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In vivo oocyte hydration in Atlantic halibut (*Hippoglossus hippoglossus*); proteolytic liberation of free amino acids, and ion transport, are driving forces for osmotic water influx

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Summary

The *in vivo* swelling and hydration of maturing oocytes of Atlantic halibut *Hippoglossus hippoglossus* were studied in order to characterise the osmotic mechanism underlying oocyte hydration in oviparous marine teleosts that spawn pelagic eggs. Sequential biopsies from two females, spanning four hydration cycles, were examined by osmometry, solute analysis and electrophoresis of dissected hydrating oocytes and ovulated eggs. The hydration cycle of the biopsied halibuts lasted 33–54 h. The majority of ovarian oocytes existed in a pre-hydrated condition (individual wet mass approx. 3.7 mg, diameter approx. 1.87 mm, 63 % H_2O) with easily visible, non-coalesced, yolk platelets. Group-synchronous batches of the pre-hydrated oocytes increased in individual wet mass, diameter and water content to reach the ovulated egg stage of approximately 15 mg, 3.0 mm and 90 % H_2O, respectively. The yolk osmolality of the hydrating oocytes was transiently hyperosmotic to the ovarian fluid (range 305–350 mOsmol l^{-1}) with a peak osmolality of about 450 mOsmol l^{-1} in oocytes of 6–8 mg individual wet mass. The transient hyperosmolality was well accounted for by the increase in oocyte content of free amino acids (FAAs; approx. 2300 nmol oocyte^{-1}), K^+ (approx. 750 nmol oocyte^{-1}), Cl^- (approx. 900 nmol oocyte^{-1}), total ammonium (approx. 300 nmol oocyte^{-1}) and inorganic phosphate (P_i; approx. 200 nmol oocyte^{-1}) when relating to the increase in cellular water. The oocyte content of Na^+ did not increase during the hydration phase. Extensive proteolysis of yolk proteins, in particular a 110 kDa protein, correlated with the increase in the FAA pool, although the latter increased by approx. 20 % more than could be accounted for by the decrease in the oocyte protein content. Both indispensable and dispensable amino acids increased in the FAA pool, and particularly serine, alanine, leucine, lysine, glutamine and glutamate. Taurine content remained stable at approx. 70 nmol oocyte^{-1} during oocyte hydration. The results show that final hydration of Atlantic halibut oocytes is caused by an osmotic water uptake in which FAAs, derived mainly from the hydrolysis of a 110 kDa yolk protein, contribute approximately 50 % of the yolk osmolality and ions (Cl^-, K^+, P_i, NH_4^+) make up the balance.

Key words: Atlantic halibut, oocyte hydration, oocyte maturation, proteolysis, yolk protein, free amino acid, ions, osmolality.

Introduction

The hyposmotic state of the body fluids of marine teleost body fluids (Evans, 1993) is reflected in their eggs (Davenport et al., 1981; Riis-Vestergaard, 1982; Watanabe and Kuo, 1986; Mangor-Jensen, 1987; LaFleur and Thomas, 1991). However, these eggs have none of the advantages of the adult osmoregulatory organs associated with homeostasis, and the typical drinking activity seems to start at, or shortly before, hatching (Guggino, 1980; Mangor-Jensen and Adoff, 1987; Riis-Vestergaard 1987; Tytler et al., 1993). This is also true for Atlantic halibut *Hippoglossus hippoglossus* embryos (Tytler and Blaxter, 1988). Thus, like other marine teleosts, the eggs of Atlantic halibut, while still protected within the maternal ovary, must be osmotically pre-adapted to the marine environment prior to spawning. The present study therefore examines the intra-ovarian changes that occur in maturing marine teleost oocytes that pre-adapt them to the hyperosmotic condition of sea water in which they will be spawned.

It was noted more than a century ago (Fulton, 1898; Milroy, 1898) that the oocytes of marine teleosts undergo a dramatic increase in water content during final maturation. The increase was seen to be greater in species that spawned pelagic eggs (pelagophils) compared to those that spawned benthic eggs (benthophils), and the higher water content of the pelagophil eggs was thought to be the major reason for their flotation (Fulton, 1898; Craik and Harvey, 1987; Mellinger 1994). The observations of Milroy (1898) implicated inorganic ions,

particularly Cl^-, as important osmolytes in the hydration process, but he also argued that protein and phosphate levels were elevated in the mature ova. As noted by Alderdice (1988), there is an impressive lack of investigative data relating to the ionic regulation of teleost oocytes. Exceptions are the studies of the pelagophils *Mugil cephalus* (Watanabe and Kuo, 1986), *Micropogonias undulatus* and *Cynoscion nebulosus* (LaFleur and Thomas, 1991) and the benthophil *Fundulus heteroclitus* (Greeley et al., 1986, 1991; Wallace et al., 1992). The analytical studies of Craik (1982) and Craik and Harvey (1984, 1986, 1987) mainly concerned the phosphate-containing fractions of the ovaries and mature eggs of different teleosts, but they also noted increased levels of K^+ in the marine pelagic eggs. Using an indirect method Craik and Harvey (1987) further argued that free amino acids (FAA), derived from proteolysis of yolk protein, were involved in the hydration process. This was later confirmed by Thorsen and Fyhn (1991, 1996) and Matsubara and Koya (1997).

A series of investigations have shown the protein levels decrease in maturing oocytes of marine teleosts, especially in pelagophils (Wallace and Selman, 1985; McPherson et al., 1987, 1989; Greeley et al., 1986, 1991; Norberg, 1987; Thorsen and Fyhn, 1991, 1996; Carnevali et al., 1992, 1993, 1999; Fyhn, 1993; Matsubara and Sawano, 1995; Matsubara and Koya, 1997; Okumura et al., 1995; Matsubara et al., 1995, 1999, 2000; Finn et al., 2000; Reith et al., 2001). Similarly, it is well documented that the newly spawned pelagophil teleost eggs contain a large pool of FAA with a profile that varies little between fishes of different taxa (Thorsen and Fyhn, 1991; Finn et al., 1991; 1995a,b, 1996, 2000; Fyhn, 1989; Fyhn et al., 1999; Rønnestad et al., 1996, 1999; Wright and Fyhn, 2001). The physiological significance, however, of these changes for the large oocyte swelling observed in pelagophil teleosts is still not clear. In this study we examine the *in vivo* oocyte swelling of Atlantic halibut, which is a good model because its eggs are exceptionally large. We studied sequential biopsies obtained during several maturation cycles.

Materials and methods

Sample collection

Two female Atlantic halibut *Hippoglossus hippoglossus* (A, 60 kg; and B, 40 kg) reared at the Austevoll Aquaculture Station, were used for the study. Atlantic halibut are multiple batch spawners, releasing up to 13 portions of pelagic eggs while kept in captivity during their spring spawning season (Holmefjord, 1996). The females were kept in circular tanks (7 m diameter) at 7 °C and 33–35 ‰ salinity during the study period. Ovary biopsies were taken regularly after the females had reached stable ovulatory rhythms (Norberg et al., 1991). Over a 7 day period, the fish were biopsied through the genital pore using a silicon tube (i.d. 4.0 mm) attached to a 10 ml syringe for negative pressure. The fish were not anaesthetised, in accordance with the guidelines for multiple batch spawners at the Austevoll Aquaculture Station. Each female was raised out of the water on a spawning table and the biopsy completed within 2 min. The females were then submerged and released in the holding tank. Biopsies were placed in Eppendorf tubes and transported (at 7 °C, approx. 2 h) to the Department of Zoology for dissection and analyses. All dissections were conducted in a cold room at a temperature of 6–9 °C.

Staging of the oocytes was established by size and degree of transparency (see Fig. 1). Oocytes were classified as oogonia, which were clear round orbs lacking yolk platelets and had a mean diameter of 0.21±0.08 mm; pre-hydrated (PH) oocytes, with a mean diameter of 1.87±0.06 mm; hydrating oocytes, which showed greater degrees of transparency compared to the PH oocytes, and ranged in diameter from 1.88 to 2.86 mm; and ovulated eggs, which had separated from their follicular layers and were greater than 2.86 mm in diameter.

Individual oocytes were isolated and freed from adhering tissues using fine forceps, while ovulated eggs were either rolled out of the biopsy, or stripped from the females. The oocytes and eggs were freed for adhering fluid with lint-free filter paper (Kimwipes®), and the individual wet mass determined in sealed, pre-weighed 1.5 ml Eppendorf tubes using a Sartorius top balance (accurate to ±0.1 mg). Samples were then immediately frozen at –80 °C, and stored at –30 °C until lyophilisation or extraction. Oocyte and egg dry masses were subsequently determined on a Cahn 25 Automatic Electrobalance (accurate to ±1 μg) after lyophilisation for 24 h. Water contents (% of wet mass, and mg oocyte^{-1}) were calculated from the difference between wet and dry mass.

