Paper I

Major osmolyte changes during oocyte hydration of a clupeocephalan marine benthophil: Atlantic herring (*Clupea harengus*)

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Abstract

The major inorganic and organic osmolytes responsible for hydrating the oocytes during pre-ovulatory meiotic maturation in autumn- and spring-spawning stocks of Atlantic herring are examined. Despite the ovulated eggs of spring-spawning herring being 1.6 to 2-fold larger than the autumn-spawning stock, the GSI (27 ± 3%) and degree of oocyte hydration (70 - 72% water) were similar. Normalising the data with respect to dry mass revealed that the physiological mechanisms underlying the maturational influx of water were the same for both classes of egg. Cl^-, K^+ and P_i together with a small pool of free amino acids (FAA) represented the driving forces for oocyte hydration. K^+ (autumn and spring) and P_i (spring) maintained their concentrations in the ovulated eggs, while all other ions, including Cl^-, Na^+, NH_4^+ and Mg^{2+} were significantly diluted. In contrast the FAA concentration increased during the hydration process. Amongst the inorganic ions, Cl^- showed the greatest increase in the ovulated eggs. The FAA content doubled from 1.5% to 3.3% of dry mass during oocyte hydration and accounted for 29% to the calculated ovoplasmic osmolality in the ovulated eggs from both autumn- and spring-spawners. This significant osmotic effect of the small pool of FAA was due to the low water content of the benthic eggs. The differential movement of the inorganic and organic osmolytes that underly oocyte hydration in Atlantic herring are discussed in relation to current models of transmembrane ion flux.

Key words

Oocyte hydration; meiotic maturation; osmolality; osmoregulation; ionoregulation; yolk proteolysis; egg yolk proteins; endosome; lysosome; marine teleost

Introduction

Oocyte hydration is a cellular event that coincides with meiotic maturation in marine or brackish water teleosts prior to ovulation. The two processes are closely, but independently regulated by extra- and inter-cellular signalling cascades that involve pituitary hormones and follicular steroids, intracellular control of acid hydrolases, and the differential proteolysis of yolk proteins (Selman et al. 2001; Carnevali et al. 2006; Finn et al. 2007a). Water influx via specialised aquaporins (Fabra et al. 2005; 2006; Cerdà et al. 2007) involves the generation of a transient intra-oocytic osmotic potential through depolymerisation of mostly VtgAa-derived yolk proteins and/or transmembrane ion fluxes (Finn et al. 2002a; Finn 2007a).

Earlier studies have shown that the degree of hydration differs between species that spawn pelagic and benthic eggs, pelagophils and benthophils, respectively (Oshiro & Hibiya 1981a, b; Craik & Harvey 1984, 1987; Thorsen & Fyhn 1991; Thorsen et al. 1996; Finn et al. 2002a; b; Cerdà et al. 2007). In pelagophils oocyte hydration reaches levels of >90%, and is primarily driven by organic osmolytes in the form of free amino acids (FAA) that are liberated mainly due to the differential proteolysis of VtgAa type yolk proteins (Matsubara et al. 1999; Reith et al. 2001; Finn et al. 2002a; b; Sawaguchi et al. 2006a; Finn 2007a; b; Finn & Kristoffersen 2007; Kolarevic et al. 2008).

Inorganic ions also play an osmotic role in these species, but the ions involved appear to vary according to species. In certain pelagophils, K^+ but not Na^+ is argued to play a major osmotic role in the oocyte hydration of plaice (*Pleuronectes platessa*) (Craik & Harvey 1984; Thorsen & Fyhn 1991), lemon sole (*Microstomus kitt*) (Thorsen and Fyhn 1991), Atlantic croaker (*Micropogonias undulates*) (LaFeur & Thomas, 1991), spotted seatrout (*Cynoscion nebulosus*) (LaFleur & Thomas 1991), black seabass (*Centopristis striata*) (Selman et al. 2001) and gilthead seabream (*Sparus aurata*) (Fabra et al. 2006). In other pelagophils, Cl^- plays a greater osmotic role during the hydration phase in grey mullet (*Mugil cephalus*) (Watanabe & Kuo 1986), Baltic and Atlantic cod (*Gadus morhua*) (Thorsen et al. 1996), and Atlantic halibut (*Hippoglossus hippoglossus*) (Finn et al. 2002a). In these latter species K^+ also increases, but the increase is not equimolar with Cl^- until the final stages of hydration. Furthermore, in contrast to other pelagophils studied, grey mullet has a high Na^+

content, although its content does not change during oocyte hydration (Watanabe & Kuo 1986).

In marine benthophils oocyte hydration is more modest, and may or may not involve limited proteolysis of VtgAa-type yolk proteins (Finn et al. 2002b; LaFleur et al. 2005). For species such as common mummichog (*Fundulus heteroclitus*), corkwing (*Crenilabrus melops*) or ayu (*Plecoglossus altivelis*) limited yolk proteolysis occurs resulting in the liberation of a small pool of FAA (Greeley et al. 1986; McPherson et al. 1989; Finn et al. 2002b; Chen et al. 2003; LaFleur et al. 2005; Raldúa et al. 2006). In other marine benthophils, such as ballan wrasse (*Labrus bergylta*), and cuckoo wrasse (*Labrus mixtus*) no such depolymerisation of yolk proteins is observed (Finn et al. 2002b). Oocyte hydration in such species appears to be driven by the differential movement of inorganic ions across the plasma membrane. In common mummichog, K^+ is regarded to be the major inorganic osmolyte with Na^+ playing a lesser role in the hydration process (Greeley et al. 1991). In contrast Na^+ has been shown to be the dominant cationic osmolyte in ayu (Chen et al. 2003). Few studies, however, have examined the simultaneous changes in multiple ions and organic osmolytes during oocyte hydration in teleosts. The present study provides such data for Atlantic herring, a clupeocephalan marine benthophil.

