Review Article

Aquaporins: Structure and Physiology in Fish

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Abstract: Aquaporins are a superfamily of transmembrane channel proteins that transport water and solutes across membranes and is found throughout the living biota. The protein exists as a homotetramer in the cell membrane. Each monomer consists of six membrane spanning alpha-helices that have a central water-transporting pore. Both C and N termini of the AQP protein face the cytosol. Most of the aquaporins consist of a conserved NPA motif that is the characteristic feature of this family. Aquaporins play a vital role in the survival of fishes as their natural environment comprises of water. Both marine and fresh water fishes express aquaporins which are regulated in a way to allow them to survive in their respective environment with different levels of salinity. Cellular localization, tissue distribution pattern and experimental studies imply that the physiological roles of piscine aquaporins extend to osmoregulation, reproduction, early development and ontogeny of fish. Most studies address on the type of AQP isoforms present in the fish, their localization in the body, their expression pattern under experimental conditions, in elucidating the structural paradigms and assigning specific functional properties; but every aspects of piscine aquaporins have not been solved yet. In this review our aim is to discuss the discovery, structure, function, localization, and diversification of the aquaporin superfamily with special reference to teleost aquaporins. This review emphasizes on discussing the types of aquaporin isoforms present in the osmoregulatory organs like gills, gastrointestinal tract and kidney of different fishes and the various techniques employed by researchers in studying them.

Key words: Aquaporin, Channel protein, Fish, Homeostasis, Osmoregulation, Paralogs

Introduction

Aquaporins (AQPs) are a superfamily of small, approximately 30 kDa Transmembrane Channel Proteins that selectively allow rapid passage of water and small solutes into and out of the cell across biological membranes. These integral membrane proteins have been identified in almost all life forms, ranging from Viruses to Archaea (Kozono *et al.,* 2003) to primates (King *et al.,* 2004). Aquaporins are diverse in their functions, and not all of them are devoted to water transport only. Some isoforms are also involved in the transport of several other molecules like ammonia, gases, urea, anions, metals etc. Since it is involved in osmoregulation by transport of water and ions inside and outside of the cell, it plays a significant role in maintaining homeostasis; water transport in kidney and exocrine glands, nutrient uptake in plants, regulate cell volume in the body, fluid secretion, fluid absorption and the others not directly related to water transport such as cell adhesion, migration, proliferation and differentiation (Agre *et al.,*1998); and glycerol transport in fat metabolism and in skin moisture etc. Hence, there is a positive selection pressure for proteins associated with intracellular fluid homeostasis as it is the prime driving factor for normal body physiology in all organisms.

However, several researchers have reported alteration or regulation of aquaporin expressions in animals and plants during certain environmental stresses, providing much insight

into the role of this membrane protein in maintaining the internal milieu of an organism even under physiological stress. Since its discovery, alongside its myriads of physiological roles in the body, aquaporins have been shown to take up roles in the pathogenesis of certain diseases like brain edema, polycystic ovarian syndrome (PCOS) etc. and the most astonishing fact discovered in the last decade is that of being its association with human ovarian cancer and brain tumors (Frede *et. al.,* 2013, Saadoun *et al.,* 2002). Aquaporins have also been speculated to be associated with autoimmune diseases in human like the Systemic Lupus Erythemetosus (SLE). Astrocyte water channel aquaporin 4 in the central nervous system is a target for serum IgG which is a biomarker for Neuromyelitis Optica Spectrum Disease (Asgari *et. al.,* 2018, Hirt *et al.,* 2018). Other aquaporins found in the Central Nervous System includes AQP1, 3, 5, 8, and 9 most of which are linked with several neurological disorders (Friscourt and Badaut 2018). Snuggs *et al.,* 2018 have identified several aquaporins (AQP 0, 1, 2, 3, 4, 5, 6, 7, and 9) in the Intra Vertebral Discs of human and canine vertebrae through investigations with qPCR and Immunohistochemistry. Interestingly, aquaporin has also been found to be expressed in blastocysts, placenta and fetal