On the first day of sample collection, groups of stripped eggs from each female were also incubated at 7 °C in 34.5‰ sea water (0.2 μm filtered) for 3 h (female A) and 8 h (female B). The water-hardened eggs were rinsed for 2 min in double-distilled water (ddH_2O) and sampled for wet mass, dry mass and diameter measurements before being frozen and stored for further analyses, as for the oocytes.

Oocyte and egg diameters were measured by placing separate sub-samples of the biopsies, in FO medium (Wallace and Selman, 1978), in order to avoid gravitational flattening and desiccation. On the first day of sampling, approximately 500 oocytes from female A were measured to determine the size-frequency distribution, otherwise diameters were determined for 10–30 individual oocytes or eggs in each size class with a calibrated Wild binocular microscope at 25–50× magnification. No further analyses were performed on the sub-samples in FO medium.

Ovarian fluid was taken from stripped egg samples or from the biopsies after slow centrifugation (180 ***g***, 1 min, 4 °C). Urine was collected by applying pressure to the female's abdomen and catching the resulting stream of urine in 1.5 ml Eppendorf tubes. On the last day of sampling approximately 4 ml blood were collected from the caudal artery of female B using a heparinised syringe. The plasma was immediately separated by centrifugation (5000 ***g***, 5 min, 4 °C) and transported with the biopsy at 7 °C for analysis at the Department of Zoology.

Analytical procedures

Osmometry

Osmolalities of yolk, ovarian fluid, urine, plasma and sea water were determined on unfrozen samples for each day of collection using a vapour pressure osmometer (Wescor Inc., model 5100C) calibrated between 290–1000 mOsmol kg^{-1} using commercial standards and 8 µl of sample applied in triplicate. For determination of yolk osmolality, oocytes of each size class were dissected out of the biopsies and external fluid removed as described above. The pooled group of oocytes (5–20 collected in sealed Eppendorf tubes on ice) were then crushed and centrifuged (14000 ***g***, 5 min, 7 °C) and sub-samples (8 µl) of the upper layers of yolk applied to the osmometer.

Solute analyses

Frozen samples were extracted directly (24 h, rotated at 4 °C) in their original Eppendorf tubes with ice-cold 6 % trichloroacetic acid (TCA). The extracts were then centrifuged (14000 ***g***, 10 min, 4 °C) and the supernatants used for quantitation of free amino acids (FAA), Cl^-, Na^+, K^+ and inorganic phosphate (P_i). Cl^- content was determined using a CMT10 chloride titrator (Radiometer Copenhagen) on triplicate sub-samples (4 µl) of the TCA extracts using 20 mmol l^{-1} NaCl (dried) as standard. Blank TCA did not affect the measurements. Contents of Na^+ and K^+ were determined by emission flame photometry (Pye Unicam, SP 192 atomic absorption spectrometer) calibrated between 0–30 µmol l^{-1} Na^+ and 0–30 µmol l^{-1} K^+. Free P_i contents were determined on dilutions (1:200) of the TCA extracts using an assay kit (Sigma Diagnostics, Cat. no. 670-C), micro-modified for the small volumes available. Following incubation in darkness at room temperature for 2 h, assays were read at a wavelength of 650 nm in quadruplicate in a Pye Unicam SP8-100 spectrophotometer, calibrated between 0 and 40 µmol l^{-1} P_i (Sigma Diagnostics, Cat. no. 661-9).

FAAs were determined by reversed-phase chromatography using a Gilson HPLC connected to an ASTED sample robot, fluorimetric detection (OPA and FMOC reagents), Inertsil C3 column (thermostatted at 30 °C), and compared to external standards (a mixture of 24 amino acids) every tenth sample. The indispensable and dispensable terminology of Harper (1983) for amino acids is used.

For calculating the total amount of solute per individual

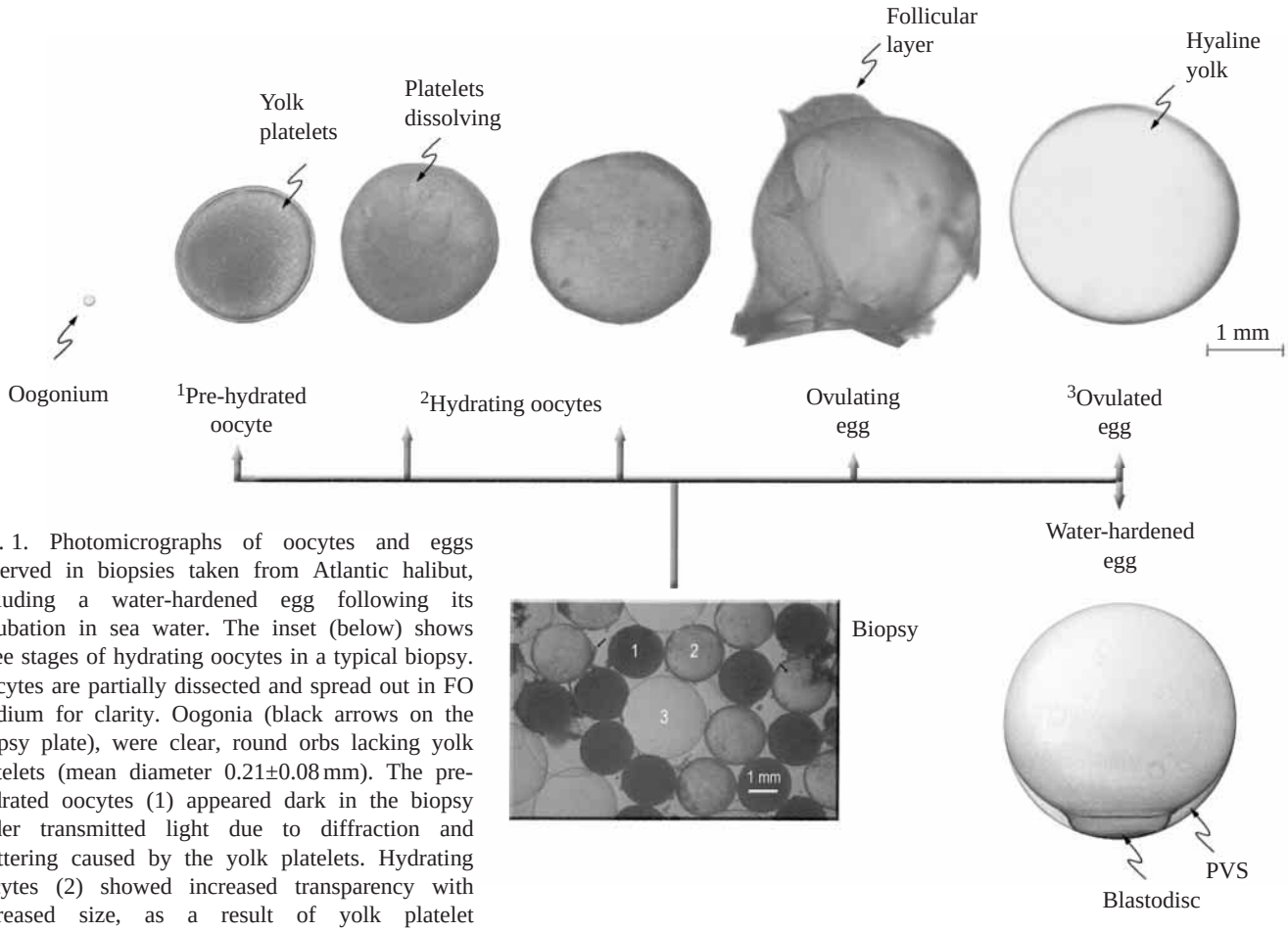


Fig. 1. Photomicrographs of oocytes and eggs observed in biopsies taken from Atlantic halibut, including a water-hardened egg following its incubation in sea water. The inset (below) shows three stages of hydrating oocytes in a typical biopsy. Oocytes are partially dissected and spread out in FO medium for clarity. Oogonia (black arrows on the biopsy plate), were clear, round orbs lacking yolk platelets (mean diameter 0.21±0.08 mm). The pre-hydrated oocytes (1) appeared dark in the biopsy under transmitted light due to diffraction and scattering caused by the yolk platelets. Hydrating oocytes (2) showed increased transparency with increased size, as a result of yolk platelet coalescence. The ovulated eggs (3) were fully separated from their follicular layers, and were highly transparent. The water-hardened egg shows the newly formed perivitelline space (PVS) and blastodisc resulting from cytoplasmic streaming to the animal pole upon water activation. The eggs were not fertilised.

oocyte, the water content (mg oocyte^{-1}) was converted to units of volume assuming a specific gravity of 1.0 kg l^{-1} and added to the TCA extract volume as shown in equation (1):

$$n = cf(V_{TCA}+V_{H_2O}) / N, \quad (1)$$

where n is the amount of solute (nmol oocyte^{-1}), c is the concentration (mmol l^{-1}) of the solute determined from the relevant standard, f is the appropriate dilution factor applied, V_{TCA} is the volume (in µl) of 6 % TCA applied to the sample, V_{H_2O} is the calculated water volume (in µl) of the sample, and N is the number of oocytes. For estimation of the molal solute concentration (mmol kg^{-1}), the determined quantities of solute (nmol oocyte^{-1}) were divided by the water content of the oocyte (mg oocyte^{-1}).