As a group, the Clupeiformes are intriguing since they were amongst the earliest extant fishes to reinvade the oceans during teleost evolution (Finn & Kristoffersen 2007). About 150 fossil species of Clupeiformes have been described, with marine forms that date back to the early Cretaceous (Maissey 1996). They are basal members of the larger rank Clupeocephala, which includes the sister-group Ostariophysi (Lecointre & Nelson 1996; Nelson 2006). This latter group (mainly carps, characins, loaches and catfishes) did not reinvade the oceans, but with ~8000 species among 68 families, is the most species abundant group of freshwater teleosts (>98% live in freshwater). We have previously argued that oocyte hydration was a key event in the adaptive evolution of the Acanthomorpha to a marine life (Finn & Kristoffersen 2007). The aim of the present study was therefore to understand how more ancestral marine benthophils solved the problem of hydrating their eggs prior to oviposition in the hyper-osmotic spawning environment of the ocean.

Materials and methods

Samples

Sampling of adult females (N = 19) was conducted using gill nets during the autumn (September – October) and spring (March) spawning seasons in coastal waters near Bergen, Norway. Females were euthenised (blow to the head) and transported on ice to the Institute of Biology, University of Bergen where biometric data (fork length and total length; ± 1 mm), and gravimetric data (female whole body, liver and ovary wet masses; ± 0.1 g) were determined. Gonadosomatic index (GSI) was calculated as the ovarian fraction of the female wet body mass. Transport to the laboratory lasted <12 hr, a procedure that does not compromise egg viability. We have found that wild-caught Atlantic herring kept on ice maintain fertilisable eggs up to 24 hr post mortem.

Vitellogenic oocytes (Vtg ooc; N = 10-15 per tube), pre-hydrated oocytes (PH ooc; N = 15-20 per tube) and ovulated eggs (OV eggs; N = 15-25 per tube) were individually dissected from two, four, and eight autumn-caught females, respectively. OV eggs (N = 12-17 per tube) were also individually dissected from five spring-caught females (Table 1). All dissections of oocytes and eggs occurred following ovary excision in a cold room (6ºC). Oocyte and egg samples were analysed for wet mass (Sartorius top balance, ± 0.1 mg) after removal of excess ovarian fluid, immediately frozen in liquid nitrogen, and stored at -80ºC until lyophilisation or extraction. Separate samples (N = 30-40) were placed in FO medium (Finn et al. 2002b) for major (d_1) and minor (d_2) diameter measurements using a calibrated binocular microscope. Oocyte and egg volumes (V) were calculated according to Alderdice et al. (1979a) using the following formula:

$$V = 4/3\ \pi\ (d_2/2)^2\ (d_1/2)$$

Mean diameters (D) were estimated from the volumes using the following formula:

$$D = 2(3V/4\pi)^{1/3}$$

After lyophilisation, sample dry mass was determined for 2 - 8 tubes from each female (Cahn 25 Automatic Electrobalance, ± 1 µg). Oocyte and egg stages were

classified by size, visual inspection of the degree of yolk transparency and retention in ovarian follicles. Ovulated eggs were free-running within the ovarian lumen.

Sample treatment for ion and FAA analyses

Frozen samples were extracted directly in their Eppendorf tubes using a 1:1 mix (v/v) of ice-cold acetonitrile (AcN) and glass distilled, ion exchanged (Millipore Milli-Q) water (ddH_2O). After sonication (15 min) samples were allowed to extract under rotation (Hetomix) for 24 hr at 4°C. Samples were then centrifuged (10 000x *g*, 5 min, 4°C) and the supernatant appropriately diluted in ddH_2O for ion analyses and borate buffer for amino acid analyses (see below).

Inorganic ion analyses

Inorganic ions were analysed using a Dionex DX-120 Ion Chromatograph set up for cation and anion analyses (CS12A & AS9-HC columns including pre-columns) with CSRS and ASRS Ultra II suppressors. Samples were automatically processed using a Gilson 221 XL Liquid Handler and a Gilson 402 Syringe pump. Chromeleon (version 6.60) software from Dionex was used for data acquisition and integration. The eluents (flow of 1.0 mL min^{-1}) were 10 mM H_2SO_4 for cations and 9 mM Na_2CO_3 for anions. External standards (Dionex five anion standard, part # 37157 and Dionex six cation-II standard; part # 46070) were run at four concentration levels prior to- and subsequent to each run, with ddH_2O as blank. All samples, standards and blanks were analysed in duplicate. The supernatants were appropriately diluted in ddH_2O before analyses depending on the sample. The analytical reproducibility based on repetitive analyses of standards was <1.5% for all ions except phosphate (P_i) at 100 μM, which showed a slightly higher variability (≈2.5%).

Amino acid and protein analyses

Free amino acids (FAA) were determined by reversed phase liquid chromatography by a Gilson HPLC automated with an ASTED robot using fluorometric detection (orthophtal-dialdehyde, OPA and 9-fluorenyl-methoxy-carbonyl, FMOC reagents). A

Varian Inertsil 3 ODS-3 (3 µm), 150 x 3 mm column, thermostated at 30ºC, and ChromSep guard column SS 10 x 2 mm, were used. The mobile phase was a mixture of solution A (10 mM phosphate buffer, pH 6.6) and solution B (volume mixture of solution A:AcN:methanol = 2:3:5). Column flow increased from 0.5 mL min^{-1} (start of run) to 0.7 mL min^{-1} (end of run), starting at 100 % solution A and ending at 100 % solution B. Gilson Unipoint Software, version 2.10 was used for sample integration. Blanks and external standards (mixture of 20 amino acids at 25 µM (125 µM for Pro and Lys) made from Sigma LAA21-IKT kit containing Tau, or Pierce amino acid standard H at 25 µM) were run for every eighth sample. Based on previous tests the yield for Gln as standard was estimated as 94% of the equivalent amount of Tau. The supernatants were appropriately diluted in borate buffer (100 mM; pH 10.4) prior to analyses. The analytical reproducibility based on repetitive analyses of standards was <1% for all amino acids except Pro (4%).

Protein content was determined in quadruplicate with the Lowry technique following precipitation and washing with trichloro-acetic acid as described by Finn et al. (2002a).

Statistical treatment of the data

Statistical differences at the 5% level were determined via ANOVA or the Student *t*-test according to the procedures of Sokal & Rohlf (2000).

