An Analysis of Aquaporin in the Oocyte Maturation of Teleosts, *Clarias gariepinus* and *Channa punctatus*

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Abstract

Aquaporin or water channels have been reported to play a significant role in reproduction of marine and freshwater fishes. This study deals with the elucidation of the role of aquaporins 1 and 3 during oocyte maturation and hydration in benthophil and pelagophil eggs of fishes. Partial sequencing of *aqp1 and aqp3* genes of *Clarias gariepinus* and *Channa punctatus* was carried out. Expression of aquaporin genes (*aqp1* and *aqp3*) was assessed, and a direct correlation was observed between the transcript number of *aqp1* and water content of oocytes during oocyte maturation and ovulation in demersal eggs of *C. gariepinus*. Treatment of oocytes with channel blockers under in vitro conditions elucidated the role of aquaporins in hydration. It alludes the role of aquaporins presumably associated with the hydration at the time of oocyte maturation. In silico characterisation of aquaporin 1 of *Clarias magur*, a congeneric species of *C. gariepinus* and aquaporin 3 of *C. gariepinus*, established the presence of conserved NPA (asparagine-proline-alanine sequences) motifs in them. The comparison of expression pattern for both the aquaporins 1 and 3 were also observed in the preliminary study of pelagophil eggs collected from *C. punctatus*. Elucidation of the role of aquaporins in the movement of fluids in teleost gonads and gametes may help to enhance the success rate of oocyte cryopreservation and thus will help in improving the breeding programs of cultured fishes and also the protection of endangered fishes.

Keywords: aquaporin, dihydroxy-4-pregnen-3-one (DHP), cortisol, teleost's egg, reproduction, maturation

Introduction

Aquaporins, a family of highly conserved, small, hydrophobic membrane proteins, are involved in conduct of water and small solutes. The protein involved in the transportation of water, glycerol, urea and ammonia (Agre et al., 2002; Litman et al., 2009; Cerdà and Finn, 2010; Tingaud-Sequeira et al., 2010). It has been categorised into two major groups, namely, water selective aquaporins (Aqp 0, 1, 2, 4 and 5) and aquaglyceroporins (Aqp 3, 7, 9 and 10). These channels are further classified as classical water-selective aquaporin (CAQP); glycerol-permeable aquaglyceroporin (AQGP); and supergene channel super-aquaporin (SAQP) based on the sequence around a highly conserved pore-forming NPA motif (Ishibashi et al., 2017). The two conservative NPA (Asn—Pro—Ala) motifs form short hydrophobic helices and constitute the first filter region in substrate selectivity (Ikeda et al., 2011). The ar/R selectivity filter, located in conserved motif 1, is also pivotal for the specificity of molecular substrates passing through the pore (Murata et al., 2000; Sui et al., 2001). These conserved motifs exist in most of the members of the aquaporin family and create two restriction sites that allow water and some other small solutes to pass (Madsen et al., 2015).

In vertebrates the highest copy number of aquaporin is found in teleosts with up to 18 paralogs reported in zebrafish (Tingaud-Sequeira et al., 2010). A perusal of literature suggests the involvement of aqp1b in oocyte hydration whereas aqp3 plays a major role in osmoregulation in fishes (Cerda 2009; Skoblina 2009;

Chauvigné et al., 2011; Zapater et al., 2011; Cerda et al., 2013). The teleost *aqp1* genes (formerly annotated as *aqp1a* and *aqp1b*) are indeed tandem duplicates and have been renamed *aqp1aa* and *aqp1ab*, respectively, to match the *aqp8* terminology (Finn and Cerda, 2011). Aquaporin-1b gene (*aqp1b*; initially named AQP1o) is teleost specific and originated by local duplication of a vertebrate AQP1 ancestor (Tingaud-Sequeira et al., 2008).

Water selective AQP1 is ubiquitously distributed in kidney, gills and intestine of fishes (Fabra et al., 2005; Raldua et al., 2007). Aquaporin-3 (Aqp3) has been detected in chloride cells and epithelial cells of gills wherein it performs various functions viz. prevention of dehydration, osmoregulation, water balance (Cutler and Cramb, 2002; Lignot et al., 2002; Hirata et al., 2003; Watanabe et al., 2005; Chng et al., 2016). Aqp3 is expressed in the renal tubules of marine teleost (Cutler et al., 2007), in prolactin cells of tilapia for the mediation of osmoreception (Watanabe et al., 2009) and on the embryonic enveloping layer in killifish to control the water loss (Tingaud-Sequeira et al., 2009). The ubiquitous expression of Aqp3 in various tissues such as gills, brain, pituitary, skin, intestine, oesophagus, eye (Cutler and Cramb, 2002; Watanabe et al., 2005) indicates its constitutive role in trans cellular fluid transport.

Multiple physiological roles of aquaporin in reproduction in marine fishes have been reported. Cerda et al., (2017) have shown the specific temporal insertion of Aqp1ab channels in the oocyte plasma membrane to mediate the hydration of egg in preparation for life in the oceanic environment, as well as insertion of Aqp1aa channels in the spermatozoon flagellar membrane to activate motility. These molecular adaptations are meant to cope up with the external osmotic challenges which the gametes face in the absence of osmoregulatory organs. Hydration of oocytes in some benthophil species, this also facilitates the survival of early embryos exposed to transient periods of environmental desiccation (Fyhn et al., 1999; Podrabsky et al., 2010). Diffusion through water channel is also associated with the process of hydration. The extent of hydrolysis of stored yolk-proteins during oocyte maturation is correlated with the increase in the oocyte volume and water content (Wallace and Begovac, 1985; Wallace and Selman, 1985; Greeley et al., 1986; Matsubara and Sawano, 1995; Matsubara et al., 1995; Thorsen and Fyhn, 1996; Matsubara and Koya, 1997; Selman et al., 2001; Finn et al., 2002a, 2002b; Sharma et al., 2022). With these backgrounds the study was restricted to observe the expression pattern of aqp1 and aqp3 in both the fishes during oocyte maturation.

Aqp1b paralogues have also been found in some freshwater and anadromous teleost that show less pronounced oocyte hydration (Tingaud-Sequeira et al., 2009, 2010). Limited information related to the role of aquaporins in oocyte hydration in freshwater fish is available in literature. Nonetheless, they have been reported to be constitutively expressed aqp1b in the ovary of zebrafish, *Danio rerio* (Knight et al., 2011), in the oocytes of rainbow trout (Bobe et al., 2006) and in the ovary and brain of the Indian catfish, *Heteropneustes fossilis* (Chaube et al., 2011). The paucity of research concerned with the role of aquaporins in hydration of oocytes in the freshwater fishes makes it imperative to conduct more studies using genetic and molecular approaches. An understanding of the involvement of aquaporins in the movement of fluids in the teleost gonads and gametes may also lead to formulation of improved cryopreservation protocols to assist in breeding and conservation of endangered species.

The previous study on *C. gariepinus* suggested significant oocyte hydration during oocytes maturation and ovulation (Sharma et al., 2022). Based on that, the present works were used to investigate the role of aquaporins 1 and aquaporin 3 in the benthophil eggs of *C. gariepinus*. Amino acid sequences of Aqp1 and Aqp3 of *C. magur* have been employed to perform structural analysis and to establish the identity of active amino acids and demarcate the conserved motifs as well. A pilot study has been conducted using the oocytes of *C. punctatus* to compare the level of expression of *aqp1* and *aqp3* in fresh water pelagic eggs with that of demersal eggs in *C. gariepinus*.

