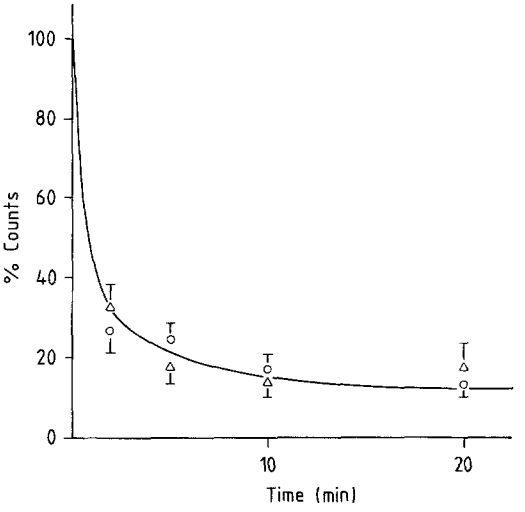

Fig. 2. Chloride efflux of eggs loaded in 8.3 mmol·l^{-1} NaCl then placed in dilute or acid media. Open circle, eggs to 0.01 mmol·l^{-1} NaCl. Open triangle, eggs to acid water pH 3.5. Values are means ± standard error

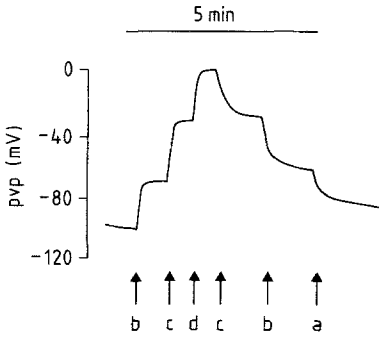

Fig. 3. The influence of external K^+ concentration on perivitelline potential (pvp). The control solution was 10^{-5} mol·l^{-1} KCl, and the solution was rapidly changed to 10^{-4} mol·l^{-1} (b), then to 10^{-3} mol·l^{-1} (c), then to 10^{-2} mol·l^{-1} (d). This procedure was then reversed until the egg was finally returned to 10^{-5} mol·l^{-1} KCl (a)

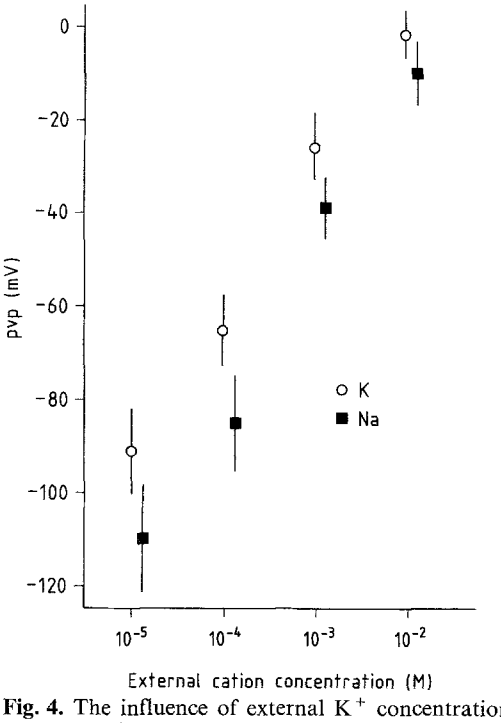

Fig. 4. The influence of external K^+ concentration (open circles) or Na^+ concentration (filled squares) on the perivitelline potential. Results are shown as means ± S.D. of 11 eggs for K^+ and of 6 eggs for Na^+

ride differed in that eggs immersed in either dilute neutral media or acid media released chloride at approximately the same rate (Fig. 2).

Potential measurements

There was a decrease in pvp when either the Na^+ or K^+ concentrations in the bathing fluid was increased above the control 10^{-5} mol·l^{-1} (Fig. 3). The relationship between pvp and log external cation concentration is shown in Fig. 4 and is

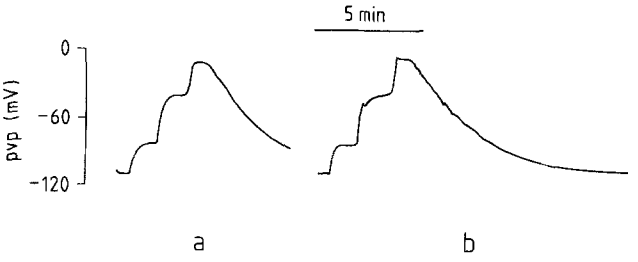

Fig. 5a, b. The influence of external cation concentration on the perivitelline potential (pvp) of a single egg. The protocol was identical to that for Fig. 3. In **a**, external K^+ was changed, whilst in **b** external Na^+ was changed with the electrode still inside the same egg. 15 min elapsed between record **a** and record **b**