Results

Gravimetry and biometry

Based on the GSI and the presence of oocytes or eggs, two populations of Atlantic herring were observed during the autumn sampling season (Fig. 1). Females that had low GSI (5.9 ± 1.8%), and Vtg ooc were classified as spring-spawners undergoing vitellogenesis. The second population consisted of mature females with either post-vitellogenic pre-hydrated oocytes (PH ooc) or ovulated eggs (OV eggs). These latter females were classified as autumn-spawners.

Table 1. Biometric and gravimetric data for females, oocytes and eggs from autumn (A) and spring (S) spawning Atlantic herring (*Clupea harengus*). For gravimetric data, n refers to the number of replicate tubes of oocytes or eggs analysed from each female, and ($\sum n_i$) for the total number of oocytes or eggs dissected. For mean diameter data, n_i refers to the number of individual oocytes or eggs measured. GSI for female 14 was not recorded due to leakage of free-running eggs. Vtg ooc: vitellogenic oocytes; PH ooc: pre-hydrated oocytes; OV egg: ovulated egg.

Female	spawning season	wM	SL	TL	GSI		wet mass (mg/ind)		dry mass (mg/ind)			diameter (mm)		
		(g)	(cm)	(cm)	(%)	Stage	n ($\sum n_i$)	Mean ± SD	n ($\sum n_i$)	Mean ± SD		n_i	Mean ± SD	
1	S	376.6	28.5	30.5	7.1	Vtg ooc	8 (80)	0.16	0.02	4 (40)	0.066	0.005	26	0.67	0.05
2	S	297.1	27.4	29.3	4.6	Vtg ooc	8 (100)	0.17	0.01	4 (59)	0.067	0.004	30	0.68	0.03
3	A	350.8	28.3	30.2	19.1	PH ooc	12 (185)	0.54	0.02	4 (65)	0.221	0.002	30	0.99	0.10
4	A	350.8	28.3	30.2	22.2	PH ooc	12 (180)	0.53	0.02	4 (60)	0.214	0.002	30	0.99	0.10
5	A	291.1	26.3	28.0	22.3	PH ooc	12 (185)	0.61	0.03	4 (65)	0.247	0.004	30	1.04	0.08
6	A	334.0	29.0	30.0	23.9	PH ooc	8 (120)	0.55	0.02	4 (60)	0.222	0.007	30	1.01	0.03
7	A	409.8	29.3	30.8	23.4	OV eggs	12 (180)	0.68	0.02	4 (60)	0.186	0.002	30	1.05	0.07
8	A	493.1	31.3	33.2	23.7	OV eggs	12 (191)	0.8	0.03	4 (61)	0.221	0.012	30	1.15	0.12
9	A	340.9	27.8	29.3	25.4	OV eggs	8 (91)	0.75	0.02	4 (40)	0.208	0.003	20	1.12	0.02
10	A	524.0	31.5	33.5	25.8	OV eggs	20 (495)	0.66	0.01	8 (100)	0.183	0.001	30	1.06	0.08
11	A	333.9	26.7	28.3	28.3	OV eggs	10 (180)	0.83	0.03	4 (40)	0.228	0.001	30	1.16	0.05
12	A	385.8	28.4	30.0	31.0	OV eggs	12 (185)	0.65	0.01	4 (60)	0.171	0.003	30	1.03	0.06
13	A	394.5	28.0	30.0	32.3	OV eggs	6 (120)	0.77	0.01	2 (40)	0.233	0.001	30	1.10	0.08
14	A	313.1	27.8	29.4	-	OV eggs	12 (179)	0.73	0.01	3 (45)	0.203	0.001	30	1.11	0.08
15	S	397.1	30.4	31.7	24.3	OV eggs	12 (177)	1.13	0.03	4 (61)	0.341	0.007	27	1.28	0.09
16	S	496.0	33.0	34.2	26.1	OV eggs	12 (182)	1.19	0.03	4 (63)	0.350	0.018	40	1.27	0.07
17	S	483.5	31.7	32.8	26.8	OV eggs	12 (182)	1.47	0.03	4 (60)	0.420	0.019	40	1.39	0.07
18	S	377.9	29.2	30.7	27.4	OV eggs	12 (175)	1.26	0.04	4 (58)	0.377	0.011	40	1.27	0.09
19	S	443.5	30.6	31.5	34.3	OV eggs	12 (160)	1.17	0.08	4 (49)	0.341	0.024	40	1.29	0.08

The largest eggs were obtained in March from the spring-spawning population of Atlantic herring (mean wet mass: 1.24 ± 0.13 mg ind^{-1}; mean diameter: 1.30 ± 0.05 mm), and were 1.6 to 2-fold (mean 1.7-fold) the size of the OV eggs from autumn-spawners (mean wet mass: 0.73 ± 0.07 mg ind^{-1}; mean diameter: 1.10 ± 0.05 mm, Table 1). The larger size of the spring-sampled OV eggs was not due to greater hydration or GSI (Fig. 1, 2). The higher GSI of both autumn- and spring-collected females with OV eggs, compared to autumn-collected females with PH ooc, was caused by the higher water load of the hydrated eggs rather than greater reproductive investment. Relative water contents did not differ significantly between Vtg ooc (58.2 ± 0.9%) and PH ooc (59.2 ± 0.7%) in the autumn-collected females.