Materials and Methods

Ethical approval

This study was approved by the Institutional Animal Ethics Committee of the Department of Zoology, University of Delhi, India, No. DU/ZOOL/IAEC/EXT-A/01/2019, dated 06 March 2019.

Fish maintenance

Adult gravid female specimens of the African catfish, *C. gariepinus* and murrel, *C. punctatus* were collected from the local fish markets and maintained in an air-conditioned laboratory at 25 ± 1 °C with a photoperiod regimen of 12L:12D. Fish were fed *ad libitum* every evening with minced beef liver. Water in the aquaria was renewed daily with dechlorinated tap water adjusted to the laboratory temperature.

Partial sequencing of aqp1 *and* aqp3 *genes*

The method of Chomczynski and Sacchi (1987) was used to extract total RNA by the single step guanidinium thiocyanate-phenol-chloroform extraction method. Trizol (Sigma) was used to extract total RNA from the oocytes in late vitellogenic stage. Two µg of total RNA was reverse transcribed and PCR was performed using primer sets designed to amplify a partial stretch of *aqp1* and *aqp3* cDNA (Table 1). The PCR products were

subjected to agarose gel electrophoresis; the single band corresponding to the amplified cDNA was extracted and purified using Wizard®SV Gel and PCR Clean-Up System (Promega, USA). The purified PCR-amplified products were cloned by pGEMT®-Easy Vector system (Promega, USA). Transformation was done by electroporation using electro competent *Escherichia coli* DH5α cells. Clones were screened by colony PCR method and plasmid DNA was isolated and sequenced. The length of *aqp1* sequence was 108 bp and that of *aqp3* was 394 bp in *C. gariepinus*. The sequences obtained for *aqp1* and *aqp3* of *C. punctatus* are 375 and 348 nucleotide long, respectively. The obtained nucleotide sequences for *aqp1* and *aqp3* were analysed for sequence similarity with sequence of genes of other fishes obtained from NCBI database.

In silico prediction

Secondary structure and domain analysis of Aqp1 and Aqp3

The nucleotide sequences of aquaporin 1 (Accession No. MH681998) and aquaporin 3 (Accession No. JX282189) mRNAs of *C. magur* and *C. gariepinus*, respectively were retrieved from NCBI database. The nucleotide sequences were translated using ExPASy translation tool (https://web.expasy.org/translate/). For secondary structure analysis, PSI-blast based secondary structure PREDiction (PSIPRED) (http://bioinf.cs.ucl.ac.uk/psipred/) software (Buchan et al., 2013) was used along with Self-Optimized Prediction Method with Alignment (SOPMA) (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html) (Geourjon and Deleage, 1995). Analysis of the conserved amino acid sequences was performed using Clustal omega alignment software (https://www.ebi.ac.uk/Tools/msa/clustalo/) (Sievers and Higgins, 2014).

Protein 3D structure prediction and model validation

The 3D PDB (protein data bank) structures of Aqp1 and Aqp3 proteins were prepared by using SWISS-MODEL (https://swissmodel.expasy.org/) and analysed using PyMOL (https://pymol.org/2/). To check the quality of the predicted PDB structure, Ramachandran plot assessment server (PROCHECK) (https://servicesn.mbi.ucla.edu/PROCHECK/) was used (Laskowski et al., 1993). WHAT IF server (https://swift.cmbi.umcn.nl/servers/html/index.html) was used to confirm the Ramachandran Z-score and the average coarse packing qualities of tertiary structures (Vriend, 1990). To validate the NMR spectroscopic and X-ray structures, ProSA web server (https://prosa.services.came.sbg.ac.at/prosa.php) was used (Wiederstein and Sippl, 2007).

Assessment of active binding sites and analysis of pores present in aquaporin

Online server Computed Atlas of Surface Topography of proteins (CASTp) 3.0 (http://sts.bioe.uic.edu/castp/index.html?1bxw) was used to predict active binding site residues of aquaporin sequences (Dundas et al., 2006). CASTp measures volume of cavities and identifies active binding residues within the 3D model. Also, MOLEonline webserver (https://mole.upol.cz/online/) was used to analyse the pores present in the aquaporins (Pravda et al., 2018).

Oocyte maturation and hydration

In vivo

Gravid catfish, *C. gariepinus*, were administered intraperitoneally with a superactive analogue of gonadotropin releasing hormone (GnRH) (Hoe766; Hoechst) at a dosage of 0.2 μg.gm^{-1}body weight (Kumari et al., 2021). Fish injected with vehicle (propylene glycol, in which hormones and its dilutions

Table 1. Primer pairs for the partial amplification of *aqp1*, *aqp3* and *β-actin* genes.

mRNA	Primer name	Primer sequence
aquaporin 1 (*Clarias gariepinus*)	Forward primer (Aqp1F1)	5' ACCTTTCAGCTGGTCCTGTG 3'
	Reverse primer (Aqp1R1)	5' AGCAGGGTTGATCCCACATC 3'
aquaporin 3 (*C. gariepinus*)	Forward primer (Aqp3F1)	5'TGGAATCTTGGTGTGTGGCCAG 3'
	Reverse primer (Aqp3R1)	5'ACACAATCAGTGCAGCTGTTC 3'
β-actin (*C. gariepinus*)	Forward primer (F1)	5' ACAACGGATCCGGTATGTGC 3'
	Reverse primer (R1)	5' TGGTGACAATACCGTGCTCG 3'
aquaporin 1 (*Channa punctatus*)	Forward primer (Aqp1F1)	5'GTTACCCTTGGGATGCTTGC 3'
	Reverse primer (Aqp1R1)	5'GGCCAGCAGGAAATCATAAA 3'
aquaporin 3 (*C. punctatus*)	Forward primer (Aqp3F1)	5'TCTAGGCACCCTCATCCTTG 3'
	Reverse primer (Aqp3R1)	5'CAGTGCTGCTGTGCCAAT 3'
β-actin (*C. punctatus*)	Forward primer	5'GGGATCCGGGTATGTGCAAG 3'
	Reverse primer	5'GGCCCATACCAACCATCACT 3'

were prepared) only, served as control. Fish were sampled at different time points i.e. 0 h (post vitellogenic oocyte), 4 h (pre-mature oocyte), 8 h (mature oocyte) and 12 h (ovulated oocyte) after GnRH administration. The samples (oocytes) were weighed for determination of wet mass. Dry mass was measured after heating the samples for 24 h at 105 °C in an oven. In addition, cluster of 20–30 oocytes were also examined at each time point under the microscope for the germinal vesicles breakdown (GVBD)(Sharma et al., 2022).