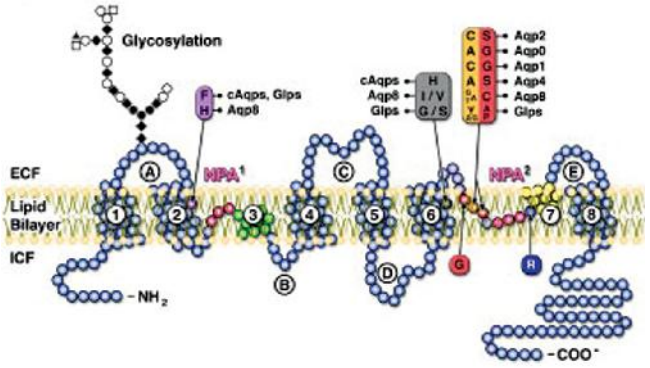

Fig. 1. General topological structure of the aquaporin molecule showing the six transmembrane ~ -helices (1, 2, 4, 5, 6, 8), the two Asn-Pro-Ala (NPA) motifs, and the five loops. The amino acid positions which define the aromatic residue/arginine (ar/R) constriction site are indicated (56, purple; 180, grey; 189, orange; 195, blue) for the different paralogs with cAqps and Glps representing classical aquaporins and aquaglyceroporins, respectively. These latter annotations also reflect the teleost -a and –b duplicates. The least conserved residues (orange and red) occur 2–3 positions upstream of the second NPA motif. The orange Cys (C) is the site for mercurial inhibition in human AQP1. (Cerdá and Finn 2010)

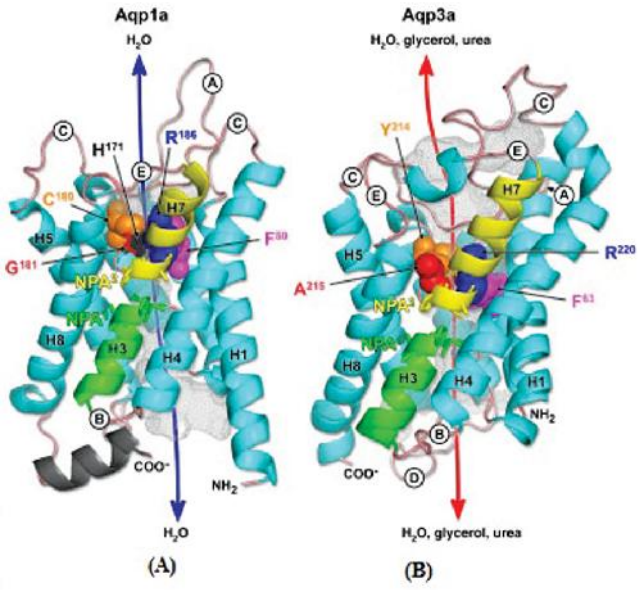

Fig. 2. 3D reconstructions of zebrafish Aqp1a (A) and Aqp3a (B) showing the paralog-specific ar/R residues (spacefill). Double-ended arrows indicate the central channels mapped through surface renders of the culled cavities. The models are based upon the structure masks of bovine (Bos Taurus) AQP1 (1J4N, Sui et al., 2001) and Escherichia coli glycerol facilitator (GlpF; 1LDF, Tajkhorshid et al., 2002) for zebrafish Aqp1a and 3a, respectively (Cerdá and Finn 2010).

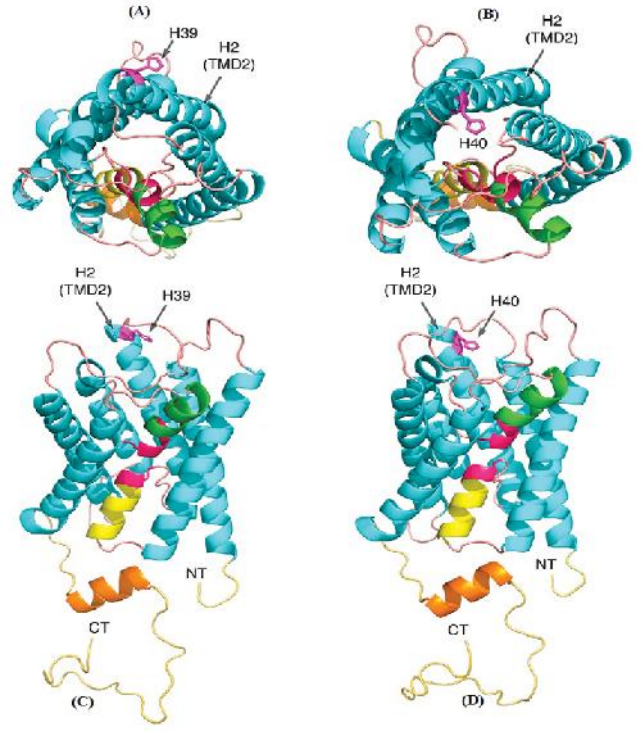

Fig. 3. 3D Structure of Atlantic salmon Aqp 0a2 (A. Extracellular view, C. Lateral view) and Aqp 0b1 (B. Extracellular view, D. Lateral view) via MacPymol (Chauvigné *et al.,* 2015)

membranes; indicating their supposed importance in normal pregnancy, fetal development and homeostasis of amniotic fluid contents and volume (Martínez and Damiano, 2017).