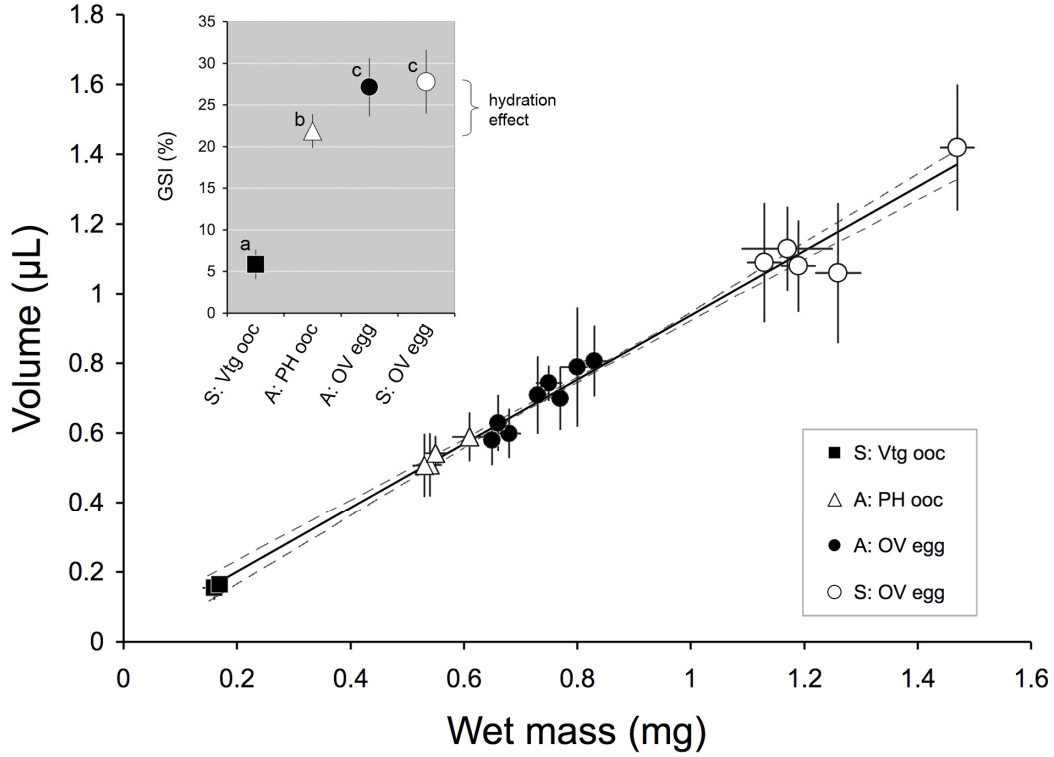

Figure 1. Relationship between volume and wet mass of oocytes and eggs of autumn- and spring-spawning Atlantic herring (*Clupea harengus*). Least squares linear regression equation (± 95% confidence intervals): y = 0.0157 (± 0.0490) + 0.9224 (± 0.0582) x; r^2 = 0.985. Inset: Gonadosomatic index (GSI) of sampled females with significant differences denoted by unequal letters. Values are means ± SD. A: autumn-spawners; S: spring-spawners; Vtg ooc: vitellogenic oocytes; PH ooc: pre-hydrated oocytes; OV egg: ovulated egg.

The mass fraction of water increased to 72.2 ± 1.04 % in OV eggs of autumn-spawners, and was 70.7 ± 0.62 % in OV eggs of spring-spawners (Fig. 2). Although these values only represent a variation of 1.5%, the difference was significant (t = 3.096; df = 11). Regression analysis of oocyte and egg volume versus wet mass showed that oocytes and eggs had a mean specific gravity of ~1.068 kg L^{-1}, i.e. denser than seawater (1.027 kg L^{-1} at 10°C; 35 ppt seawater).

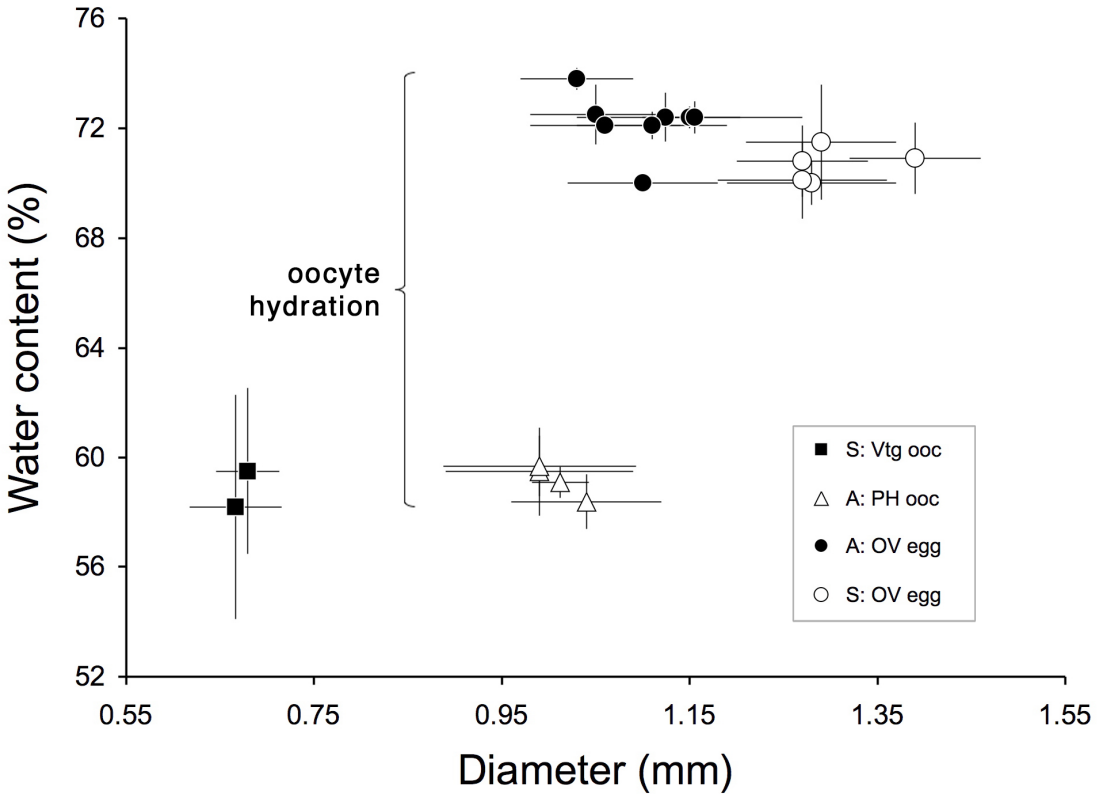

Figure 2. Relative water contents of vitellogenic oocytes (Vtg ooc), pre-hydrated oocytes (PH ooc) and ovulated eggs (OV egg) in Atlantic herring (*Clupea harengus*) as a function of oocyte diameter. Values are means ± SD. A: autumn-spawners; S: spring-spawners.