Protein quantitation and electrophoresis

The TCA precipitates were washed once in 6 % TCA, then solubilised in 1.0 mol l^{-1} NaOH under rotation for 24–48 h at room temperature. Equal volumes of ddH_2O were then added to give a final concentration of 0.5 mol l^{-1} NaOH, and solubilisation for an additional 24 h was continued prior to protein determination. Protein content was measured without the addition of surfactant using Bio-Rad's detergent compatible assay kit (Cat. no. 500-0112), which is a micro-modification of the Lowry technique (Lowry et al., 1951). Assays were conducted in quadruplicate using bovine serum albumin (BSA) as standard, and samples read at 750 nm in an Anthos Labtec HTII microplate absorption photometer. Total protein (µg oocyte^{-1}) was calculated according to equation (2):

$$n = cfV_{NaOH} / N, \quad (2)$$

where V_{NaOH} is the volume of sample in 0.5 mol l^{-1} NaOH.

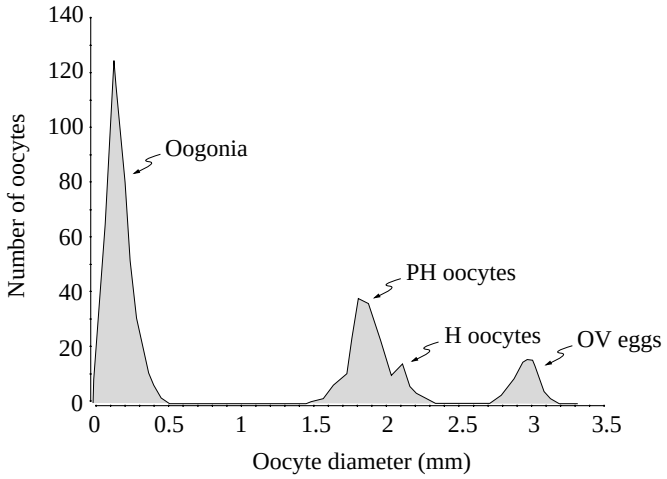


Fig. 2. Size-frequency distribution of oocytes in an ovary biopsy of Atlantic halibut (N=498, female A). PH oocyte, pre-hydrated oocytes; H oocyte, group of oocytes early in hydration; OV eggs, ovulated eggs.

For electrophoresis of yolk proteins, lyophilised samples were homogenised in ice-cold buffer (60 mmol l^{-1} Hepes, 150 mmol l^{-1} NaCl, 50 µg ml^{-1} aprotinin, pH 7.5), centrifuged at 10000 ***g***, 5 min, 4 °C, and the supernatants aspirated into clean Eppendorf tubes. The soluble protein concentration was estimated using Bio-Rad's detergent-compatible assay kit (described above) in order to apply similar amounts of protein per lane. The assays, however, were confounded by increasing amounts of FAA present during hydration (see Results), making true estimation of the soluble protein content difficult. Using the estimates of the soluble protein assays, samples were diluted to 1 µg µl^{-1} with reduced loading buffer (2.5 % 2-

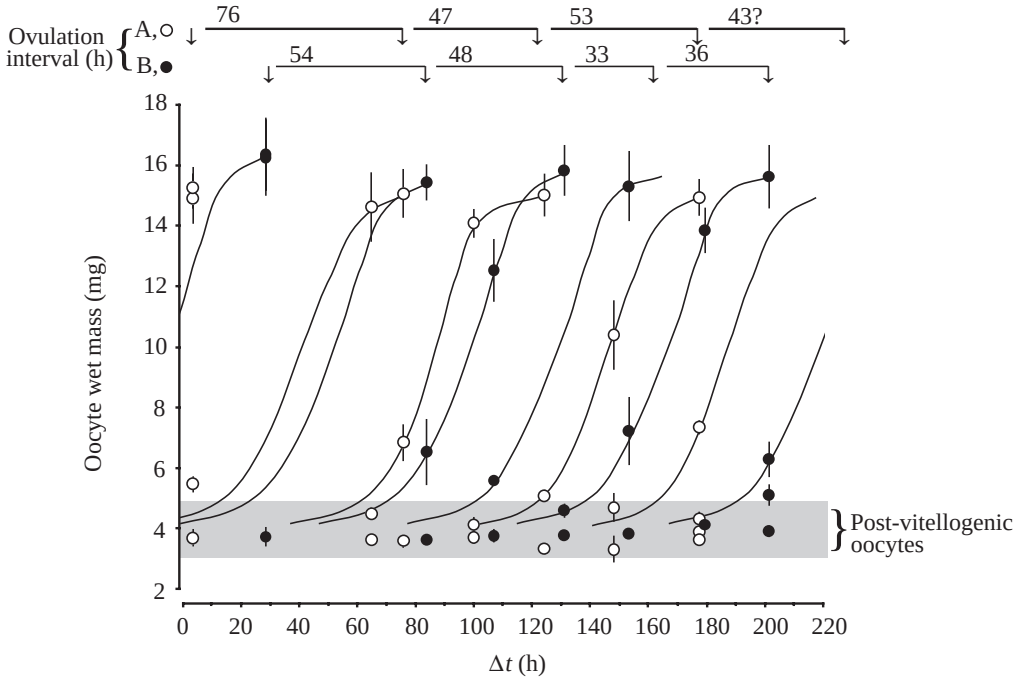


Fig. 3. Ovulation intervals of two female Atlantic halibut (female A, open circles; female B, closed circles) determined from wet mass (means ± S.D.) of oocytes at the time of biopsy sampling. Curves are fitted by eye, and the ovulation intervals calculated as the Δt (h) between the appearance of ovulated eggs in sequential biopsies.

Table 1. *Biometric and gravimetric changes occurring during hydration of Atlantic halibut oocytes*

Parameter	PH oocytes (*N*)	OV eggs (*N*)	Swell factor
Female A			
Diameter (mm)	1.87±0.026 (90)	2.95±0.08 (83)	
Volume (μl)*	3.45±0.14	13.53±1.03	3.9
Wet mass (mg)	3.64±0.17 (53)	14.92±0.65 (79)	4.1
Dry mass (mg)	1.286±0.077 (12)	1.501±0.049 (7)	
H_2O (%)	63.7±2.23	90.1±0.64	
H_2O (mg)	2.26±0.17	13.68±0.78	6.1
Female B			
Diameter (mm)	1.88±0.062 (101)	3.03±0.03 (73)	
Volume (μl)*	3.49±0.34	14.54±0.98	4.2
Wet mass (mg)	3.73±0.20 (54)	15.55±0.55 (60)	4.2
Dry mass (mg)	1.294±0.178 (4)	1.609±0.014 (4)	
H_2O (%)	62.4±1.8	89.8±0.39	
H_2O (mg)	2.16±0.47	14.24±0.049	6.6

Values are means ± S.D. for individual oocytes or eggs.

Swell factors were calculated by dividing the measured parameter of the ovulated eggs by that of the pre-hydrated oocytes.

*Volumes were calculated from diameter measurements using the formula for an elipsoid.

PH oocytes, pre-hydrated oocytes; OV eggs, ovulated eggs.

mercaptoethanol, 0.0625 mol l^{-1} Tris, 10 % glycerol, 2 % sodium dodecyl sulfate (SDS), 0.001 % Bromophenol Blue, pH 6.8) and applied to 7.5 % T, 3.3 % C homogeneous acrylamide/bis-acrylamide gels (0.75 mm) using the buffer system of Schagger and von Jagow (1987). The stacking gel consisted of 4 % T, 3.3 % C acrylamide/bis-acrylamide. Samples were electrophoresed in a Bio-Rad Protean II cell at 95 V, 50 mA per gel for 80 min, and the protein bands visualised with Coomassie Brilliant Blue G-250. For estimation of molecular masses, Bio-Rad precision prestained markers at 250, 150, 100, 75, 50, 37, 25, 15 and 10 kDa (Cat. no. 161-0372) were applied to both sides of the gel.