Oocyte maturation and hydration under in vitro conditions

Ovarian samples (20–30 oocytes) were excised and transferred to oxygenated modified Wolf and Quimby culture medium (Goswami and Sundararaj, 1971). Stock solution of steroid hormone(s) {17α, 20β-dihydroxy-4-pregnen-3-one (DHP), cortisol, deoxycorticosterone acetate (DOCA), cortisone and progesterone} of different concentration was prepared by dissolving the hormone in propylene glycol. Culture medium containing these steroids was distributed in 24-well culture plates. Each well contained 2-mL of culture medium with different concentrations of steroids or only vehicle, serving as a control. Oocytes were transferred to the culture medium containing DHP (1 $\mu g.mL^{-1}$) and cortisol (10 $\mu g.mL^{-1}$, 100 $\mu g.mL^{-1}$) alone or together in different combinations. In addition, oocytes were treated with water channel blocker ($HgCl_2$, 500 µM) or ion channel blocker (Tetra ethyl ammonium chloride at concentrations of 10 µM, 50 µM, 100 µM) along with DHP (1 $\mu g.mL^{-1}$) and/or cortisol (10 $\mu g.mL^{-1}$). Oocytes were incubated with intermittent shaking and examined for GVBD at regular intervals. At the time point in which oocytes scored the maximum maturational response, water content in the oocyte samples was determined using the gravimetric method. Oocytes were initially weighed to determine their wet mass, followed by measuring their dry mass after being heated for 24 h at 105 °C in an oven. The difference between the wet and dry mass provided an estimate of the water content within the oocytes.

Quantitative real-time PCR

All the samples were processed for extraction of total RNA to study the pattern of *aqp1* and *aqp3* expression. Transcripts of *aqp1* and *aqp3* were quantified in the ovarian samples. Relative levels of *aqp1* and *aqp3* mRNA were examined by real-time PCR. To evaluate PCR efficiency, uniformity and linear dynamic range of each real-time PCR assay, a standard curve for each gene of interest was constructed using serial dilutions for both the genes. Real-time PCR reactions consisted of 10 µL reaction containing 5 µL FG, Power SYBR Green PCR Master Mix (Applied Biosystems), 1 µL (0.5 µM) of primer pair containing both forward and reverse primers, 1.5 µL cDNA template and 2 µL RNase-free water. In no template control (NTC) cDNA was replaced with nuclease free water. Dissociation curve analysis of amplification products was performed at the end of each PCR. The beta actin gene was used as the endogenous control, and *aqp1* and *aqp3* mRNA were averaged and normalised to beta actin. PCR for all the samples was performed in triplicate. The cycle threshold (C_T) number for *aqp1* and *aqp3* from each fish was the location where sample fluorescence entered the linear portion of the amplification curve, and the C_T value was the mean of triplicate reactions. Subsequently the difference between the mean ΔC_T ($\Delta\Delta C_T$) was computed and the relative fold change in *aqp1* and *aqp3* expression was calculated ($2^{-\Delta\Delta C_T}$).

Statistical analysis

The statistical analysis of CT values and the relative changes in gene expression for *aqp1* and *aqp3* across treatments was conducted using ANOVA. Subsequently, the Student-Newman-Keuls pairwise multiple comparison test (utilising the SigmaStat software package) was employed to discern variations between the mean values of distinct treatment groups. Gravimetric analysis values were presented as mean ± SEM, and a one-way ANOVA with a significance level set at $P < 0.05$ was performed.

Results

In silico sequence analysis

Secondary structure prediction and domain analysis of Aqp1 and Aqp3 proteins of *Clarias* spp. (*C. magur* and *C. gariepinus*)

Secondary structure of the Aqp1 and Aqp3 proteins of *Clarias* spp. was analysed by SOPMA and PSIPRED software. The analysis indicated presence of more than 40 % and 39 % random coils in Aqp1 and Aqp3, respectively, followed by 38 % and 34 % alpha helices in Aqp1 and Aqp3, respectively (Fig.1a). Analysis of conserved amino acid sequences performed using Clustal omega alignment software revealed the presence of two highly conserved NPA motifs (asparagine-proline-alanine sequences) in both Aqp1 and Aqp3 protein sequences (Fig.1b). These motifs are the hallmark of the aquaporin (AQP) family which helps to bind water molecules for selective and efficient water passage.

Protein 3D structure prediction, model validation

The tertiary structures of Aqp1 and Aqp3 were generated by SWISS MODEL and the best model was selected on the basis of Qualitative Model Energy ANalysis (QMEAN) score which describes the major geometrical aspects of the model (Fig. 2). Stereo chemical and geometric evaluations of the tertiary structure were performed by PROCHECK which showed that 96.5 % residues lie in the most favoured and additional allowed regions for Aqp1, whereas for

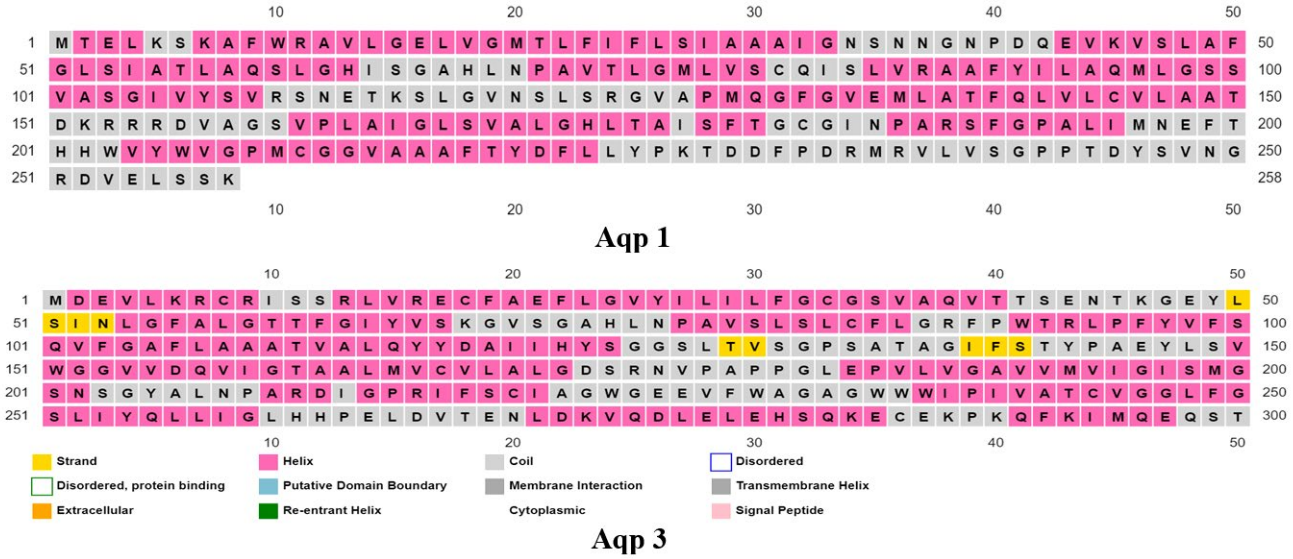


Fig. 1a. Secondary structure prediction of Aqp1 (A) and Aqp3 protein (B) of *Clarias magur* and *Clarias gariepinus*, respectively using PSIPRED software.