Piscine aquaporins have a high copy number with a huge repertoire discovered in Zebrafish (Tingaud-Sequeira *et al.*, 2010) and Atlantic salmon (Davidson *et al.*, 2010). But structural analysis and functional studies are not yet done for all the piscine aquaporins discovered so far. Computer simulation methods and *In-Silico* homology modeling are employed to predict the three dimensional structure of the protein but only a few crystal structures of fish aquaporins have been solved so far.

Different cell types express different aquaporins depending upon their function in that cell. Also, localization may vary among texa to texa. Therefore, aquaporins are widely found in almost every organ system, osmoregulatory or not.

Discovery of aquaporins

The discovery of aquaporins was a fairly pleasant serendipity. Though the existence of a water channel was long before speculated than its actual discovery, it was found on the way to the characterization of an entirely different protein. It has been assumed that for maintaining fluid homeostasis inside the body, water passes hydrophobic epithelia by transcellular and/or paracellular pathways, the former determined by the serial permeability of apical and basolateral cellular membranes, the latter being defined by the characteristics of intercellular junction complexes. It was initially considered that water transport occurred via non-specific leakage across hydrophobic membranes. The existence of specific water channels were considered after observations of increased water permeability in cells and tissues like the human erythrocytes and the urinary bladder of frogs (Agre *et al.*, 2001, Agre, 2005). Observations on highly available junctional proteins in bovine lens plasma membranes led to the eventual discovery of aquaporins (Bloemendal *et al.*, 1972; Broekhuyse and Kuhlmann, 1974). The purified junctional proteins would migrate in a gel as band III, MP26, or MP34 and collectively they were termed as the main or major intrinsic protein (MIP) of the lens fiber (Broekhuyse *et al.*, 1976; Vermorken *et al.*, 1977; Gorin *et al.*, 1984). However, the first evidence of a bonafide water channel was produced only after the study of human erythrocytes membrane proteins (Benga *et al.*, 1986a,b).

Peter Agre and his colleagues were the first to isolate aquaporins during their ongoing work on the erythrocyte Rh(D) antigen. At first, the protein that appeared as a diffuse band at 30-32 kDa molecular weight on SDS-PAGE gels were thought to be a degradation product of the Rh antigen; but it failed to cross react with a polyclonal antibody against Rh. This gel band was established to contain a novel erythrocyte membrane protein which had previously escaped detection because of its failure to stain with coomasie blue. The newly discovered protein was termed as CHannel-forming Integral Protein of 28 kDa or 'CHIP28' (Agre *et al.*, 1987) as it was thought to function as a membrane channel. It was found to exist as an oligomer in the membrane abundantly. Sequence analysis revealed that CHIP28 belonged to the Major Intrinsic Protein (MIP) family, of the lens fiber cell (Gorin *et al.*, 1984). Through *ex vivo* injection of the CHIP28 transcript in *Xenopus laevis* oocytes followed by exposure of the oocytes to hyposmotic challenge, Agre and co-workers were able to demonstrate that CHIP28 was the

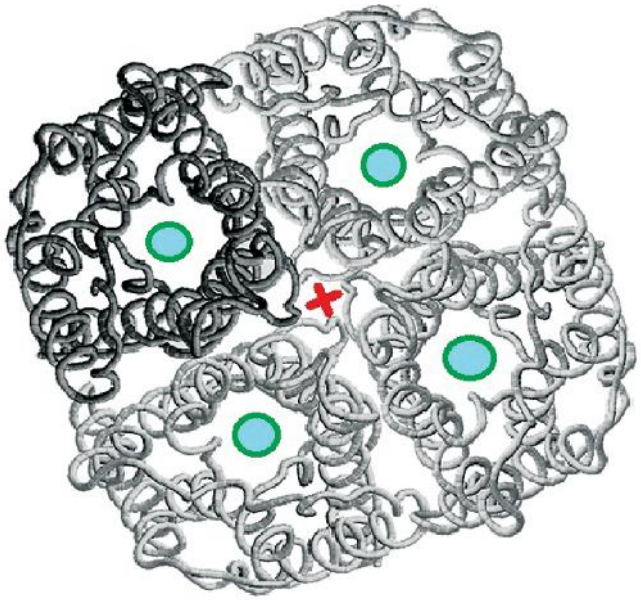

Fig. 4. Diagram of the quaternary structure of the Aqp1 tetramer. The water channels within the monomers are indicated with circles, and the supposed region of the ionic channel is marked with cross (Shapiguzov 2004).