Results

Examination of the ovary biopsies of the spawning Atlantic halibut revealed that, for any given day, there was a quadri-modal size-frequency distribution of the oocytes (Figs 1, 2), where the majority of oocytes present were oogonia with a mean diameter of 0.21±0.08 mm. Although approximately 500 oocytes were measured, it was clear that this number was subjectively insufficient to yield the true proportions. Fig. 2 does, however, illustrate the typical relative distribution of the oocytes on any given day. No mid-vitellogenic oocytes were observed in the biopsies, and the next size class present were the pre-hydrated oocytes (mean diameter, 1.87±0.06 mm; mean individual wet mass 3.6–3.7 mg; there was no statistical difference between the two tested females (Student's *t*-test, $P>0.05$). Disregarding the oogonia, pre-hydrated oocytes comprised the majority and were always present in the

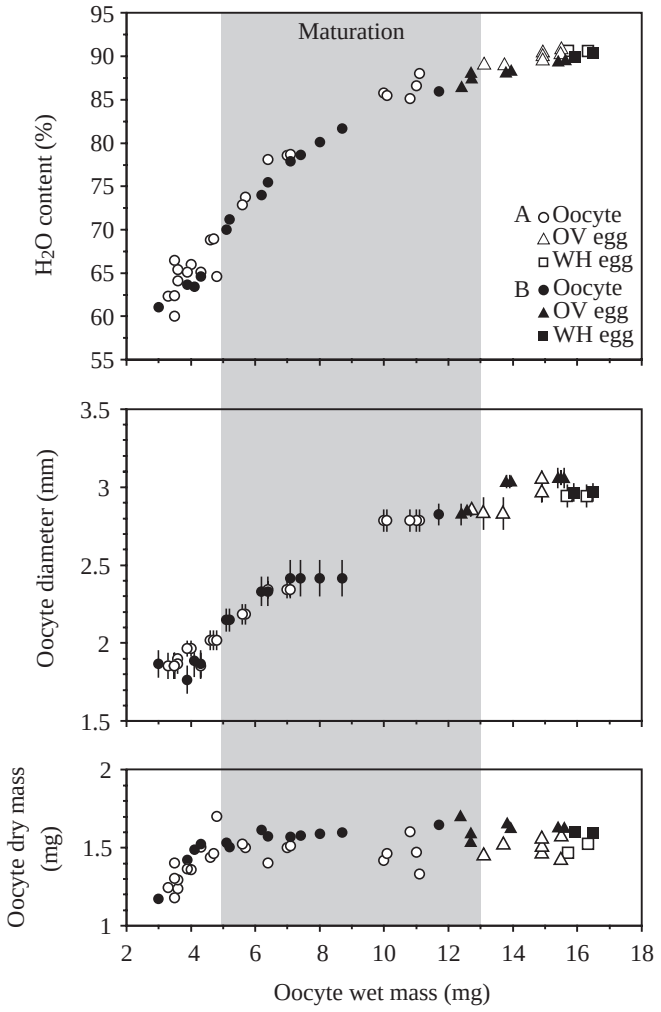


Fig. 4. Atlantic halibut oocyte water content (% wet mass), diameter and dry mass as a function of oocyte wet mass during final maturation (shaded area).

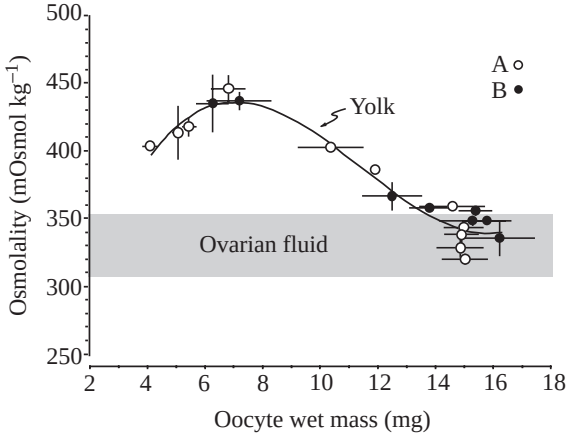


Fig. 5. Osmotic pressure (means ± S.D., vertical error bars) of yolk and ovarian fluid (shaded area denotes the total range of values) as a function of oocyte wet mass (means ± S.D. of the pooled sample used for osmometry, horizontal error bars) (N=5–20) during final oocyte maturation of Atlantic halibut.

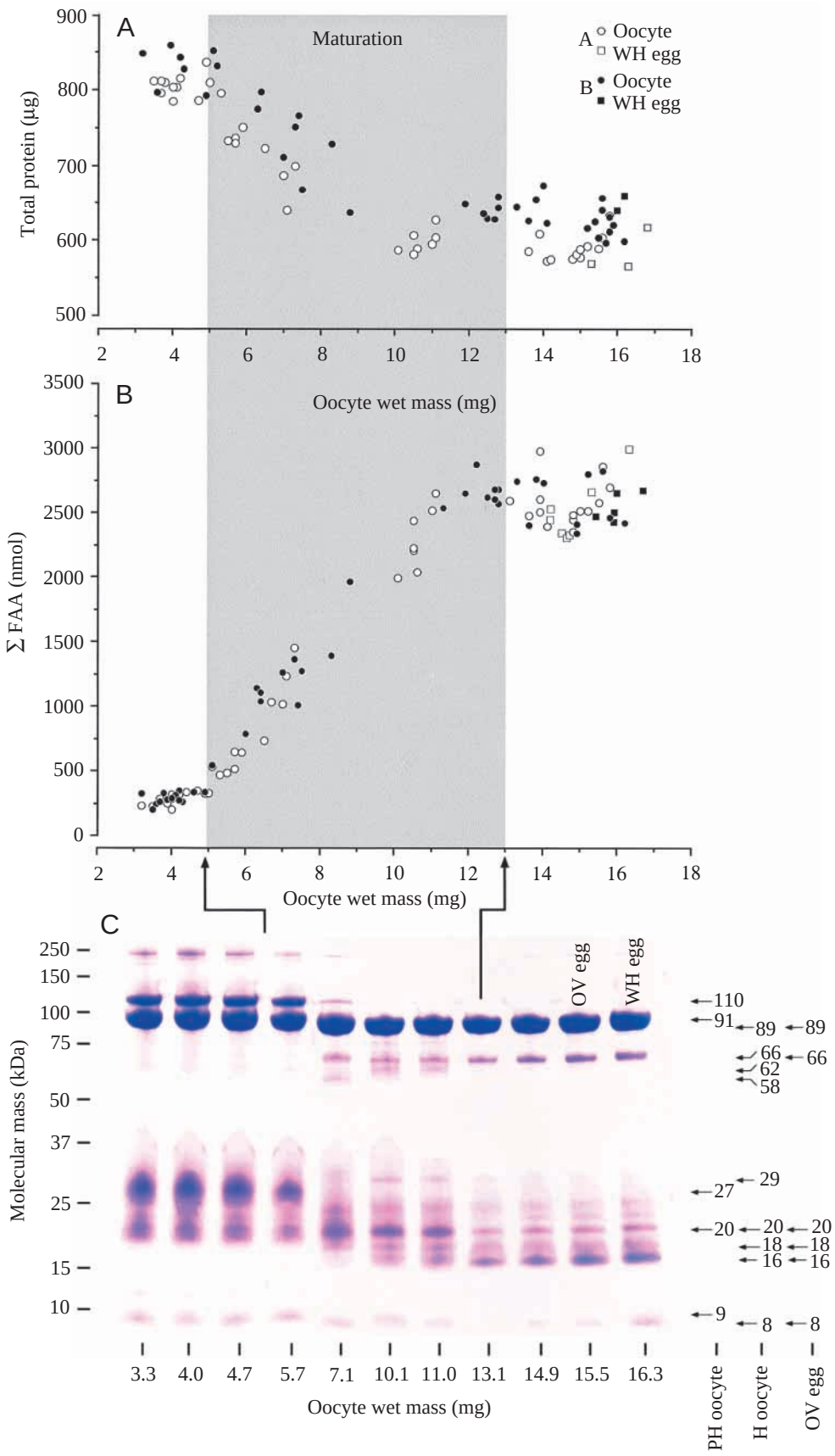


Fig. 6. Atlantic halibut individual oocyte protein content (A), free amino acid content (∑FAA) (B) and electrophoretic protein profiles (C), as functions of oocyte and egg wet mass during final maturation (shaded area). The gel in C (7.5% homogeneous SDS-PAGE gel, Coomassie Blue stained; molecular mass standards indicated at left) is organised relative to oocyte and egg wet mass with reference to the protein and amino acid data. The molecular masses of the oocyte and egg protein bands are identified to the right of the gel. PH oocyte, pre-hydrated oocytes; H oocyte, hydrating oocytes; OV egg, ovulated egg; WH egg, water-hardened egg.

biopsies. They had a clearly visible yolk platelet structure, but during maturation and hydration, they gradually fused and coalesced.