Osmolytes

Due to the synchronous nature of oogenesis in Atlantic herring, oocytes and eggs were not obtainable from the same female. In order to clarify the quantitative changes that occurred during oocyte hydration, total solute data (nmol ind^{-1}) are presented for pooled autumn-sampled PH ooc and OV eggs that did not differ significantly in dry mass, while the ovoplasmic concentration and data normalised to dry mass are presented for all females (Fig. 3). For the autumn-sampled Atlantic herring, an increase was found (in nmol ind^{-1}) for Cl^- (19.3), K^+ (16.4) and P_i (6.3) (Fig. 3a). No significant changes were noted for Na^+, NH_4^+, or Ca^{2+}, while a significant decline was observed for Mg^{2+} (0.6), and SO_4^{2-} was undetectable. The larger OV eggs of spring-sampled Atlantic herring contained significantly greater amounts of the major inorganic osmolytes Cl^-, K^+, P_i, and Na^+. Levels of NH_4^+ were also significantly elevated in these latter OV eggs.

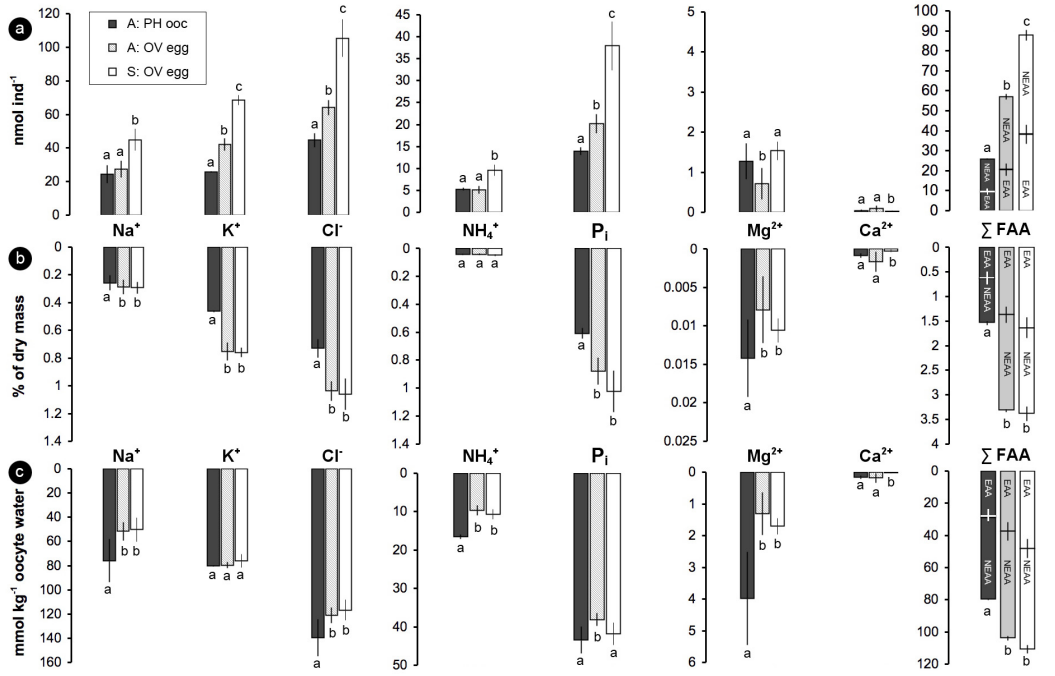

Figure 3. Changes in the inorganic ion and free amino acid (FAA) pools during oocyte hydration in Atlantic herring (*Clupea harengus*). Values are means ± SD. Panel (a): Quantitative changes in solute content determined in autumn-sampled pre-hydrated (PH ooc) and ovulated eggs (OV egg) of equal dry mass (Table 1: females 3, 4, 6, 8, 9, 11, 13 and 14), and spring-sampled OV eggs (Table 1: females 15, 16, 18 and 19). Panel (b): Fractional solute content of all oocytes and eggs. Panel (c): Solute concentrations calculated from total solute content (nmol ind^{-1}) and water content (mg ind^{-1}) of all oocytes and eggs. Significant differences are denoted by unequal letters. A: autumn-spawners; S: spring-spawners. EAA: indispensable amino acids; NEAA: dispensable amino acids.

When normalised to dry mass, no significant differences were noted between the inorganic ion contents of the OV eggs of autumn- or spring-sampled Atlantic herring, except for Ca^{2+}, which decreased (Fig. 3b). Compared to the PH ooc, both classes of OV egg (autumn and spring) contained significantly greater amounts of Cl^-, K^+, P_i, and Na^+, but lower amounts of Mg^{2+}. In terms of ovoplasmic concentration, however, only K^+ (autumn and spring) and P_i (spring) maintained their levels in the hydrated eggs, while Cl^-, Na^+, NH_4^+ and Mg^{2+} were significantly diluted (Fig. 3c).

Total FAA content doubled (Δ 31 ± 2 nmol ind^{-1}) in autumn-sampled OV eggs compared to the PH ooc, and were further significantly increased in spring-sampled OV eggs (Fig. 3a). Similar to the situation for inorganic ions, normalised FAA contents of autumn- and spring-sampled OV eggs were not significantly different (Fig. 3b). Contrary to data for the inorganic osmolytes, the concentrations of FAA

were significantly increased in both autumn- and spring-collected OV eggs compared to the PH ooc (Fig. 3c). Each FAA, except Arg and Glu, increased significantly during oocyte hydration of autumn- and spring-sampled OV eggs, although some variation was noted for Thr, Val, Lys, and Pro (Fig. 4).