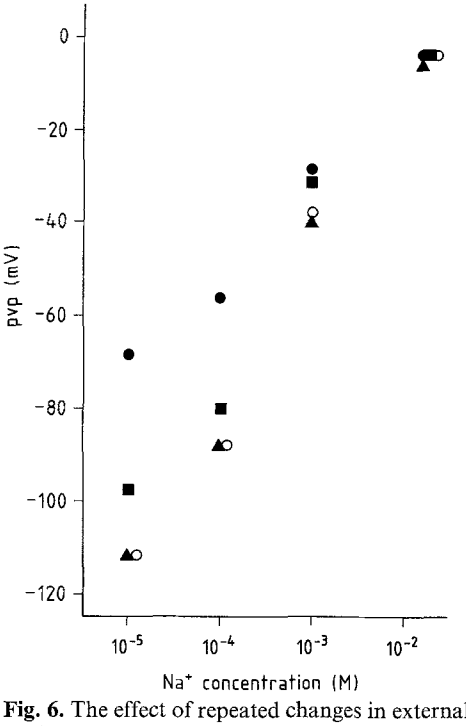

Fig. 6. The effect of repeated changes in external cation concentration on perivitelline potential (pvp). Protocol identical to Fig. 3. Results from first exposure to increasing external Na^+ shown as filled circles. The egg was then returned to 10^{-5} mol·l^{-1} NaCl for approx. 10 min, when a new increased level of pvp was attained (filled square at 10^{-5} molar Na). External Na^+ was then increased again, as in Fig. 4, and results shown as filled squares (at 10^{-4}, 10^{-3}, 10^{-2} Na^+) were obtained. Return to 10^{-5} mol·l^{-1} NaCl produced a further increase in pvp (filled triangle). Protocol then repeated to produce results shown as filled triangles. When protocol was repeated for a fourth time, no further increase in pvp occurred (open circles)

broadly in agreement with the observations of Peterson (1984), although such marked differences between the maximal slopes for Na^+ and K^+ which were 46 mV per decade change in K^+ were not obtained. In one egg, the bathing fluid was changed through the whole range of both K^+ and Na^+ concentrations with the electrode in place.

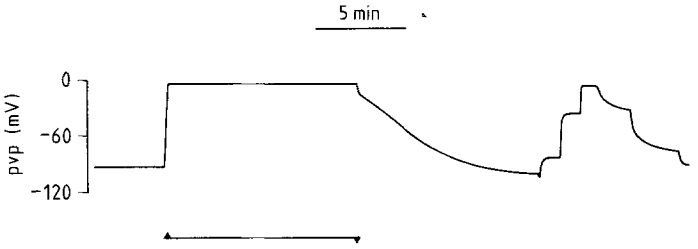

Fig. 7. The time course of repolarization of the perivitelline potential (pvp) after cation-induced depolarization. The bath concentration of NaCl was increased from 10^{-5} mol·l^{-1} to 10^{-2} mol·l^{-1} during the period shown by the lower bar. Note the fast initial phase of repolarisation on return to 10^{-5} mol·l^{-1} NaCl which was followed by a slow phase

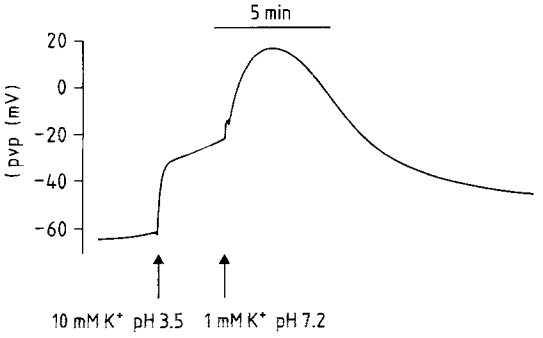

Fig. 8. The effect of protons on perivitelline potential (pvp). External cation and proton concentration was increased from 10^{-5} mol·l^{-1}, pH 7.2 as indicated by the first arrow. Note the slow depolarization superimposed upon the cation-induced depolarization. On return to 1 mmol·l^{-1} K^+ (pH 7.2) note that pvp becomes inside positive

The response of the egg was similar regardless of the nature of the monovalent cation (Fig. 5), just as would be expected if the pvp were determined by a Donnan effect across a non-selective chorion.