Depending on the time of sampling, larger oocytes were present in varying degrees of hydration. The diameter of the ovulated eggs in the biopsies was 2.86–2.96 mm (12–13 mg), while stripped eggs ranged between 2.97 and 3.06 mm (13.7–15.0 mg). Possibly the mechanical disturbance of the biopsy procedure forced late-maturing oocytes out of their follicular tissue. The ovulated eggs had lost all semblance of the yolk platelets and had clear hyaline yolks (Fig. 1). Plotting the mean wet masses of the oocytes and eggs against the time of sampling revealed the ovulatory interval of each female (Fig. 3). Both females showed a reduced interval during the sampling period, where female A declined from 76 h to approx. 43 h over four cycles, and female B declined from 54 h to 33–36 h over four cycles. This may have resulted from handling and stripping of the fish.

The ovulated eggs of female B were slightly larger than female A, but the degree of oocyte hydration was similar for both females (Table 1). The calculated swell factors characterise the size increase during oocyte maturation. For the two female Atlantic halibut the swell factors for volume and wet mass are well corroborated between 3.9–4.2, but in fact the total water (mg oocyte^{-1}) increased by a factor of 6.1–6.6. The clearest indication that the swell factors represent oocyte hydration, rather than increased size as occurs during vitellogenesis, is the change in relative water content, which increased from approximately 63 % in the pre-hydrated oocytes to 90 % in the ovulated eggs.

Fig. 4 shows the change in dry mass, percentage water content and oocyte diameter as a function of the oocyte wet mass. While the dry mass, wet mass, and thus the water content, refer to measurements on the same oocytes and eggs, the diameter measurements refer to a separate group of oocytes and eggs (see Materials and methods). The major phase of hydration occurred between a wet mass range of 5–13 mg despite there being no change in the dry mass. The relative water content increased from approximately 63 % of wet mass in the pre-hydrated oocytes to 90 % in the ovulated eggs. No difference was found in dry mass, diameter or relative water content between the stripped ovulated eggs and water-hardened eggs of either female. This applied to water-hardened eggs from both females despite the difference in egg incubation time (3 h for female A; 8 h for female B).

Osmotic measurements of the yolk showed it to be transiently hyperosmotic to the ovarian fluid, reaching levels of up to 450 mOsmol l^{-1} compared to a value of 307–353 mOsmol l^{-1} determined for the ovarian fluid of both females (Fig. 5). This transient hyperosmolality increased quickly in the early oocytes, but then gradually declined until the yolk of the ovulated eggs was apparently isosmotic with the ovarian fluid. Plasma osmolality of the single blood sample (female B) at 334 mOsmol l^{-1} was comparable to the mean urine osmolality of 325±10 mOsmol l^{-1} (N=13, both females). These measurements were determined with respect to an external seawater osmolality of 983±22 mOsmol l^{-1} for the fish holding tank.

SDS-PAGE of the oocytes and eggs showed four major protein bands (110, 91, 27 and 20 kDa) and a minor band at 9 kDa in the pre-hydrated oocytes ranging in individual size from 3.3 to 5.7 mg wet mass (Fig. 6C). A band at approx. 230 kDa was also present in pre-hydrated oocytes. The proteolytic event that led to the reduction in intensity of the 110 and 27 kDa bands in particular seems to have commenced in the oocytes of 5.7 mg wet mass (lane 4, Fig. 6), which showed reduced stain intensities compared to the oocytes of 3.3–4.7 mg wet mass. In oocytes between 5.7 and 13.1 mg wet mass the bands at 110 and 27 kDa gradually disappeared while the band at 91 kDa appeared to be shifted down to 89 kDa. Some minor bands at 62, 58 and 29 kDa made a transitory appearance during the swelling phase, until finally the profile of the ovulated eggs was established with a major band at 89 kDa, and additional smaller bands at 66, 20, 18, 16 and 8 kDa. No difference in the electrophoretic profiles of the ovulated eggs and water-hardened eggs could be detected.

During the same phase of protein band shift (5–13 mg oocyte^{-1} wet mass), the total protein content of the oocytes in both females declined by about 25 % (Fig. 6A). Female B had higher total protein content in pre-hydrated oocytes (827±28 μg oocyte^{-1}), falling to 629±21 μg oocyte^{-1} in the ovulated eggs, as compared with 805±15 μg oocyte^{-1} and 588±18 μg oocyte^{-1}, respectively, in the pre-hydrated oocytes and ovulated eggs of female A. The total decline thus varied between 197 and 217 μg oocyte^{-1} for the two females during the phase of oocyte hydration. Concomitant with the decline in total protein, the content of FAA increased from low levels (280±46 nmol oocyte^{-1} for both females) to a large pool of 2508±185 nmol oocyte^{-1} (female A) and 2620±275 nmol oocyte^{-1} (female B). The phase of FAA increase coincided with the hydration phase for oocytes between 5 and 13 mg wet mass (Fig. 6B).

Fig. 7A,B illustrates that both indispensable and dispensable FAA were responsible for the generation of the large FAA pool. All FAA increased during the swelling phase with the exception of the non-proteinic amino acid analogue taurine, which remained stable at 66±15 nmol oocyte^{-1} (female A) and 75±15 nmol oocyte^{-1} (female B, Fig. 7B). Leucine, lysine and valine, among the indispensable amino acids, and alanine, serine, glutamine and glutamate, among the dispensable amino acids, showed the largest increase during the swelling phase.

Typical profiles of the FAA in the pre-hydrated oocytes and ovulated eggs (Fig. 8) show that taurine dominates the pool of the pre-hydrated oocytes (together with the basic amino acids lysine and arginine), while the pool of the ovulated eggs was dominated by alanine, serine, leucine and lysine, followed by valine, glutamine and glutamate.

The changes in ionic content (Fig. 9) show that Cl^- and K^+ were quantitatively the dominant ions in the pre-hydrated oocytes as well as in ovulated eggs. During the swelling phase, Cl^- together with P_i increased strongly in oocytes of less than 8 mg wet mass, then remained stable until Cl^- showed increased