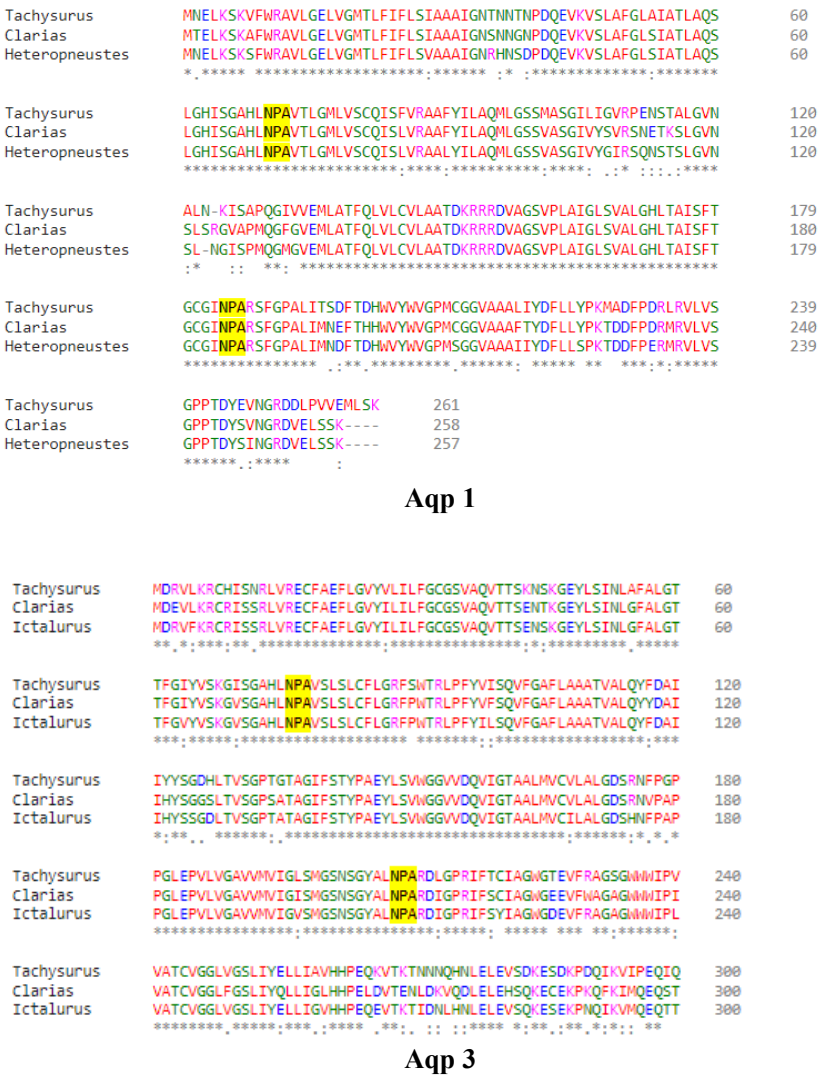


Fig.1b. Conserved amino acid sequences of the Aqp1 and Aqp3 proteins among teleost groups (*Tachysurus*, *Clarias* and *Ictalurus*). Two highly conserved NPA motifs (asparagine-proline-alanine sequences) are a hallmark of the aquaporin (AQP) family which helps to bind water molecules for selective and efficient water passage.

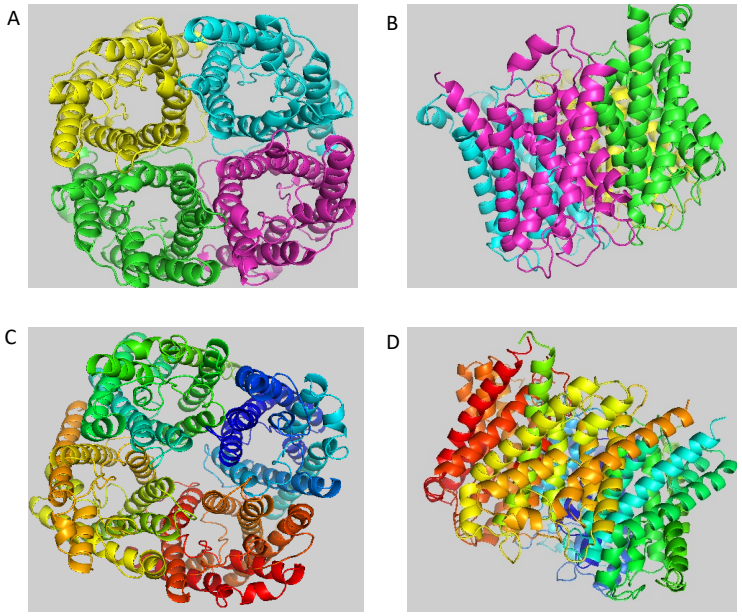


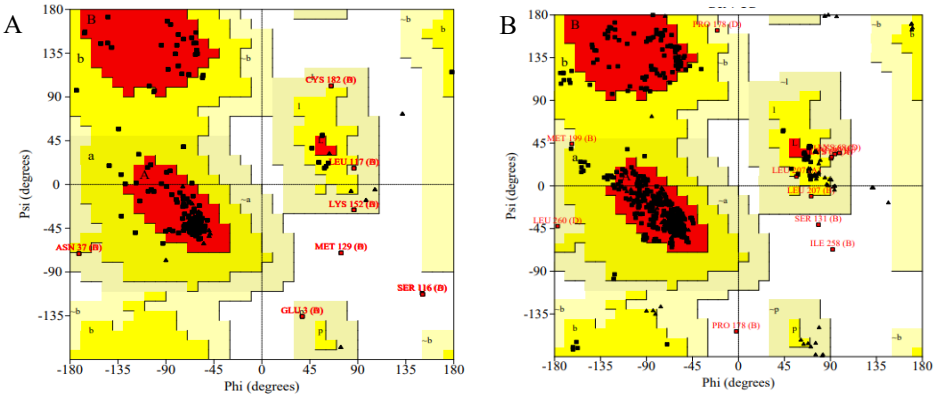
Fig. 2. The 3D PDB structures of proteins (A) Aqp1 (surface view), (B) Aqp1 (side view), (C) Aqp3 (surface view), (D) Aqp3 (side view). The PMID (2018) for the SWISS-MODEL homology modelling of protein structures is 29788355.

Aqp3, 98.8 % of the residues lie in the most favoured and additional allowed regions is 98.

Ramachandran plot statistics signifies the reliability of models with respect to their backbone conformations (Fig. 3). Furthermore, Ramachandran Z-score and structural average packing score assigned by WHAT IF server was -4.50 and -3.96, respectively for Aqp1 and Aqp3 models which describes the backbone conformation of all residues fits into the allowed region of Ramachandran plot. Fold reliability of the model was analysed by ProSA server which estimated energy profiles of the model (Z-score) employing a molecular mechanics force field whose value was -6.09 and -4.11 for Aqp1 and Aqp3, respectively, and provides additional evidence for model reliability.

Assessment of active binding sites and analysis of pores present in aquaporin

Binding residues corresponding to the Aqp1 and Aqp3 was analysed by CASTp which shows the active binding residues in the proteins (Table 2). MOLEonline web server was used to analyse the pores present in aquaporins (Fig. 4). It showed pore length 56.9 Å and bottleneck 0.8 Å for Aqp1 and pore length 48.4 Å and bottleneck 0.9 Å for Aqp3.



Ramachandran plot statistics	Aqp1 (%)	Aqp3 (%)
Residues in most favoured and additional allowed regions	96.5	98.8
Residues in generously allowed regions	2.5	1.0
Residues in generously disallowed regions	1.0	0.2

Fig. 3. Ramachandran plot analysis of Aqp1 (A) and Aqp3 (B) protein model of *Clarias magur and Clarias gariepinus*, respectively calculated using PROCHECK programme.

Table 2. The systems database information of the proteins, their active amino acids and the in-silico interaction for the prediction of bonds formed by molecular docking.