A consistent observation was that when the egg was returned to 10^{-5} mol·l^{-1} Na^+ or K^+ after a few minutes at higher cation concentrations, the pvp did not return to its previous level in 10^{-5} mol·l^{-1} cation, but rather it increased (became more negative) significantly (e.g., Fig. 6, see also Fig. 7). Return to 10^{-5} mol·l^{-1} cation after further exposure to higher concentrations produced further hyperpolarization. After several exposures to raised external cation concentrations, the pvp reached a steady state which did not change further despite further exposure to high concentrations. Eggs penetrated immediately after transference from distilled water into 10^{-5} mol·l^{-1} cation showed a steady increase in pvp with time until a maximum was reached as at the start in Fig. 3. If the eggs were exposed to 10 mmol·l^{-1} K^+ or Na^+ for 10 min before placing them in 10^{-5} mol·l^{-1} cation, pvp immediately attained the steady-state value without the steady climb. The data for Fig. 4 were obtained after the steady-state had been reached.

It was apparent that whereas the pvp decreased rapidly when external cation concentration was increased, the repolarization on return to lower concentrations followed a much longer time course (Fig. 5, Fig. 7). The rapid flow rate through the small volume bath made it unlikely that the entire repolarization time reflected a slow rate of change of bath cation concentration, particularly in view of the rapid attainment of a new steady plateau level when bath concentration was elevated. A common finding was that the repolarisation exhibited a fast and a slow component which were particularly prominent upon return to the control solution after a prolonged exposure to a higher cation concentration (Fig. 7).

If the perfusion fluid was switched from 1 mmol·l^{-1} cation pH 7.2 to 10 mmol·l^{-1} cation pH 3.5, a slow phase of depolarization was superimposed upon the rapid cation induced depolarisation (Fig. 8, cf., Figs. 3, 5, and 7). A slow depolarization ensued even if the switch was between 1 mmol·l^{-1} cation pH 7.2 and 1 mmol·l^{-1} cation pH 3.5. When the fluid was changed back to 1 mmol·l^{-1} cation pH 7.2 when the egg was depolarized by either 1 or 10 mmol·l^{-1} cation pH 3.5, a further depolarization occurred in 10 of the 12 eggs tested, such that the pvp became positive (Fig. 8). The example shown in Fig. 8 is of an egg which then repolarized, but some eggs remained inside positive for several minutes before slowly repolarizing.

Discussion

Potential measurements

Many of the findings of this study are in accord with previous reports which attribute the distribution of ions across the chorion of the salmon egg to a Donnan effect set up by the presence of non-diffusible colloids in the perivitelline space (Rudy and Potts 1969). Thus, it appears that the perivitelline potential is dependent upon the cation gradient across the chorion, and in contrast to Peterson (1984), that the egg is unable to clearly distinguish between Na^+ and K^+ (Figs. 4 and 5). The chorion therefore seems to exhibit little selectivity towards monovalent cations even though the mobilities of Na^+ and K^+ through the chorion may be different ($K^+ > Na^+$) (Peterson and Martin-Robichaud 1987). Assuming that the non-diffusible anions on the perivitelline colloids lead to the rela-

tive accumulation of cations (we have looked only at monovalent cations) within the perivitelline space, and that this creates a concentration gradient across the chorion which generates what is essentially a diffusion potential, it should therefore be expected that the pvp should decrease as the gradient is reduced. This is precisely what we find. Instead of the predicted Nernstian relationship between pvp and log external cation concentration we find, as did Peterson (1984), that the maximum slope of the relationship is less than predicted. Our maximum slope was 46 mV per decade change in cation concentration. Such a relationship could be expected if the internal cation concentration also rises as external cation concentration rises. The fact that eggs stored in distilled water slowly gained potential on exposure to 10^{-5} mol·l^{-1} cation, and that this hyperpolarization was speeded up after exposure to millimolar cation, indicates that the cation gradient steadily increases. A point seems to be reached when the cation gradient, and hence the pvp, becomes maximal as the perivitelline exchangeable cation pool becomes "saturated".