Table 1. Tissue distribution of fish aquaporins and the techniques used by various authors in their study.

Aquaporin	Fishes	Tissues													References	Techniques employed
		G	I	K	E	H	B	L	M	ST	S	P	O	T		
Aqp 0	African lungfish	nd	nd	nd	m	nd	nd	nd	nd	nd	nd	nd	nd	nd	Konno *et al.,* 2010	Cl, qPCR, WB,IMH
Aqp 0a	Atlantic killifish	nd	nd	nd	m	nd	nd	nd	nd	NI	nd	NI	nd	nd	Virkki *et al.,* 2001	Cl, NB
	Zebrafish	nd	nd	nd	m	NI	nd	nd	nd	NI	nd	NI	nd	nd	Tingaud- Sequeira *et al.,* 2010	*
	Atlantic salmon	m	m	m	m	NI	m	NI	NI	NI	NI	NI	m	m	Chauvigné *et al.,* 2015	Cl, Rt PCR, Seq, Phy A, Insilico.
Aqp 0b	Zebrafish	nd	nd	nd	m	NI	nd	nd	nd	NI	nd	NI	m	nd	Tingaud- Sequeira *et al.,* 2010	*
	Atlantic salmon	m	m	m	m	NI	m	NI	NI	NI	NI	NI	m	m	Chauvigné *et al.,* 2015	Cl, Rt PCR, Seq, Phy A, Insilico.
Aqp 1a	Gilthead Seabream	m,p	m,p	m,p	m	NI	m	m	m	NI	NI	NI	m,p	m,p	Fabra *et al.,* 2005; Raldua *et al.*, 2008; Zilli *et al.,* 2009; Cerda and Finn 2010	qPCR, WB, IMF, RT PCR
	Black Porgy	m	m	m	NI	NI	m	m	NI	NI	NI	NI	NI	m	An *et al.,* 2008	RACE PCR, sqRTPCR
	European Seabass	m	m	m	NI	NI	m	NI	NI	NI	NI	NI	NI	NI	Giffard- Mena *et al.,* 2007	PCR, Cl, Seq, RACE, Ph.A., NB, PSA
	European Eel	nd	m,p	m,p	m	m	m	nd	m	m	NI	m	NI	NI	Martinez *et al.,* 2005a,b	Cl, Seq,WB, NB, IMF, SDS PAGE
	Japanese Eel	nd	m,p	nd	NI	m	NI	nd	nd	NI	NI	NI	NI	nd	Aoki *et al.,* 2003; Kim *et al.,* 2008	RACE, NB, WB, IMC, qPCR
	Atlantic Salmon	m	m,p	m,p	NI	NI	m	m	m	m	NI	NI	NI	NI	Tipsmark *et al.,* 2010; Madsen *et al.*, 2011; Engelund and Madsen 2015	qPCR, PhA, WB, IMF, CM, SDS PAGE, PASS, IMH
	Zebrafish	m	m	m	m	NI	m	m	m	NI	m	NI	m	m	Tingaud- Sequeira *et al.,* 2010	*
	Climbing perch	m	m	m	NI	NI	NI	NI	NI	NI	m	NI	NI	NI	Ip *et al.,* 2013	PCR, RACE, qPCR, PhA
	Marine Medaka	m	m	m	m	m	m	m	m	NI	NI	NI	m	m	Kim *et al.,* 2014	RT PCR, PhA
	Atlantic halibut	NI	NI	m,p	m,p	NI	NI	NI	NI	m	NI	NI	m,p	m,p	Zapater *et. al.*,2011	Cl, Seq, qPCR, Ph A, IMF, IEM, SDS PAGE
	Japanese Medaka	m	m,p	m	NI	NI	m	m	m	NI	m	NI	m	m	Madsen *et al.,* 2014	WB, IMF, EP
	Rainbow wrasse	p	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	Brunelli *et al.,* 2010	LM, EM, CM, IMF
	Rainbow trout	NI	m, p	m, p	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	Engelund and Madsen 2011; Madsen *et al.,* 2014	WB, IMF, EP
	River pufferfish	m	m	m	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	Jeong *et al.,* 2014	qPCR, Cl
	Sea Bass	m	m, p	m	NI	NI	m	NI	NI	NI	NI	NI	NI	NI	Giffard- Mena *et al.,* 2007, 2011	PCR, Cl, NB, qPCR, IMF, Seq, RACE, Ph.A., PSA
Aqp 1ab	Silver Seabream	m	m	m	NI	m	nd	m	NI	NI	NI	NI	NI	NI	Deane *et al.,* 2011	Cl, Seq An, S q RTPCR
	European Eel	NI	NI	m,p	nd	nd	nd	nd	nd	nd	NI	nd	NI	NI	Martinez *et al.*, 2005a, b	Cl, Seq,WB, NB, IMF, SDS-PAGE
	Gilthead Seabream	NI	m,p	NI	m	NI	NI	NI	NI	NI	NI	NI	m	NI	Raldua *et al.,* 2008; Fabra *et al.,* 2005	qPCR, WB, IMF
	Japanese eel	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	m	NI	Kagawa *et al.*, 2009	In vitro experiments
	Atlantic halibut	NI	NI	m,p	m,p	NI	NI	NI	NI	NI	NI	NI	m,p	m,p	Zapater *et al.*, 2011	Cl, Seq, qPCR, Ph A, IMF, IEM, SDS PAGE
	Stinging Catfish	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	m	NI	Chaube *et al.,* 2011	Cl, Seq, Ph A, IMF, qPCR