S. No.	Protein model	NCBI Accession No.	Predicted binding residues	Area (A^{o2})	Volume (A^{o3})
1	Aqp1	QBQ04082.1	23 PHE, 34 ASN, 39 ASN, 40 PRO, 41 ASP, 42 GLN, 43 GLU, 44 VAL, 45 LYS, 47 SER, 48 LEU, 50 PHE, 51 GLY, 52 LEU, 54 ILE, 55 ALA, 56 THR, 57 LEU, 58 ALA, 59 GLN, 60 SER, 62 GLY, 63 HIS, 66 GLY, 67 ALA, 68 HIS, 69 LEU, 70 ASN, 73 VAL, 77 MET, 142 LEU, 145 CYS, 146 VAL, 148 ALA, 149 ALA, 150 THR, 151 ASP, 152 LYS, 154 ARG, 155 ARG, 156 ASP, 157 VAL, 158 ALA, 159 GLY, 160 SER, 161 VAL, 162 PRO, 163 LEU, 164 ALA, 165 ILE, 166 GLY, 167 LEU, 168 SER, 169 VAL, 170 ALA, 171 LEU, 173 HIS, 174 LEU, 182 CYC, 183 GLY, 184 ILE, 185 ASN, 188 ARG	4857.79	5347.77
2	Aqp3	AGC25940.1	36 VAL, 39 VAL, 40 THR, 44 ASN, 52 ILE, 123 TYR, 130 VAL, 131 SER, 132 GLY, 133 PRO, 135 ALA, 138 GLY, 142 THR, 143 TYR144 PRO, 145 ALA, 146 GLU, 147 TYR, 204 GLY	231.17	196.02

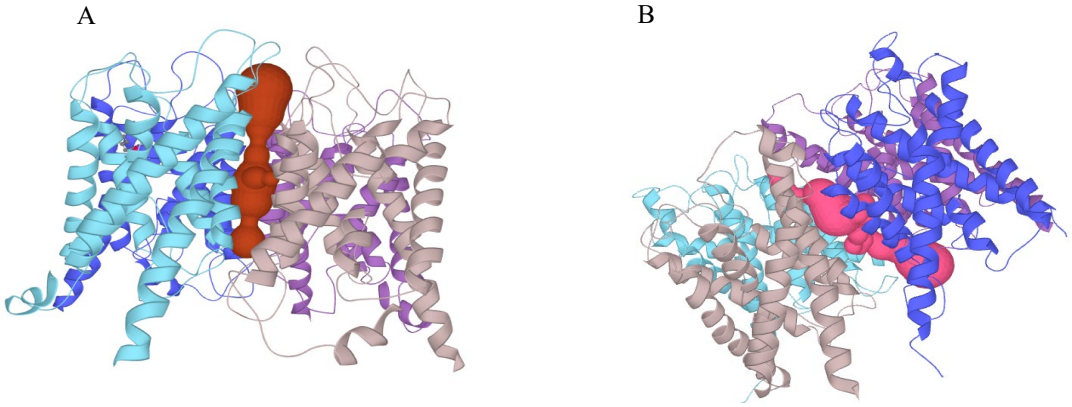


Fig. 4. Analysis of pore of Aqp1 (A) and Aqp3 (B) using pdb files of *Clarias magur and Clarias gariepinus*, respectively using MOLEonline programme.

Gravimetric analysis of oocytes during maturation and ovulation

In vivo

GnRH is effective in inducing oocyte maturation and ovulation in *C. gariepinus*. A substantial increase in the water content of oocytes was observed within 4 h after GnRH administration, which enhanced further at 8 h (maturation) and 12 h (ovulation). These time points represented oocytes at various stages, viz. 0 h - post vitellogenic, 4 h - premature, 8 h - mature and 12 h - ovulated oocytes. In gravid African catfish, exogenous administration of GnRH is effective in inducing in vivo oocyte maturation, ovulation and spawning. The oocytes mature after 7–8 h of GnRH injection and approximately 10–12 % oocyte hydration occurs during ovulation in the demersal eggs of the catfish (Sharma et al., 2022).

In vitro

Well-known indicators of oocyte maturation namely germinal vesicle breakdown and clearance of cytoplasm were employed in the present investigation (Sundararaj and Goswami, 1977; Haider and Rao, 1992). DHP (1 $\mu g.mL^{-1}$) and cortisol (10 $\mu g.mL^{-1}$) were found to be potent in inducing oocyte maturation under in vitro conditions (Table 3). Concomitant with maturation process under short-term culture conditions, water content of the oocytes also increased considerably (10 %), indicating that both steroids (DHP and Cortisol) alone or in combination are competent to stimulate GVBD as well as hydration process.

Significant increase in water content of oocytes was observed progressively from vitellogenic to maturation and ovulation stage, both under in vitro and in vivo conditions.

Table 3. Percentage change in water content of oocytes undergoing maturation with dihydroxy-4-pregnen-3-one (DHP) (1 µg.mL^{-1}) and cortisol (COR) (10, 100 µg.mL^{-1}) alone or in combination, in *Clarias gariepinus*.

Time (h)	DHP (µg.mL^{-1})	COR (µg.mL^{-1})		DHP + COR (µg.mL^{-1})		Vehicle (µL)
	1	10	100	(1 + 10)	(1 + 100)	20
	Percentage water content					
22	50.24 ± 2.20 (8)	51.60 ± 1.60 (7)	49.20 ± 0.90 (8)	54.48 ± 1.14* (6)	55.79 ± 0.84* (7)	51.29 ± 1.06 (7)
27	54.87 ± 1.68* (7)	54.90 ± 2.2* (7)	51.80 ± 1.1** (7)	53.66 ± 1.10* (10)	54.76 ± 1.59* (10)	47.50 ± 1.68 (7)
	Percentage germinal vesicle breakdown (% GVBD)					
22	60 ± 3 (5)	65 ± 5 (4)	62 ± 4 (3)	59 ± 3 (4)	55 ± 1 (3)	5 ± 1 (3)
27	76 ± 5 (4)	83 ± 1 (3)	73 ± 3 (3)	75 ± 4 (4)	70 ± 1 (2)	6 ± 1 (2)

Gravimetric analysis was done for each dose and percentage increase in water content was calculated. Values are expressed as mean ± SEM. Statistical analysis was done by one way ANOVA (* $P < 0.05$; ** $P < 0.001$).
Values in parentheses represent the number of replicates.
Vehicle: Propylene glycol.

Oocyte maturation and hydration with channel blockers

Maturational response in the oocytes of *C. gariepinus* cultured under in vitro conditions and treated with DHP and cortisol was 73 % and 78 %, respectively (Figs. 5a and 5b). It was reduced to 47 % in the presence of ion channel blocker and 55 % in the presence of water channel blocker. Water content in these oocytes was lower than the control (Fig. 5). A dose-dependent reduction in the water content of the oocytes as well as maturation response seen with tetraethylammonium (TEA) indicated that ion channel blocker was more effective as compared to water channel blocker ($HgCl_2$). Although we found the inhibiting effect of $HgCl_2$ is poisonous as many oocytes found to be atretic in addition to the matured oocytes.