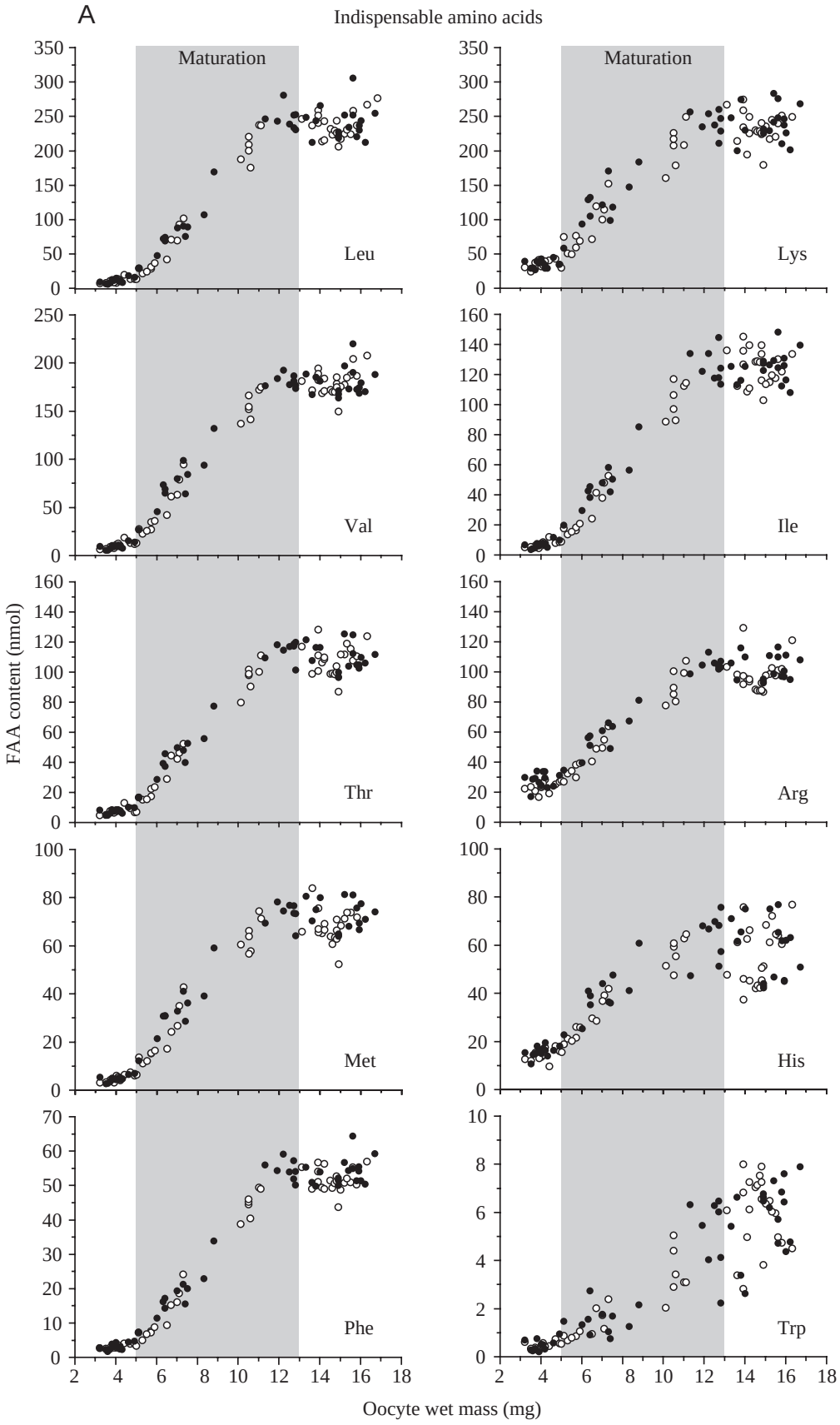
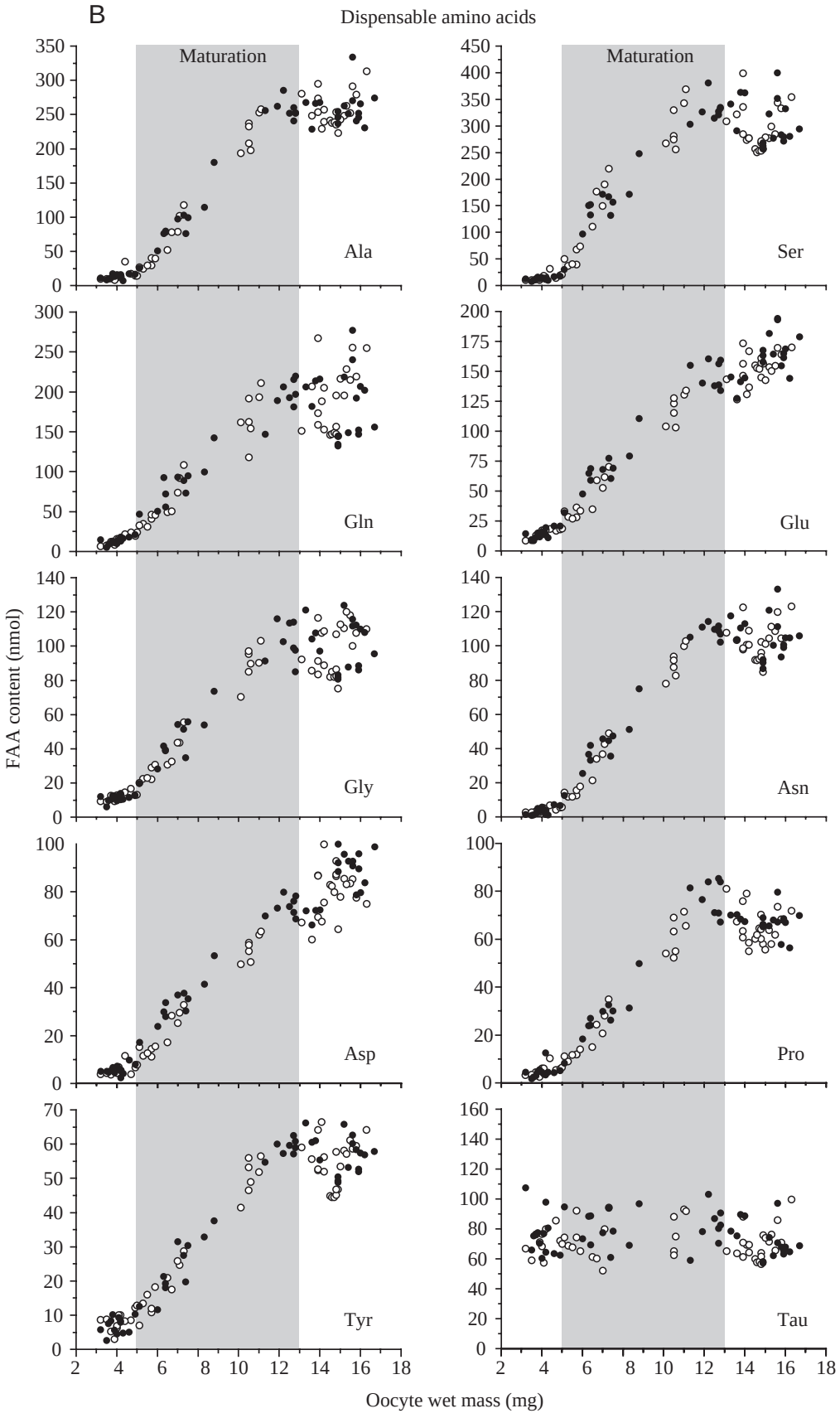


Fig. 7. Atlantic halibut oocyte content of (A) indispensable and (B) dispensable free amino acids as a function of oocyte and egg wet mass during final oocyte maturation in the two females (A, open circles; B, closed circles). Leu, leucine; Lys, lysine; Val, valine; Ile, isoleucine; Thr,



threonine; Arg, arginine; Met, methionine; Phe, phenylalanine; Trp, tryptophan; Ala, alanine; Ser, serine; Gln, glutamine; Glu, glutamate; Gly, glycine; Asn, asparagine; Asp, aspartate; Pro, proline; Tyr, tyrosine; Tau, taurine.

levels following ovulation. K^+, on the other hand, showed a small, but steady increase during the swelling phase, and a stronger increase after ovulation. Among the OV and WH eggs, the data variability for Cl^- and K^+ was especially large. The Na^+ content remained unchanged throughout oocyte maturation with a mean value of 135±55 nmol oocyte^{-1} (for both females).

Discussion

The results presented here suggest that the driving force for the uptake of water by the maturing oocytes is a transient hyperosmolality of the yolk in relation to the blood plasma and ovarian fluid (Fig. 5). The osmolality of the ovulated eggs at 320–360 mOsmol l^{-1} is slightly lower than that determined for newly spawned water-hardened eggs of Atlantic halibut (Riis-Vestergaard, 1982), but similar to those measured in ovulated eggs of Atlantic cod taken directly from the ovarian fluid (Mangor-Jensen, 1987). The yolk osmolality of the Atlantic halibut oocytes also agrees with the data of Watanabe and Kuo (1986) for the pelagophil *Mugil cephalus*, which showed a peak osmolality of 421±17 mOsmol l^{-1} falling to 313±15 mOsmol l^{-1} in the ovulated eggs. An increase in yolk osmolyte content occurring faster than the ensuing water influx indicates a limited membrane permeability, and will form the basis for the hyperosmolality.

The yolk osmolality curve for Atlantic halibut oocytes can be assessed by calculating the molal concentrations from the contents of inorganic and organic solutes (Figs 6–8) and oocyte water content (mg oocyte^{-1}; from Fig. 4). The calculations are shown graphically in Fig. 10. Data for NH_4^+ refer to analyses of the same material, but are published elsewhere (Terjesen et al., 2001) (pre-hydrated oocytes, 45±5 nmol oocyte^{-1}; ovulated eggs, 365±10 nmol egg^{-1}). Applying an average osmotic coefficient of 0.9 to the solutes to take account of solute–water interactions shows that the total solute concentration of the measured osmolytes matches the shape of the osmolality curve shown in Fig. 5. The match for the early oocytes and ovulated eggs is close, but yolk

