Materials and methods

Fish. Adult gilthead seabream were obtained from local fishermen, and Senegal sole (*Solea senegalensis*) and sea bass (*Dicentrarchus labrax*) were purchased from commercial fish farms. Wild-type zebrafish (*Danio rerio*) were obtained from a local pet store. Fish were sacrificed by stunning and decapitation, and the ovary and other tissues were used for isolation of ovarian follicles, RNA extraction and immunocytochemistry. All procedures for the sampling of fish and sacrifice were approved by the Ethical Committee from IRTA (Spain).

Water content measurement, culture of ovarian follicles *in vitro*, and SDS-PAGE of yolk proteins. Seabream gonads were placed in 75% Leivovitz L-15 culture medium with L-glutamine (Sigma), and fully-grown, follicle-enclosed oocytes were manually isolated as described (*S1*). Water content of fully-grown and mature oocytes and eggs (*n* = 200) was measured gravimetrically to constant weight at 60°C. Water content increased from 64.8% in fully-grown oocytes to 91.5% in mature oocytes and eggs (% in weight). For induction of oocyte maturation and hydration *in vitro*, fully-grown ovarian follicles were incubated in culture medium with 0.1 µg ml⁻¹ of the maturation-inducing steroid (MIS) 17α,20β-dihydroxy-4-pregnen-3-one up to 48h at 18°C. To evaluate the effect of mercury on oocyte hydration, groups of follicles undergoing maturation in vitro (“stippled” stages), not fully hydrated, were incubated with HgCl₂ for 1h. The effect of HgCl₂ was reversed by immediate post-incubation of HgCl₂-treated oocytes with 5 mM β-mercaptoethanol (βME) for 15 min. Changes in oocyte volume were calculated from the oocyte diameter measured with an ocular stereomicroscope to the nearest 0.01 mm until full hydration was observed. Yolk
proteins extracts from follicles undergoing maturation and hydration were analyzed by SDS-PAGE as described (S2).

**Cloning of fish aquaporins and phylogenetic analysis.** Full-length complementary DNAs (cDNAs) of SaAQP1o and SsAQP were cloned from total RNA extracted from the ovary (Qiagen) using RT-PCR and degenerate primers corresponding to the highly conserved regions of the major intrinsic protein (MIP) gene family (S3). Rapid amplification of cDNA ends (RACE, Gibco) was further employed to isolate the 5’ and 3’ ends, followed by a final amplification of the full-lenght cDNAs with a high-fidelity polymerase (Pwo, Roche). Full-length DrAQP1 and SaAQP1 cDNAs were isolated by RT-PCR using a deposited expressed sequence tag (GenBank accession number BQ783573), and by screening a kidney cDNA library (S4), respectively. The partial DlAQP1 cDNA was cloned from kidney total RNA using the forward degenerate primer initially employed and a reverse primer (AUAP) specific for the adapter region of the oligo d(T) used for cDNA synthesis (Gibco). Multiple amino acid sequence alignments and phylogenetic analyses using the neighbour-joining method were performed with the Clustal W multiple sequence alignment program (www.ebi.ac.uk/clustalw) and drawn with the Phylodendron application (www.es.embnet.org/Doc/phylodendron/treeprint-form.html).

**Gene expression analysis.** The abundance of SaAQP1o and SaAQP1 transcripts in different adult tissues was determined by RT-PCR on oligo dT-synthesised cDNAs using PCR cycles (32, and 30, respectively) that generated half-maximal amounts of PCR product (not shown). The forward and reverse oligonucleotide primers for SaAQP1o were: 5’-GGCGGCTCTTATCTACGATTT-3’ and 5’-TGAAAGCTTTTCTGCAACTCA-3’, respectively, whereas for SaAQP1 these were: 5’-GGCTCTCACGTACGATTTCC-3’, and 5’-TCTGTGTGGGACTATTTTGACG-3’. The PCR for β-actin (24 cycles) was performed to control the variation in mRNA concentration in the RT reaction using the following forward and reverse primers, respectively: 5’-
GCAAAACAACCACACCACAC-3’, and 5’-GGCGAATACGTGGTCTGAACA-3’ (GenBank accession number X89920).

**Functional expression in Xenopus laevis oocytes.** Complementary RNA production (cRNA), expression of SaAQP1o in X. laevis oocytes and osmotic water permeability ($P_i$) measurements, in the presence or absence of HgCl$_2$ and βME, were performed as described (55).