Analysis of aqp1 *and* aqp3 *partial nucleotide sequence*

The amplicon length produced by cloning PCR products of *aqp1* (GenBank accession No: JX282188.1) and *aqp3* (GenBank accession No: JX282189.1) was 108bp and 394bp, respectively. The partial nucleotide

0 sequences of *aqp1* and *aqp3* from *C. gariepinus* showed high similarity with sequences of *aqp1* and *aqp3* from *C. magur*. Since the aquaporin genes are highly conserved across taxa, the available complete nucleotide sequences of *aqp1* and *aqp3* from *C. magur* from were used for further analysis.

Analysis of aqp1 *and* aqp3 *expression*

Both *aqp1* and *aqp3* were expressed in the oocytes. The quantitative analysis of *aqp1b* transcripts in oocytes of *C. gariepinus* after GnRH administration is depicted in Figure 6. Relative change in transcript levels was calculated with reference to yolky oocytes (0 h). The result shows that relative transcripts progressively increased to 2.7 ± 0.09 fold in stimulated oocytes undergoing maturation (4 h) to 5.7 ± 0.08 fold in mature oocytes (8 h). In murrel, *C. punctatus* the relative transcripts of *aqp1* 0.066 ±0.10 in vitellogenic oocytes and increased to 2.47 ± 0.035 in ovulated oocytes whereas the relative expression of *aqp3* increased remarkably after ovulation and reached from 0.2 ± 0.1 to 2.19 ± 0.038 after 48 h of GnRH injection (post ovulation) in Figure 7.

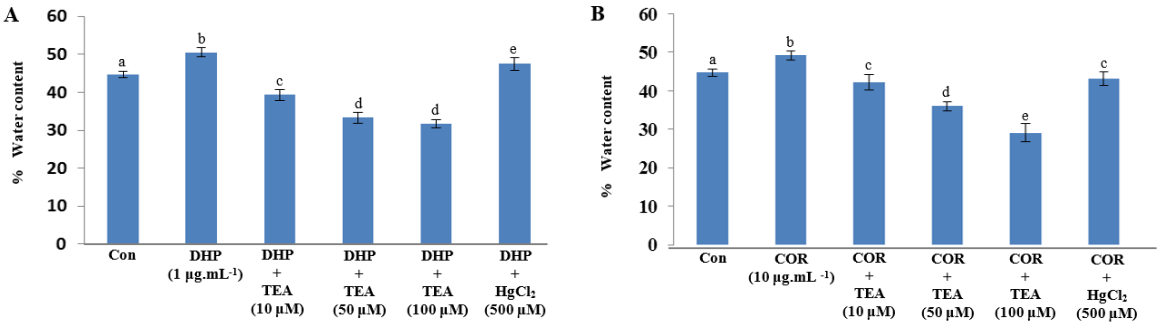


Fig. 5. Effect of maturation inducing steroid (MIS) and channel blockers tetraethylammonium (TEA) or $HgCl_2$ on water content percentage in oocytes cultured in vitro. Oocytes were cultured in a medium with a constant dose of (A) dihydroxy-4-pregnen-3-one (DHP) or (B) cortisol (COR), with and without varying concentrations of TEA and $HgCl_2$. Values are presented as Mean ± SEM (n = 6). All pairwise multiple comparison procedures were conducted using the Student-Newman-Keuls method, indicating significance at $P \leq 0.001$. Error bars labelled with different superscripts (a, b, c, d, e) indicate significant differences.

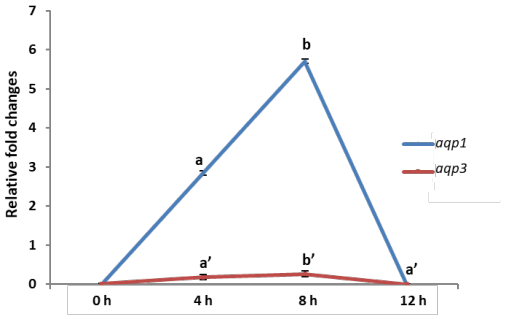


Fig. 6. Quantitative expression of *aqp1* and *aqp3* in oocytes of *Clarias gariepinus* at different time points after GnRH administration in relation to β- actin (value at 0 h was used for calibrating the data). Values are expressed as Mean ± SEM. The bars bearing different superscripts (a-b and a′-b′) differ significantly (Student-Newman-Keuls pairwise multiple comparison test, $P < 0.05$).

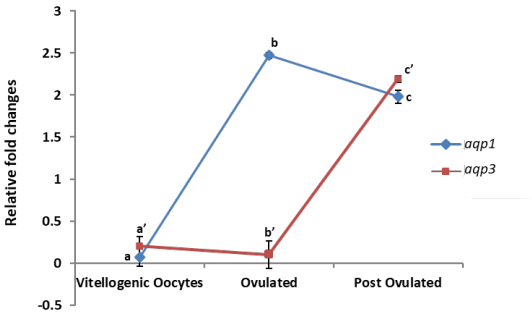


Fig. 7. Quantitative expression of *aqp1* and *aqp3* in oocytes of *Channa punctatus* at different time points (0 h i.e. vitellogenic, 28 h or ovulated and 48 h or post ovulated) after GnRH administration in relation to β- actin (value at 0 h was used for calibrating the data). Values are expressed as Mean ± SEM. The bars bearing different superscripts (a-c and a′-c′) differ significantly (Student-Newman-Keuls pairwise multiple comparison test, $P < 0.05$).

Discussion

This study mainly focuses on the expression of the two aquaporin paralogs *aqp1* and a*qp3* during oocyte maturation in freshwater fish. A prerequisite for understanding the role of aquaporins is to identify the transmembrane and surface regions of membrane proteins. The structural information along with the functional aspects of aquaporin 1 and 3 has not been researched in air breathing catfish, *Clarias* spp. Hence, the 3D model of both the proteins has been prepared using homology modelling approach and various Bioinformatics tools have been used to authenticate the reliability of the models. Secondary structure prediction by SOPMA and PSIRED software reveal the helix and coils in the secondary structures of Aqp1 and Aqp3 (Ghosh et al., 2019). Asparagine-Proline-Alanine (NPA) motif is a hallmark of the aquaporin superfamily (Finn and Cerdà, 2011). To identify the NPA motif in Aqp1 and Aqp3, amino acid sequences from *Tachysurus* sp., *Ictalurus* sp. and *Heteropneustes* sp. have been used. In both the proteins, two NPA motifs are present in all the aligned teleost sequences which further confirm the conserved nature of aquaporins in teleost. These motifs help to bind the water molecules for selective and efficient water passage (Kruse et al., 2006; Ghosh et al., 2019).

Three-dimensional structure of aquaporin proteins has been prepared using SWISS MODEL webserver that shows structural similarity with the aquaporin of zebrafish which has alpha helix and random coils. Identification of active sites is very important while performing assessment of sites for drug binding. MOLEonline webserver that identifies the presence of pores in the aquaporins, shows the presence of pores in both the aquaporins. These pores may play a crucial role in facilitating and controlling the transport of water, ions and molecules across biomembranes.