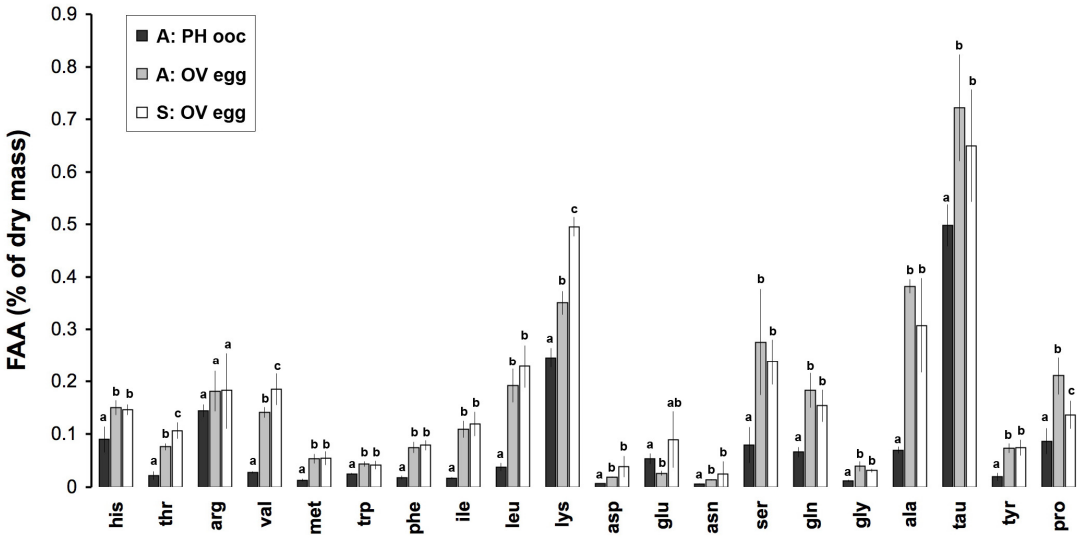

Figure 4. Free amino acid (FAA) profiles for pre-hydrated oocytes (PH ooc) and ovulated eggs (OV eggs) of Atlantic herring (*Clupea harengus*) normalised as % of dry mass. Values are means ± SD. Indispensable amino acids (EAA) are His, Thr, Arg, Val, Met, Trp, Phe, Ile, Leu, Lys. Dispensable amino acids (NEAA) are Asp, Glu, Cys, Asn, Ser, Gln, Gly, Ala, Tyr, and Pro. Tau is an amino acid analogue not found in proteins. Significant differences are denoted by unequal letters. A: autumn-spawners; S: spring-spawners.

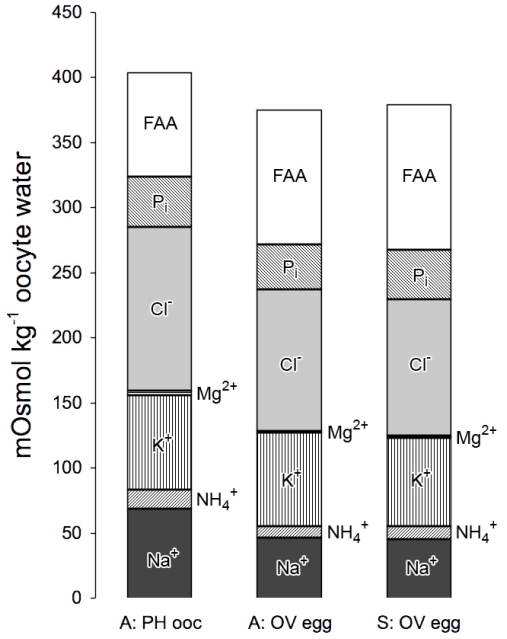

Figure 5. Composite views of the major solutes that contribute to the osmolality of pre-hydrated oocytes (PH ooc) and ovulated eggs (OV eggs) in autumn- (A) and spring- (S) spawning Atlantic herring (*Clupea harengus*). Calculated osmolality is based on total solute content (nmol ind^{-1}) and water content (mg ind^{-1}) and an osmotic coefficient of 0.9 for monovalent ions.

Fractional protein content did not differ significantly (t = 0.37; df = 11) between the PH ooc and OV eggs with values of 62.5 ± 4.2 and 61.5 ± 2.4% of dry mass, respectively.

Discussion

In the present study, we have for the first time determined the changes in the major osmolytes responsible for oocyte hydration in Atlantic herring. Although PH ooc were not obtained for the spring-spawning stock of Atlantic herring, the dry mass-normalised data for spring-sampled OV eggs showed that the underlying mechanisms of oocyte hydration were the same. Both autumn- and spring-spawning Atlantic herring had similar degrees of egg hydration (70 – 72 %) despite their 1.6- to 2-fold difference in size. The greater size of the spring- compared to the autumn-collected OV eggs is corroborated by previous reports (Farran 1938; Baxter 1959; Hempel & Blaxter, 1967). The current findings of equivalent GSI in the autumn- and spring-spawners with OV eggs are further in line with the notion that an atretic reduction in fecundity occurs during over-wintering in this species (Óskarsson et al. 2002; Kurita et al. 2003).

The fractional content of normalised osmolyte data revealed that the major changes that took place during oocyte hydration were an increase in FAA content followed by Cl^-, K^+ and P_i. From a physiological perspective, however, Cl^- (28.4%) and FAA (28.6%) were the dominant osmolytes followed by K^+ (18.6%), Na^+ (12.1%), P_i (9.5%) and NH_4^+ (2.5%) that contributed to the osmotic potential of the OV eggs (Fig. 5B). Assuming an osmotic coefficient of 0.9 for monovalent inorganic ions (Washburn 1926-1930; 2003), the osmolality of autumn-sampled PH ooc was 404 mOsmol kg^{-1} oocyte water, while that of autumn- and spring-sampled OV eggs was 376 and 379 mOsmol kg^{-1} oocyte water, respectively.

The degrees of hydration for the OV eggs agree with the data of Kurita *et al*. (2003), while the calculated osmolalities are lower than previously published data for the yolk osmolality of unfertilised eggs of Atlantic herring (425 - 460 mOsm; (Hølleland & Fyhn 1986). Our data, however, are close to the yolk osmolalities of Pacific herring held in 35 ppt seawater (390 mOsm; Alderdice et al. 1979b), and represent slightly

hyper-osmotic values in relation to the blood plasma previously determined for Atlantic herring (325 mOsm; Herbert & Steffensen 2006). Our current data are closer to the 30 minute post-exercise values of 400 mOsm determined for Atlantic herring by Herbert & Steffensen (2006). All data, however, show that the ovoplasm and extracellular fluids of the adults are strongly hypo-osmotic to the spawning environment, a condition also reported for other marine teleosts (Lasker & Theilacker 1962; Davenport et al. 1981; Riis-Vestergaard 1982; Kjørsvik et al. 1984; Watanabe & Kuo 1986; Mangor-Jensen 1987; Finn et al. 2002a).