**Antibody production and immunofluorescence.** A peptide was synthesized corresponding to the C-terminal 20 amino acids of SaAQP1o (PREGNSSPGGPSQGPSQWPKH) and injected into rabbits (Cambridge Research Biochemicals). The antisera were affinity-purified on thiopropyl sepharose 6B coupled to the synthetic peptide. Immunofluorescence on paraplast (Sigma) embedded tissue sections was carried out on tissues fixed either in 4% paraformaldehyde or Bouin’s without acetic using standard protocols. The anti-SaAQP1o antibody (1:300-500) was applied for 1 h at room temperature or overnight at 4ºC. Using the pre-immune serum or pre-incubation of the antibody with the synthetic peptide for 1h at 37ºC previous to its application onto the sections did not reveal any staining (not shown), which demonstrated the specificity of the signals. Pictures were taken using a confocal laser-scanning microscope.

**Data analysis.** Data from in vitro incubations of sea bream oocytes and $P_i$ values from X. laevis oocytes expressing SaAQP1o were statistically analyzed by the Student T-test. Differences were considered significant at $P < 0.05$.

**GenBank accession numbers.** The teleost aquaporin sequences described in this paper have been deposited in GenBank under the accession numbers: SaAQP1o (AY626938), SaAQP1 (AY626939), S. senegalensis AQP1 (AY626941), D. labrax AQP1 (AY626940), D. rerio AQP1 (AY626937).
The phylogenetic tree depicted in fig. S2C shows that the branches of ovarian AQP1o proteins are considerably larger than those of normal fish aquaporin-1 proteins. This suggests a faster evolution of the AQP1o proteins, which is often observed for proteins with a specific function (as SaAQP1o shown here). This rapid evolution might be helped by the genome duplication early in evolution in teleosts, because this allowed a fast development of one copy into a specialized function, while the old function will be maintained by the remaining aquaporin-1. During evolution, fish evolved from seawater to freshwater, but later in evolution fish are thought to have switched sometimes between seawater and fresh water (S6). This means that AQP1o proteins might have developed for generating egg buoyancy in particular fish species when they were in seawater, while this function might have been lost later with the switch of these fish species to freshwater. Therefore, in freshwater fish, in which eggs do not become buoyant, AQP1o might be still present. Indeed, catfish, which encode a SaAQP1o-related protein (lpAQP1o), is a freshwater species. In these fish, AQP1o might have a similar function, might be redundant, or might have obtained another osmoregulatory function in the ovary. In salmonids and other “primitive” freshwater teleosts, such as cyprinodontiforms and siluriforms (e.g., catfish), a continuous hydration of the yolk and/or the oocyte during meiotic maturation and ovulation, resulting in a low increase in oocyte volume (5-10%), has been described (S7-S10). Therefore, AQP1o proteins in freshwater fish might have a similar function as in pelagic marine fish (e.g., mediating oocyte/yolk hydration), but resulting in non-buoyant eggs.
Supporting References and Notes


Legends to the Supporting Figures

Fig. S1. Time-course of oocyte hydration (A) and proteolysis of major yolk proteins (B) during MIS-activated meiotic maturation of seabream oocytes in vitro. (A) Representative volume changes over time of a single oocyte (n = 8) treated with 0.1 mg ml⁻¹ of MIS (closed circles) or ethanol vehicle (Control, open circles). (B) SDS-PAGE of follicle protein extracts (5 µg/lane) at the specific hydration stages (1 to 5) indicated in (A) (the arrowhead indicates degradation of a major yolk protein of approximately 100 kDa).

Fig. S2. (A) Osmotic water permeability (Pᵢ; mean ± SEM; n = 9) of X. laevis oocytes injected with water or 1 ng of SaAQP1o cRNA, and its inhibition and recovery by 1 mM HgCl₂ and 5 mM βME, respectively. Asterisks denote significant differences (P = 0.05). (B) Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of SaAQP1o and SaAQP1 transcripts in adult tissues (minus indicates absence of RT during cDNA synthesis). (C) Phylogenetic relationships of vertebrate aquaporin-1 (AQP1)-like water channels. The GenBank accession numbers of the sequences used were: human (Homo sapiens; Hs; NP_000376), mouse (Mus musculus; Mm; BC007125), rat (Rattus norvegicus; Rn; NM_012778), sheep (Ovies aries; Oa; AF009037), frog (Hyla japonica; Hj; AB073315), edible frog (Rana esculenta; Re; L24754), toad (Bufo marinus; Bm; AF020620), catfish (Ictalurus punctatus; Ip; CK418363), Senegal sole (S. senegalensis; Ss; AY626941), gilthead seabream (S. aurata; Sa; AY626938 and AY626939), sea bass (D. labrax; Dl; AY626940), black sea bass (Centropristis striata; Cs; AY626936), puffer fish (Fugu rubripes; Fr; FRUP00000130258), Japanese eel (Anguilla japonica; Aj; AB094501), and zebrafish (D. rerio; Dr; AY626937).
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Fabra et al. Fig. S2