Aquaporin	Fishes	Tissues													References	Techniques employed
		G	I	K	E	H	B	L	M	ST	S	P	O	T		
Aqp 1b	Gilthead Seabream	m	m,p	m	nd	NI	nd	nd	nd	NI	NI	NI	m,p	nd	Fabra *et al.*,2005; Raldua *et al.*, 2008	qPCR, WB, IMF
	European Eel	nd	m	m	nd	nd	nd	nd	nd	nd	NI	nd	m	nd	Martinez *et al.,* 2005 b Tingaud-Sequeira *et al.,* 2008	Cl, Seq,WB, NB, IMF, SDS PAGE, Ph A
	Japanese Eel	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	m	NI	Kagawa *et al.,* 2009	Invitro experiments
	Senegalese Sole	nd	nd	nd	NI	NI	nd	nd	NI	NI	NI	NI	m	nd	Tingaud-Sequeira *et al.,* 2008	Cl, Seq, Ph A, RT PCR, RACE, WB, IMF
	Atlantic Salmon	m	m	m	NI	NI	m	m	nd	m	NI	NI	NI	NI	Tipsmark *et al.,* 2010	qPCR, PhA
	Zebrafish	nd	nd	nd	nd	NI	m	nd	nd	NI	nd	NI	m	m	Tingaud-Sequeira *et al.,* 2008, 2010	Cl, Seq, Ph A, RT PCR, RACE, WB, IMF ; *
Aqp 2	African lungfish	nd	nd	m,p	nd	nd	nd	nd	nd	nd	NI	NI	nd	nd	Konno *et al.,* 2010	Cl, qPCR, WB,IMH
Aqp 3a	European Seabass	m	m	m	NI	NI	nd	NI	NI	NI	NI	NI	NI	NI	Giffard- Mena *et al.,* 2007	PCR, Cl, Seq, RACE, Ph.A., NB, PSA
	Atlantic Killifish	m,p	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	Tingaud-Sequeira *et al.,* 2009; Jung *et al.*, 2012	Cl, SDS PAGE, WMIH, IMF, qPCR, IMC
	Zebrafish	m	m	m	m	m	m	m	m	NI	m	NI	m	m	Hamdi *et al.,* 2009 Tingaud-Sequeira *et al.,* 2010	RT PCR,Cl, Oocyte exp., *
	Atlantic Salmon	m,p	m	m,p	NI	NI	m	nd	m	m	NI	NI	NI	NI	Tipsmark *et al.,* 2010b; Engelund and Madsen 2015	qPCR, PhA, IMF, CM, SDS PAGE, PASS, IMH
	European Eel	m	m	m	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	MacIver *et al.,* 2009	Cl, Xenopus oocyte exp.,TA
	Japanese Medaka	m	m	m	NI	NI	m	nd	nd	m	m	NI	m	m	Madsen *et al.,* 2014	WB, IMF, EP
	Marine Medaka	m	m	m	m	m	m	m	m	NI	NI	NI	m	m	Kim *et al.,* 2014	RT PCR, PhA
	Mozambique tilapia	m,p	m	m	m	NI	m	nd	NI	NI	m	NI	NI	NI	Watanabe *et al.*, 2005	Mol Cl, RACE, NB, RT PCR, oocyte exp, WB, FM, TEM
	Rainbow wrasse	p	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	Brunelli *et al.,* 2010	LM,EM, CM, IMH
	River pufferfish	m	m	m	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	Jeong *et al.,* 2014	PCR, Cl, qPCR
	Sea Bass	m	m	m	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	Giffard- Mena *et al.*, 2007, 2008	PCR, Cl, Seq, RACE, Ph.A., NB, PSA, Hist.
	Silver Seabream	m,p	NI	m,p	NI	m,p	m	m,p	NI	NI	NI	NI	NI	NI	Deane and Woo 2006	Cl, SDS PAGE, ImBlott.
	Sockeye Salmon	m	m	m	NI	NI	nd	nd	NI	nd	NI	NI	NI	NI	Choi *et al.*, 2013	TC, qPCR, WB
Aqp 3b	European Eel	m,p	m,p	m,p	m	nd	nd	nd	NI	nd	NI	nd	NI	NI	Cutler and Cramb 2002; Lignot *et al.*, 2002; Cutler *et al.,* 2007	Cl, Seq, NB, WB, RIA, IMFLM,CLSM, TEM, IGEM
	Zebrafish	m	nd	nd	m	nd	nd	nd	m	NI	m	NI	nd	m	Hamdi *et al.,* 2009 Tingaud-Sequeira *et al.,* 2010	RT PCR,Cl, Oocyte exp., *
	Japanese Eel	m	m	nd	NI	NI	nd	NI	NI	m	NI	NI	NI	NI	Kim *et al.,* 2010	Mol Cl, qPCR, RACE
Aqp 4	Zebrafish	m	nd	nd	m	NI	m	nd	m	NI	m	NI	m	m	Tingaud-Sequeira *et al.,* 2010	*
Aqp 7	Japanese Medaka	m	m	m	NI	NI	m	m	nd	nd	NI	NI	m	m	Madsen *et al.,* 2014	WB, IMF, EP
	Zebrafish	m	m	m	nd	NI	nd	nd	nd	NI	m	NI	m	m	Tingaud-Sequeira *et al.,* 2010	*