Table 2. The molar charge of cations (Na^+, K^+, NH_4^+, Mg^{2+}, Ca^{2+}), anions (Cl^-, H_2PO_4) and free amino acids between pH 5-6 for pre-hydrated oocytes (PH ooc) and ovulated eggs (OV egg) of autumn (A) and spring (S) spawning Atlantic herring (*Clupea harengus*). Values are calculated from solute concentrations (Fig. 3c and 4). At pH 5 - 6, free P_i is present as $H_2PO_4^-$, while other ions are fully dissociated.

	A: PH ooc	A: OV egg	S: OV egg
	mM ± SD	mM ± SD	mM ± SD
Asp	-0.3 ± 0.0	-0.6 ± 0.0	-1.2 ± 0.6
Glu	-2.5 ± 0.4	-0.7 ± 0.1	-2.5 ± 1.5
His	3.9 ± 1.0	3.8 ± 0.5	3.9 ± 0.3
Arg	5.6 ± 0.3	4.1 ± 1.0	4.4 ± 1.7
Lys	11.4 ± 0.6	9.3 ± 1.0	14.1 ± 0.9
Cations	176.9	142.5	138.6
Anions	-183.1	-159.3	-158.5
∑ mM	12.1	-0.9	-1.2

Normalising the data with respect to dry mass showed that the major osmolytes that could drive hydration of the oocytes in both classes (autumn and spring) of Atlantic herring were FAA, Cl^-, K^+ and P_i. Conversely, Na^+, NH_4^+, and Mg^{2+} were not effective osmolytes since each was strongly diluted by the inflow of water. Despite the largest increase noted for Cl^- in the autumn-sampled females, in relative terms Cl^- increased 1.4-fold, while K^+ increased 1.6-fold, and P_i increased 1.5-fold. Only K^+ and P_i (spring eggs) remained undiluted following hydration, while all other ions, including Cl^- were diluted. An explanation for this arises from the doubling of FAA

content, which represents the only pool of osmolytes that showed increased concentration in the OV eggs. At a physiological pH of 5 - 6, which has been reported for maturing oocytes of marine teleosts (Matsubara et al. 2003), only certain FAA are charged. To determine whether our measurements represent physiologically relevant values, the charge equivalence of the oocytes and eggs was calculated (Table 2). These data show that the total pool of charged solutes in the OV eggs is close to electroneutrality at a pH of 5 – 6.

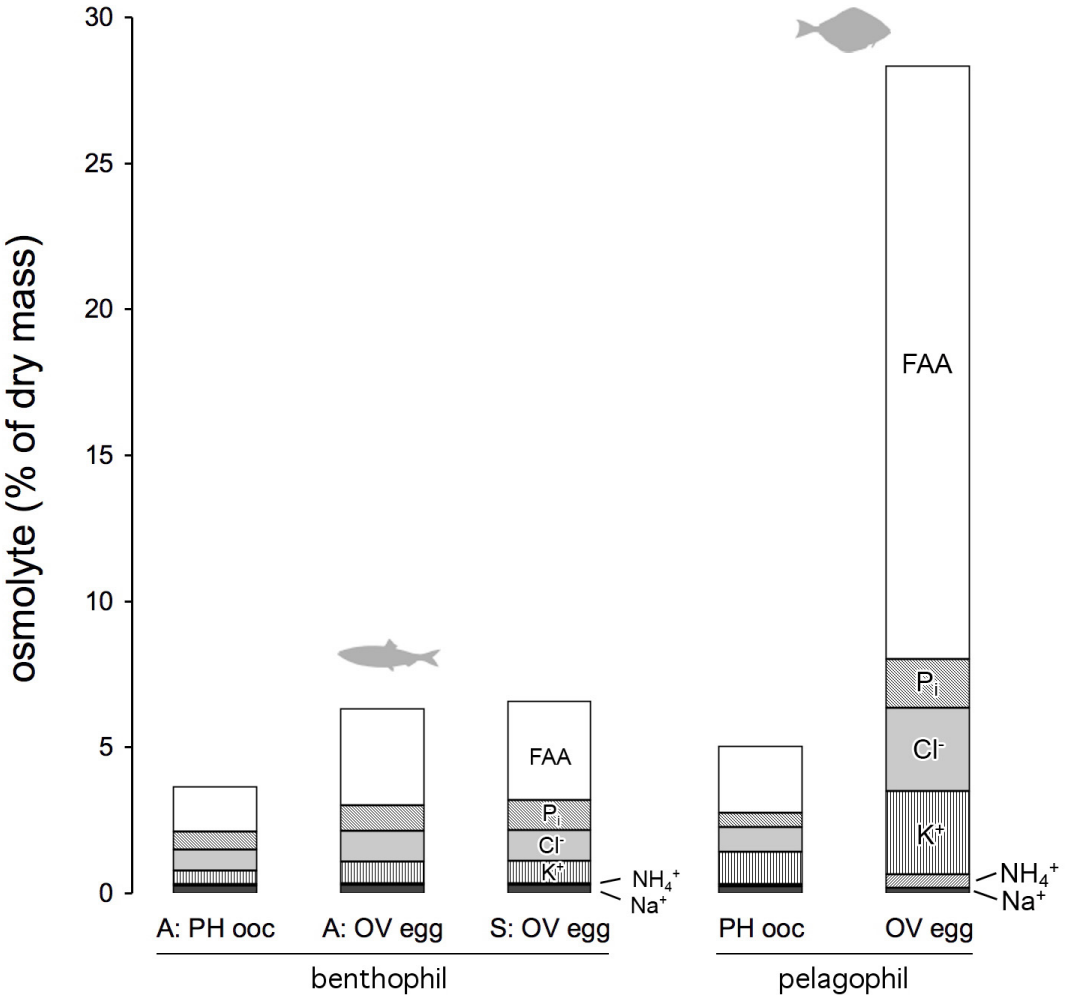

Figure 6. Comparison of the major solutes normalised to dry mass in the oocytes and eggs of the marine benthophil Atlantic herring (*Clupea harengus*) and a marine pelagophil Atlantic halibut (*Hippoglossus hippoglossus*) after Finn et al. (2002a). See legend to Fig. 1 for the description of acronyms.