The previous understanding of oocyte maturation in the Indian catfish suggests that corticosteroid sensitises the oocytes gonadotropin action. The synthesis of MIS greatly stimulated by gonadotropins strongly suggests the existence of pituitary-interrenal –ovarian axis in the maturation of catfish oocytes (Sundararaj and Goswami, 1977). Hydrocortisone and deoxycorticosterone have been reported to be the most effective maturation inducing agents (Sundararaj and Goswami, 1977). In Asian walking catfish, *Clarias batrachus*, dihydroxy progesterone (DHP) and deoxycorticosterone (DOCA) are shown to be equally effective inducer of oocyte maturation (Haider and Rao, 1992). In the present study, the doses as well as effectiveness of both the ovarian and inter-renal steroids to initiate meiotic resumption in the African catfish, *C. gariepinus* was standardised. Hormone of ovarian origin (DHP, 1 µg. mL^{-1}) and hormones of inter-renal origin (cortisol and DOCA, 10 µg.mL^{-1}) were effective in inducing maturation under in vitro conditions. Clearance of cytoplasm and germinal vesicle breakdown has been taken as an indicator of oocyte maturation. A significant increase in the number of oocytes showing GVBD (70 %) has been observed after 26–28 h of oocyte culture. After 28 h of culture, oocytes exhibited atresia. Further, cortisol is equivalent to DHP in inducing oocyte maturation in the African catfish. But the combinations of DHP and Cortisol (1 and 10 µg.mL^{-1}) showed no significant changes with respect to oocyte maturation as well as hydration have also been observed. In contrast, DHP has been shown to be one of the most potent steroids for inducing oocyte maturation in teleosts (Kagawa et al., 1983; Van-der Kraak., 1985; Levavi-Zermonsky and Yaon, 1986; Upadhyaya and Haider, 1986; Kobayashi et al., 1987; Sakai et al., 1987; Petrino et al., 1989; Miura et al., 2007).

In benthophil fish (marine and freshwater) laying demersal eggs, oocyte hydration is comparatively less (5 % to 20 %) as compared to marine pelagophil fish (15 % to 40 %), where significant oocyte hydration occurs during oocyte maturation (Table 4). Sharma et

al. (2022) have reported approximately 10–12 % oocyte hydration in demersal eggs of the African catfish. The extent of oocyte hydration during the maturation or the role of water in contributing to the pelagic nature of the egg needs to be understood. Pelagic eggs are mainly floating type, smaller in size compared to demersal eggs. They are buoyant; the buoyancy is maintained by single oil globule. If the oil globule is not there, high percentage of water is present which helps in floating. The demersal eggs are generally larger than pelagic eggs which may be laid in masses or singly. These eggs are heavy or dense. Since they are heavy, they link to the bottom. As the *C. punctatus* eggs are pelagic one, we want to study the presence of aquaporins and to know is there any change observed at mRNA level from vitellogenic stage to ovulated stage. Therefore, pilot study has been conducted with the pelagic eggs of *C. punctatus* which have a large oil globule and exceptionally high lipid content (Prakash et al., 2013). Relatively low expression of *aqp* transcripts in *C. punctatus* as compared to *C. gariepinus* indicates that the pelagic nature of egg might be because of lipid and not because of oocyte hydration. The present investigation confirms the expression of *aqp1b* and *aqp3* in oocytes of freshwater gravid African catfish (*C. gariepinus*) as well as murrel (*C. punctatus*).

It has been observed that in *C. gariepinus*, GnRH-induced oocyte maturation, specifically up-regulates *aqp1* expression in ovaries. An attempt has been made to assess temporal expression of *aqp1* and *aqp3* during meiotic resumption in the ovary of catfish and murrel. Both the aquaporins (1 and 3) are up-regulated in mature oocytes and are later down-regulated at the time of ovulation in the African catfish, when GnRH-induced meiotic resumption occurs. Transcripts of *aqp1* progressively increased from the late vitellogenic stage, reached maximum during the maturation and then decreased at the time of ovulation. These observations are in accordance with that of the catfish, *H. fossilis*, where *aqp1b* transcript levels are relatively low in both resting and preparatory stage, but later increased significantly and are at maximum level in the pre-spawning fish (Chaube et al., 2011). In case of *C. punctatus* the number of the transcripts of *aqp1* show a progressive increase and attain maximal levels in ovulatory stage and decline in the postovulatory stage. However, in Zebrafish, expression of *aqp1b* is significantly higher

Table 4. Relative change in percentage water content during meiotic maturation in oocytes of teleost.

Species	Water content in vitellogenic oocytes (%)	Water content in mature oocytes (%)	Increase in water content (%)	References
Freshwater benthophil				
Oryzias latipes	76	81	5	Hirose (1976)
Carassius auratus	68	75	7	Clemens and Grant (1965); Greeley et al. (1986)
Plecoglossus altivelis	60	79	19	Chen et al. (2003)
Heteropneustes fossilis	65	85	20	Singh and Joy (2010)
Oncorhynchus mykiss			27	Milla et al. (2006)
Clarias gariepinus	30	42	12	Sharma et al. (2022)
Marine benthophil				
Labrus bergylta	71	76	5	Finn et al. (2002b)
Gasterosteus aculeatus	74	80	6	Craik and Harvey (1986)
Limanda yokohamae	69	82	13	Oshiro and Hibiya (1981b)
Fundulus heteroclitus	61	81	20	Greeley et al. (1991)
Marine pelagophil				
Mugil cephalus	60	85	15	Watanabe and Kuo (1986)
Gadus morhua	71	92	21	Thorsen and Fyhn (1996)
Verasper moseri	69	91	22	Matsubara and Koya (1997)
Hippoglossus hippoglossus	63	90	27	Finn et al. (2002a)
Sparus aurata	63	92	29	Fabra et al. (2005)
Ctenolabrus rupestris	62	93	31	Finn et al. (2002b)
Centropristis striata	54	93	39	Selman et al. (2001)

in mid-vitellogenic follicles as compared to full grown follicles and the level of expression remains constant during oocyte maturation, suggesting that *aqp1b* is regulated at a post-transcriptional level in this fish (Knight et al., 2011). Fabra et al. (2005, 2006) have shown that in seabream, *Sparus aurata*, oocyte

hydration involves an aquaporin 1-like protein, which is regulated at the post-translational level since no significant variation in the amount of *aqp1b* mRNA has been detected during vitellogenic growth. Accumulation of these transcripts in the oocytes as maternal messengers which are required for early development, suggests a coordinated role of different aquaporins in ovarian fluid homeostasis (Chauvigné et al., 2011).

Six-fold increase in the transcript levels of *aqp1* as compared to *aqp3* during the oocyte maturation indicates that *aqp1* plays a major role in the process of hydration of oocytes from *C. gariepinus*. Functional relevance of Aqp1 in water uptake into the fish oocyte during meiotic maturation is evident because aquaporin channel blockers (tetra ethyl ammonium and mercuric chloride) inhibited water uptake in steroid (DHP or cortisol) induced oocyte maturation in the catfish. In addition, reduction in the oocyte maturational response in the presence of aquaporin blocker (TEA and $HgCl_2$) with the reduction in the oocyte water content pertinent the role of hydration at the time of oocyte maturation. It is also supported experimentally by the observation that the swelling of oocytes is blocked by aquaporin inhibitors such as mercury and tetraethylammonium in marine teleosts (Kagawa et al., 1983; Fabra et al., 2005, 2006). Aquaporin inhibitors prevent pre-ovulatory oocyte swelling in a dose-dependent manner in in vitro incubations of ovarian follicles undergoing oocyte maturation in *Sparus aurata* (Fabra et al., 2005, 2006) and *Anguilla japonica* (Horiuchi et al., 2008). Site-directed mutagenesis has identified Cys189 as a site of the mercurial inhibition of AQP1 (Hasegawa et al., 1993; Zhang et al., 1993). Hg++ is a nonspecific and toxic AQP1 blocker. Many compounds reported with AQP1 inhibition activity, including DMSO, Au+++, Ag+, tetraethylammonium and acetazolamide but no one is very specific and there is need to identify new aquaporin inhibitors (Yang et al., 2006).