The observation that repolarization after exposure to elevated external cation concentrations was very slow in comparison to the preceeding depolarization (see Figs. 5 and 7) may be attributed to a diffusion potential which slowly declines. An alternative explanation could be that there is an additional slowly exchanging cation pool. When the external cation concentration is subsequently suddenly reduced, it is as though the saturated pool postulated above is prevented from seeing the sudden large increase in trans-chorionic gradient because of the intervention of a layer in which the cation concentration is only slowly declining. One possibility is that the chorion itself is acting as this buffer between the outside of the egg and the perivitelline fluid – it is possible that cation rapidly enters the chorion (by anionic attraction?), but that washout is rather more difficult. In other words, it is suggested that some part of the egg, perhaps the chorion, is acting as a poorly-stirred layer.

The slow depolarization that ensues when external proton concentration is raised (Fig. 8) is of unknown origin at present but may be a diffusion potential originating as a result of greater mobility of hydrogen ions over chloride ions (Peterson and Martin-Robichaud 1987). Preliminary experiments indicate that switching to alkaline solutions (pH 9) had little effect on pvp but this needs more rigorous investigation. The rapid and large depolarization which is shown in Fig. 8 upon returning to 1 mol·l^{-1} K^+ (pH 7.2) after a period in 10 mol·l^{-1} K^+ (pH 3.5) was surprising in view of the repolarization which normally occurs when external cation concentration is reduced. Furthermore, this depolarization also occurred when pH was increased again with no change in external K^+ concentration. Clues as to the origin of this seemingly anomalous depolarization can be obtained if this electrophysiological data is considered in parallel to the data shown in Fig. 1. When eggs are placed in acid conditions, there is a rapid and large Na^+ efflux such that approximately 50% of exchangeable Na^+ comes out within 2 min. Increased pH leads to a re-uptake of Na^+ (Fig. 1 and Results). The unexpected depolarization may therefore be explained as follows: on exposure to acid conditions there is a fast cation efflux. Efflux is much faster and greater into acid conditions than into a lower cation concentration of unchanged pH (Fig. 1), and this may indicate that the postulated "buffer" zone (see above) also has titratable negative charge on it. Internal cation concentration is thereby rapidly reduced. On return to an elevated pH, a "back-titration" of anionic sites occurs, so that there is suddenly a large excess of unoccupied sites. The depolarization observed at this time may thus represent an inward cation current down an enormous electrochemical gradient.

An extensive investigation of the involvement of chloride ions in these events has not been carried out. Peterson and Martin-Robichaud (1986) have shown that the anion of the test salt is unimportant in the generation of the pvp. Figure 2 shows that a part of the internal chloride pool of the eggs is exchangeable with the outside, and that washout seems to be indifferent to the presence of acid, unlike the washout of cation. We cannot rule out the possibility that changes in chloride fluxes and concentrations contribute significantly to the potential changes reported here, and this area requires much more investigation.

Possible origin of unstirred layers

The chorion of several salmonid species was investigated by Groot and Alderdice (1985). It is a non-living protective layer and, dependent on species, is approximately 25–50 μm thick penetrated by pores 0.5–0.8 μm in diameter. There is a thin external layer 0.15–0.3 μm thick were the external entrances to the pores are plugged. This outer layer appears to act as a semipermeable membrane allowing diffusion of small molecules only (Groot and Alderdice 1985) and establishement of a Donnan equilibrium for sodium ions (though not a extreme concentrations) as suggested by Rudy and

Potts (1969). Large molecules such as the perivitelline colloids (molecular wt. 9 kDa) are unable to escape and while occupying the pores effectively form an unstirred layer within the chorion. Thus, the negatively charged perivitelline fluid will readily attract external cations and when the eggs are placed in dilute media outward diffusion of cations will be retarded not only by the electrostatic attraction of the bulk perivitelline fluid but also by the relatively immobile perivitelline fluid in the pores. This may explain the slow repolarisation seen when cation loaded eggs are transferred to dilute media (Fig. 8), and the effects of acid media previously mentioned (Fig. 1). Anionic groups on the chorion molecules per se also appear to have ion exchange capacity (Peterson and Martin-Robichaud 1986).

Environmental aspects

Naturally spawned salmon eggs will establish cationic equilibrium between the perivitelline fluid and the surrounding water (Eddy and Talbot 1985); should this balance be upset by an acid episode then cation (mainly sodium) will be lost but upon restoration of normal conditions external cations will rapidly be taken up by the perivitelline fluid. In this way normal sodium levels may be restored but other external cations which may be present e.g., heavy metals, aluminium etc. will readily enter the perivitelline fluid, offering the opportunity for interaction with the previously unexposed yolk and embryo unless the chorion's ability to bind metals effectively protects the embryo from sudden changes.

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