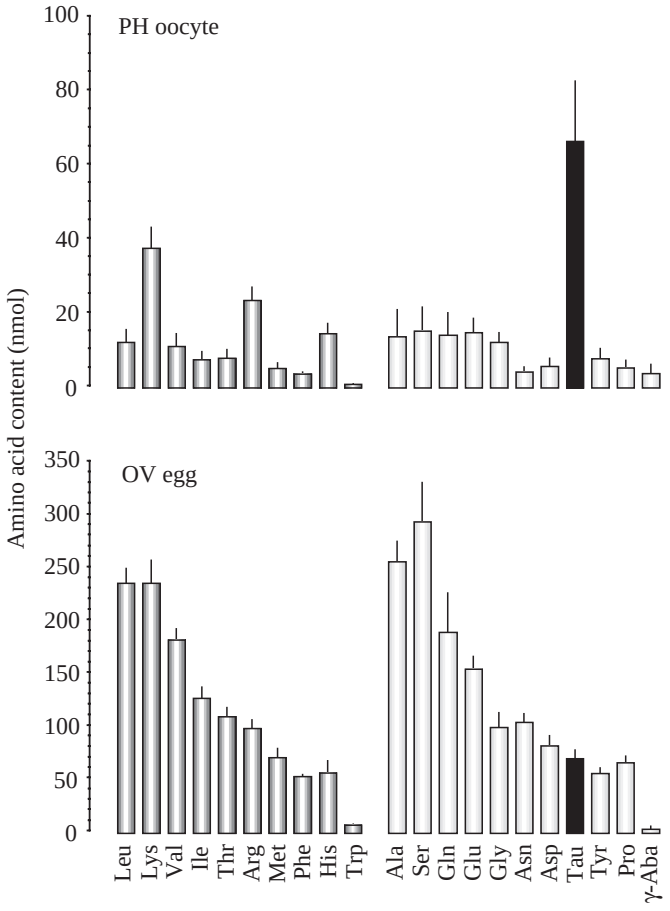


Fig. 8. Amino acid profiles for the pre-hydrated oocytes (PH oocyte) and ovulated eggs (OV egg) of Atlantic halibut. Values are means ± S.D. (*N*=25 for PH oocyte, *N*=24 for OV egg). Amino acid abbreviations as in Fig. 7.

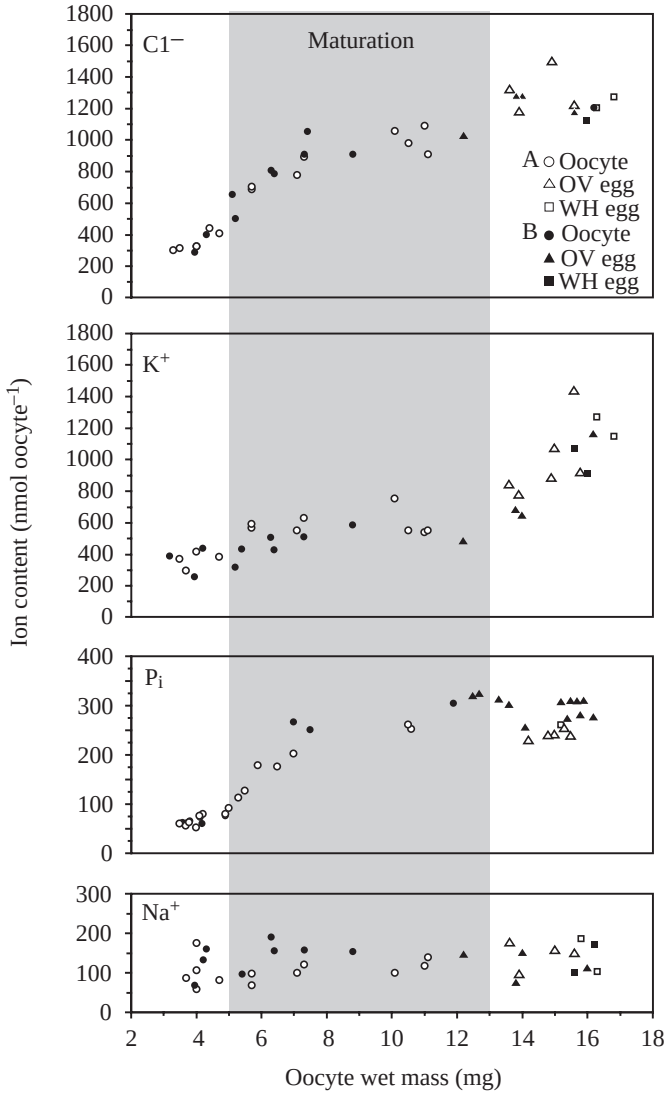


Fig. 9. Atlantic halibut individual oocyte contents (nmol) of chloride (Cl^-), potassium (K^+), inorganic phosphate (P_i) and sodium (Na^+) as a function of oocyte and egg wet mass during final oocyte maturation (shaded area). OV egg, ovulated egg; WH egg, water-hardened egg.

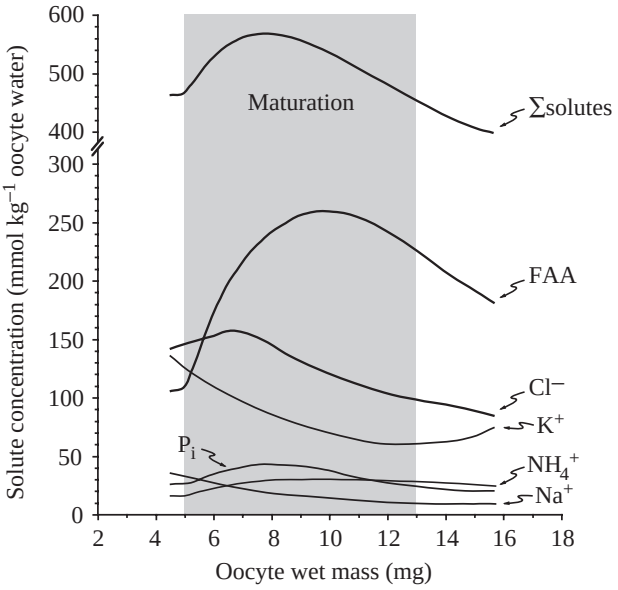


Fig. 10. Solute concentrations (mmol kg^{-1} H_2O) in the hydrating oocytes of Atlantic halibut as calculated from solute contents (nmol oocyte^{-1}, Figs 6, 9) and water content (mg oocyte^{-1}, from Fig. 4) as a function of oocyte and egg wet mass. The curves are polynomials. Data for NH_4^+ ions refer to the present oocyte material, as published in Terjesen et al. (2001). Σsolutes, sum of measured solutes; FAA, free amino acids.

osmolality may have been underestimated during peak solute generation at a wet mass of 8 mg oocyte^{-1}. Despite this discrepancy, Fig. 10 does corroborate the transient hyperosmolality of the yolk during oocyte maturation.

The major part of the driving force for water influx appears to be the generation of the large FAA pool, which reaches a peak concentration of 250 mmol kg^{-1} at a wet mass of 8–11 mg oocyte^{-1}, coinciding with the period of maximal oocyte swelling (Fig. 3). By ovulation, the FAA concentrations had declined to the levels commonly found for the pelagic eggs of marine fish (Rønnestad and Fyhn, 1993; Matsubara and Koya, 1997; Rønnestad et al., 1999; Finn et al., 2000; Gunasekera and De Silva, 2000; Reith et al., 2001; Wright and Fyhn, 2001). Taken together, the FAAs, which occur almost exclusively in the yolk compartment of Atlantic halibut (Rønnestad et al., 1993; Finn et al., 1995a), contribute 50 % of the yolk osmotic pressure, while the sum of the inorganic ions contributes the other half. This supports earlier work, arguing that neither inorganic solutes (Watanabe and Kuo, 1986; LaFleur and Thomas, 1991; Wallace et al., 1992) nor organic solutes (Greeley et al., 1986; Thorsen et al., 1993) are sufficient on their own to generate the observed degree of oocyte hydration in the pelagic eggs of marine teleosts.

Of the inorganic solutes, Cl^- and K^+ have been implicated as major contributors to the water influx in maturing oocytes of marine pelagophils with Na^+ also contributing in the hydrating oocytes of the marine benthophil *Fundulus heteroclitus* (Watanabe and Kuo, 1986; Craik and Harvey, 1987; Thorsen and Fyhn, 1991; LaFleur and Thomas, 1991; Greeley et al., 1991; Wallace et al., 1992). The present data on Atlantic halibut verify the earlier studies of marine pelagophils and, furthermore, our sequential studies on the individual oocytes from the biopsies show that the movement of K^+ in relation to Cl^- occurs in less than equimolar proportions during the major hydration phase. The Cl^- as well as P_i contents increase quite rapidly during the early phase of oocyte hydration, while K^+ lags behind, and increases mainly after ovulation. A similar phase-shift with an increase of K^+ in relation to Cl^- was shown for the hydrating oocytes of *M. cephalus* (Watanabe and Kuo, 1986). This differential movement of K^+ and Cl^- is intriguing and requires further investigation. It may imply the presence of different types of ATP-powered pumps such as the P-class and V-class pumps. The former involve ATP hydrolysis and a regulatory phosphorylation to move H^+, Na^+, K^+ or Ca^{2+}, while the latter do not involve such phosphorylation and typically move H^+ and Cl^- across lysosomal and endosomal membranes (Lodish et al., 2000). It is interesting that the increase of free phosphate occurs early in the hydration of the Atlantic halibut oocytes, concomitant with an increase in free serine (see below). The newly generated P_i pool may also reflect an increased activity of Na^+/K^+-ATPase (a P-class pump), as found during oocyte hydration of *M. undulatus* and *C. nebulosus* (LaFleur and Thomas, 1991). The increasing Cl^- concentration early in the hydration phase of the Atlantic halibut oocytes (Fig. 10) suggests a possible role for V-class pumps, which essentially acidify vesicles during lysosome formation.