It has been reported that limited proteolysis occurs in the yolk protein of oocytes associated with hydration at the time of oocyte maturation and ovulation in benthophil catfish *C. gariepinus*. It also explains the transient increase in the enzymatic activity of cathepsin B at the time of maturation (Sharma et al., 2022).

The comparison between *aqp1* and *aqp3* transcript numbers in *C. punctatus* and *C. gariepinus* clearly elucidated the role of aquaporin 1 during the maturation and ovulation of oocytes and further suggests notable explanation behind the mechanism of oocyte hydration at the time of oocyte maturation in *C. gariepinus*. In *C. punctatus* transcripts of *aqp3* is slight high in vitellogenic stage as compared to ovulated stage and increases further after ovulation. Presence of water channel to perform osmoregulation is not a surprising fact but a temporal increase in transcript numbers at specific time points suggests specific roles for different aquaporins.

Conclusion

Oocyte hydration is a common phenomenon in marine fishes laying pelagic eggs, however to a lesser extent hydration is also observed in the benthic eggs of *C. gariepinus*. Temporal increase of *aqp1b* transcripts and increase in oocyte hydration during the maturation suggests the feasible role of *aqp1* in *C. gariepinus*. The possible reason of the pelagic nature of *C. punctatus* egg is the lipid globules as the relative expression of these aquaporins genes were comparatively lesser in *C. punctatus* to that of demersal egg of *C. gariepinus*. To conclude, the significant expression of aquaporin during the oocyte maturation in both the fishes suggests its possible roles.

The elucidation of the role of these proteins is essential to understand the reproductive physiology of these species. It can be concluded that the molecular mechanism of hydration is conserved in both marine and freshwater species. The knowledge of the presence of aquaporins on the gametes can be implemented for the globally use methods for the long-term preservation of gametes cryopreservation. The expression of *aqp1* and *aqp3* in the pelagic oocytes of *C. punctatus* needs to be correlated with the function of these proteins in the maturation of oocytes by using specific inhibitors.

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Author contributions: Luni Sharma: Designing and conducting experiments. Pooja Kaushik: Conducting experiments and analysing data. Ila Singh: Conducting experiments and analysing data. Neeta Sehgal: Conceptualisation. Varunendra Singh Rawat: Conceptualisation.

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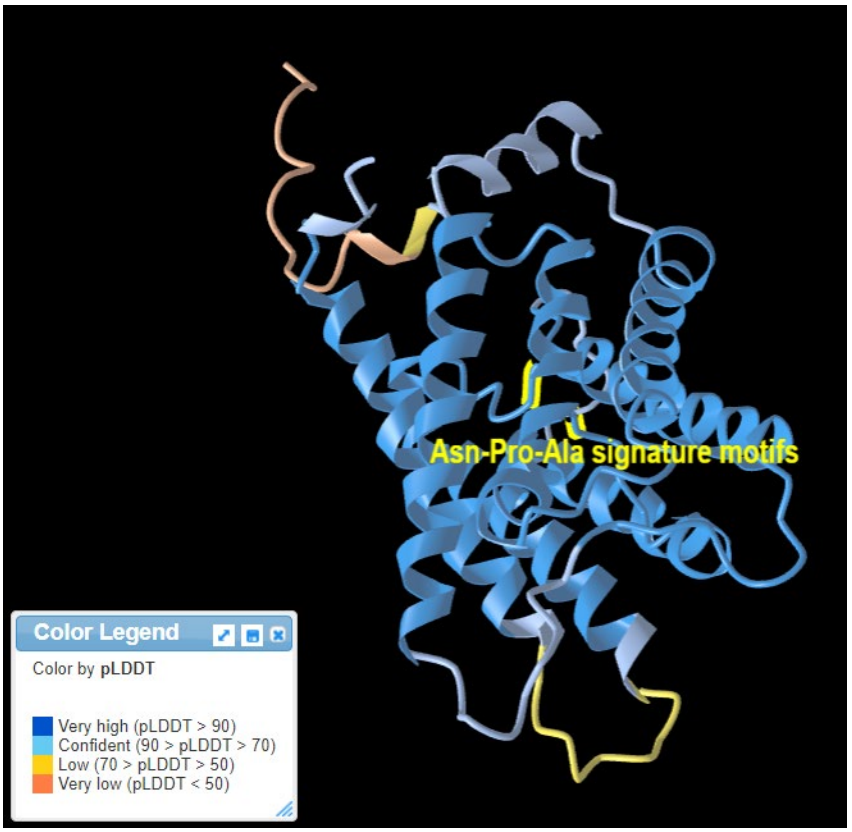
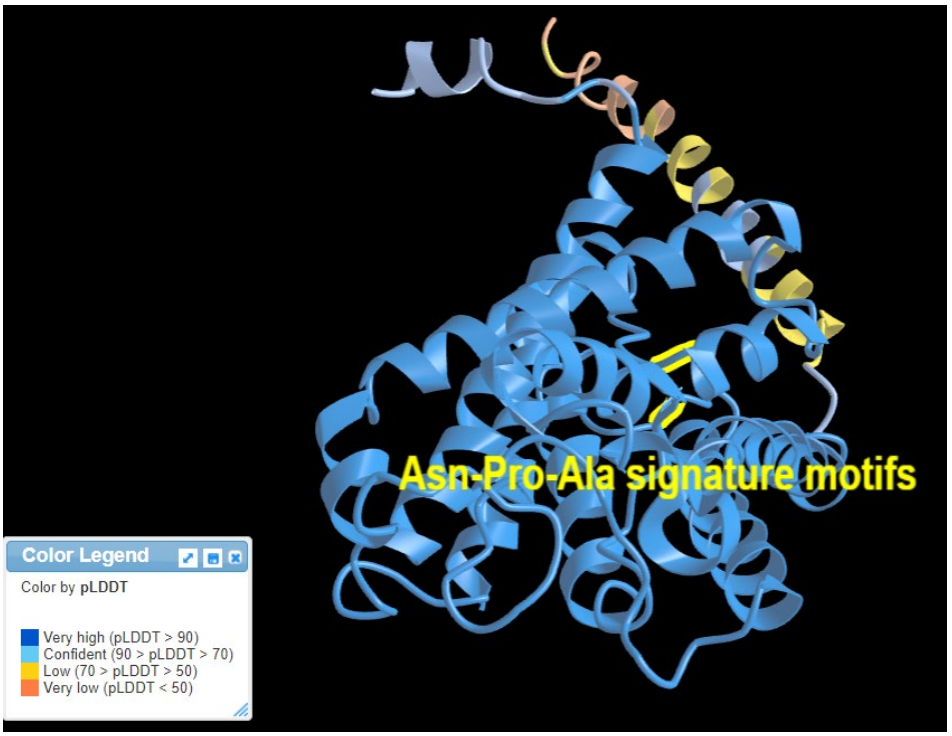
Aquaporin 1



Aquaporin 3



Supplementary Fig. 1. Three Dimensional structural views by Alphafold of Protein Aqp1 and Aqp3.