The doubling of the FAA pool during oocyte hydration only represented a fractional increase of 1.8% of dry mass. This is considerably less than observed in pelagophils

(Fig. 6). Nevertheless, the modest increase in FAA in Atlantic herring still contributed 29% to the osmotic pool of egg solutes (Fig. 5). This significant contribution to the ovoplasmic osmolality in Atlantic herring OV eggs is due to the lower water content of the benthic eggs (70 - 72%) compared to pelagic eggs (>90%). The increase in the FAA pool further suggests that limited proteolysis of yolk proteins occurs in this species. With a protein content of 62% of dry mass, the increase in the FAA pool in the autumn-sampled OV eggs would imply hydrolysis of a small fraction of the yolk protein pool (<2.5%).

The increased P_i content in the OV eggs is likely to derive from dephosphorylation of phosvitin (Pv). Evidence for this notion derives from our unpublished observations that Pv bands disappear from the protein profiles of hydrating Atlantic herring oocytes, and the current observations of a concomitant rise in free Ser (Fig. 4). Recent studies have also demonstrated that acid phosphatases catalyse the dephosphorylation of Pv in barfin flounder (Sawaguchi et al. 2006b), while other studies have shown a synchronous rise in P_i and Ser during the disappearance of Pv bands in Atlantic halibut (Finn 2007a). Ion channels for the inward movement of P_i in hydrating oocytes may thus not be necessary, but in addition to the previously proposed gap-junctional transport (Wallace et al. 1992; Cerdà et al. 1993; 2007) are the likely basis for the increased contents of K^+ and Cl^-.

Two ATP-dependent electrogenic ion pumps are known to be activated during oocyte maturation in pelagophil and benthophil marine teleosts: An acidifying V-class proton pump (vH^+) (Selman et al. 2001; Raldúa et al. 2006) and a Na^+,K^+-ATPase (NKA) (LaFleur and Thomas 1991; Wallace et al. 1992). The vH^+ is associated with the acidification of vacuolar endosomes/lysosmes in plants and animals in order to initiate receptor-ligand events, membrane trafficking and luminal activation of degradative enzymes within the lysosomal vesicle. Other physiological roles include regulation of pH, volume homeostasis, organic solute transport, cell migration, cell proliferation and differentiation (Beyenbach & Wieczorek 2006; Hurtado-Lorenzo et al. 2006). The neutralising influx of Cl^- is also associated with the electrogenic activity of vH^+, but current models implicate separate channels such as the CFTR or ClC family for this purpose (Jentsch et al. 2005; Jentsch 2007; Suzuki et al. 2006). To our knowledge, no information exists for the cellular or sub-cellular localisation of the

vH^+, CFTR or ClCs in developing oocytes and eggs of teleosts, although variants of each type of channel are expressed in the plasma membrane and endosomal/lysosomal membranes of other organisms (Beyenbach & Wieczorek 2006; Di et al. 2006; Hurtado-Lorenzo et al. 2006; Swanson 2006; Suzuki et al. 2006; Jentsch 2007). Earlier studies have shown that the vesicular formation of yolk in teleosts and other animals represents a specialisation of the endosomal-lysosomal pathway (Busson-Mabillot 1984; Wall & Maleka 1985; Wall & Patel 1987; Sire et al. 1994; Fagotto 1995), and the maturational acidification involving vH^+ is crucial to this process (Selman et al. 2001; Matsubara et al. 2003; Carnevali et al. 2006; Raldúa et al. 2006). Our current data for the increase in Cl^- as the major inorganic osmolyte involved in hydrating the oocytes of Atlantic herring supports the notion that chloride channels are likely regulators of the hydration process.

Current models for the transmembrane conductance of Cl^- argue that cationic proton gradients from vH^+ activity are the major driving forces for Cl^- influx (Di et al. 2006; Swanson 2006; Jentsch 2007). However, for electroneutral, equimolar translocation of Cl^-, our data for the increase in Cl^- during oocyte hydration of autumn-spawning Atlantic herring (Δ19.3 nmol$\cdot$ind^{-1}) would necessitate an equal amount of H^+ for this purpose. Since the pH of teleost PH ooc is reported to be ~6, but decreases almost a full unit to ~5.1 during oocyte maturation/hydration (Matsubara et al. 2003), it is possible to calculate the total H^+ influx necessary to elicit this acidification from our data. The total water content of autumn-spawning PH ooc of Atlantic herring in the present study was 333 ± 26 µg$\cdot$ind^{-1}, while that of the OV egg was 515 ± 40 µg$\cdot$ind^{-1} (Table 1). Hence only 3.8 pmol H^+ would suffice to illicit this pH reduction. Although such an influx in H^+ represents an almost 12-fold increase in the H^+ content, it only represents 0.02% of the observed change in Cl^- content. This analysis suggests that for equimolar H^+/Cl^- transport to occur, >99.98% of the pumped protons would have to be buffered. A more likely scenario is the ClC-mediated back-transport of H^+ in exchange for Cl^- augmented by a plasma membrane H^+/K^+ exchanger (Jentsch 2007). This latter electroneutral cation exchanger possibly in conjunction with a NKA that is activated early in the oocyte maturational cycle of pelagophil and benthophil teleosts (LaFleur & Thomas 1991; Wallace et al. 1992) could explain the observed influx of K^+ and dilution of Na^+ in the hydrating oocytes of Atlantic herring in the present study and of several teleosts in other studies (see introduction). However, the earlier

observations of an ouabain-sensitve NKA in pelagophils (LaFleur & Thomas 1991), but an ouabain-insensitve NKA in benthophils (Wallace et al. 1992), and the delayed increase in K^+ compared to Cl^- in certain teleosts (Watanabe & Kuo 1986; Finn et al. 2002a) suggest that ion transport mechanisms and stoichiometry involved in oocyte hydration of marine teleosts are more complex.

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