The destabilisation, fusion and coalescence of the yolk platelets (Fig. 1) during hydration of maturing marine teleost oocytes has often been noted in the literature (Wallace and Selman, 1978; Oshiro and Hibiya, 1981; Selman and Wallace, 1989; Kjesbu and Kryvi, 1989, 1993; Cerdá et al., 1996; Matsubara and Koya, 1997; Mylonas et al., 1997; Yueh and Chang, 2000; Itano, 2000). Yolk platelet fusion has also been associated with acidification in the yolk vesicles of invertebrates (Mallya et al., 1992), amphibians (Fagotto and Maxfield, 1994; Yoshizaki and Yonezawa, 1998; Komazaki and Hiruma, 1999), birds (Causeret et al., 1991, 1992; Nordin et al., 1991) and mammals (Ichimura et al., 1997; Jerala et al., 1998; Turk et al., 1999), with acid hydrolysis of yolk proteins being a common proposal (Bonnier and Baert, 1992; Nussenzveig et al., 1992; Sire et al., 1994; Fagotto, 1995; Carnevali et al., 1999, 2001). The disappearance of the major protein bands of the pre-hydrated oocytes during the maturation phase of Atlantic halibut (Fig. 6) conforms with this hypothesis. Indeed, yolk proteolysis associated with oocyte hydration in marine teleosts seems to be a common phenomenon (Wallace and Selman, 1985; Greeley et al., 1986; McPherson et al., 1987, 1989; Norberg, 1987; Carnevali et al., 1993; Okumura et al., 1995; Matsubara and Koya, 1997; Thorsen and Fyhn, 1996; Matsubara et al., 1995, 1999, 2000; Finn et al., 2000; Reith et al., 2001).

It is only more recently that the appearance of the large FAA pool, predicted by Craik and Harvey (1984, 1987), Watanabe and Kuo (1986) and Greeley et al. (1986), but confirmed by Thorsen and Fyhn (1991, 1996), Thorsen et al. (1993) and, later,

by Matsubara and Koya (1997), was thought to account for the missing osmolytes that fulfilled the degree of hydration observed in ovulated eggs of pelagophils. The data for individual FAAs shown in Fig. 7A,B illustrate that indispensable and dispensable amino acids are equally responsible for causing the transient hyperosmolality of the yolk of Atlantic halibut. The non-protein amino acid analogue taurine, however, is an exception. Taurine dominates the FAA profile in the pre-hydrated oocytes, contributing up to 28 % (35 mmol kg^{-1}) of the total pool, but shows no increase during hydration and becomes considerably diluted in the ovulated egg. The role of taurine seems therefore to be related to functions other than cellular swelling in Atlantic halibut oocytes. A high taurine content is typical for oocytes and eggs of marine benthophil fishes (Thorsen et al., 1993; Rønnestad et al., 1996). These fishes show considerably less hydration during oocyte final maturation, and the integrity of the yolk platelet membranes remains essentially intact. In this respect, the antioxidant and membrane-stabilisation properties of taurine may be more relevant (Huxtable, 1992; Nakamura et al., 1993; Timbrell et al., 1995).

Earlier studies have demonstrated that the rise in the indispensable and dispensable amino acids during oocyte hydration of marine pelagophils is not due to their transport from an extracellular source (Thorsen and Fyhn, 1996). This implies that the FAAs of the hydrating Atlantic halibut oocytes are derived from degradation of the protein bands that disappear during the maturation phase, particularly the 110 kDa protein in the pre-hydrated oocytes (Fig. 6). Quantitatively the decline in total protein (197–217 μg $oocyte^{-1}$) is less than the increase in the FAA pool (240–250 μg $oocyte^{-1}$) when using a mean content of 103 g mol^{-1} for the polymerised amino acids. This suggests that proteins continue to be sequestered, but are rapidly hydrolysed to FAA, up to ovulation, when the oocytes lose intimate contact with the follicular layers. The stable protein levels but increasing FAA contents of oocytes greater than 10 mg, and the lack of the 110 kDa band in these oocytes, lend weight to this argument. A sequestering of oocyte proteins during the maturation phase agrees with previous proposals for the pelagophils *Scophthalmus maximus*, *Pleuronectes platessa*, *Gadus thori* and *Gadus morhua* (Thorsen and Fyhn 1991; Thorsen et al., 1993; Kjesbu et al., 1996), and for the benthophil *Fundulus heteroclitus* (Wallace and Selman, 1985; Thorsen et al., 1993).

The identity of the 110 kDa protein has not yet been confirmed, but is probably homologous to the heavy chain of lipovitellin A found in the oocytes of *Verasper moseri* (Matsubara et al., 1999). Based on immunoblot studies, these authors argued that lipovitellin A is a major source of the egg FAA pool in *V. moseri*. The small shift in the 91 kDa band to 89 (Fig. 6) has also been reported for two other Pleuronectiformes; *Paralichthys olivaceus* (Matsubara et al., 1996) and *V. moseri* (Matsubara and Sawano, 1995; Matsubara and Koya, 1997), and this protein is likely to be homologous to the heavy chain of lipovitellin B of *V. moseri* (Matsubara et al., 1999). Similar changes have also been observed in Gadidae pelagophils, although their A and B heavy chain lipovitellins are almost equal in size (Matsubara et al., 2000; Reith et al., 2001). Western immunoblots of the Atlantic halibut pre-hydrated oocyte proteins indicate that the lipovitellin light chains are located in the 27 kDa band, while the β-component is the 20 kDa band (Fig. 6; T. Matsubara, personal communication). Staining of the phosvitins was not achieved with Coomassie or silver stains (silver stains not shown) and their gel location is currently being investigated. The appearance of the free phosphate pool, however, implies partial or full degradation of phosvitins during the hydration phase. Phosvitins are heavily phosphorylated, serine-rich derivatives of vitellogenin (Wallace and Bergovac, 1985) and their degradation observed in *V. moseri* (Matsubara et al., 1999) should imply a relationship between the increases of free phosphate and free serine in Atlantic halibut. Based on the present data (Figs 7B, 9) the appearance of free P_i is faster than that of free serine during the early stages of hydration. This indicates that phosphatases are activated prior to hydrolases in the maturing oocytes of Atlantic halibut, but further investigation is required.

The differential processing of the yolk proteins during the maturation of teleost oocytes has recently been attributed to the presence of multiple forms of vitellogenin (Matsubara et al., 1999, 2000; Reith et al., 2001). As discussed by Reith et al. (2001), multiple forms of vitellogenin have been detected in only a few vertebrates, and are therefore of considerable interest with regard to the evolution of the teleosts. It has been hypothesised (Fyhn et al., 1999) that the hydrolysis of the yolk proteins resulting in the appearance of the FAA pool may have been a key event that permitted the radiation of the teleosts in the oceans during the Cretaceaous period. The remarkable similarity of the FAA pool (Fig. 8) demonstrated in all pelagic eggs investigated to date (Rønnestad et al., 1999) lends weight to this hypothesis, but more comparative studies of teleost vitellogenins and the subsequent processing of oocyte and egg proteins are needed.

In conclusion, these studies examined the physiological mechanisms underlying the *in vivo* hydration of oocytes of Atlantic halibut, a deep-water marine teleost that spawns pelagic eggs. Sequential biopsies revealed that group-synchronous batches of oocytes underwent a rhythmic cycle of hydration, such that the water content of the pre-hydrated oocytes increased from approximately 63 % of wet mass to 90 % in the ovulated eggs. The driving force of the oocyte hydration is a transient hyperosmolality of the yolk, which is due mainly to the liberation of FAAs by extensive hydrolysis of, predominantly, a 110 kDa yolk protein, but inorganic ions (Cl^-, K^+, P_i and NH_4^+) also participate, with Cl^- being the dominant species. Taken together, FAAs contribute 50 % of the increase in yolk osmolality, and inorganic ions make up the balance. We argue that these mechanisms of oocyte hydration are responsible for pre-adapting the pelagic eggs of teleosts to the hyperosmotic condition of sea water in which they will be spawned.

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