Aquaporin	Fishes	Tissues													References	Techniques employed
		G	I	K	E	H	B	L	M	ST	S	P	O	T		
Aqp 8aa	Zebrafish	m	m	m	m	NI	nd	m	nd	NI	nd	NI	m	m	Tingaud-Sequeira *et al.*, 2010	*
	Atlantic Salmon	n.d	m	m	NI	NI	nd	m	nd	nd	NI	NI	NI	NI	Engelund *et al.,* 2013; Tipsmark *et al.,* 2010	Oocyte exp., qPCR, IMF, IEM, IMBlott
	European Eel	NI	m	NI											Cutler *et al.,* 2009	
	Japanese Eel	n.d	m	m	NI	NI	nd	m	NI	nd	nd	NI	NI	NI	Kim *et al.*, 2010	Mol Cl, qPCR, RACE
Aqp 8ab	Atlantic Salmon	m	m,p	n.d	NI	NI	nd	nd	nd	NI	NI	NI	NI	NI	Tipsmark *et al.*, 2010b, Madsen *et al.,* 2011; Engelund *et al.,* 2013	qPCR, PhA, WB, IMF, CM, SDS PAGE, PASS, IMH, Oocyte exp., IEM, IMBlott
	Zebrafish	nd	m	m	nd	NI	nd	nd	nd	NI	nd	NI	nd	nd	Tingaud-Sequeira *et al.*, 2010	*
	Japanese Medaka	m	m,p	m	NI	NI	m	nd	m	nd	NI	NI	nd	nd	Madsen *et al.,* 2014	WB, IMF, EP
	Marine Medaka	m	m	m	m	m	m	m	m	NI	NI	NI	m	m	Kim *et al.*, 2014	RT PCR, PhA
Aqp 8b	Atlantic Salmon	m	m,p	m,p	NI	NI	nd	nd	nd	nd	NI	NI	NI	NI	Tipsmark *et al.*, 2010a; Engelund *et al.,* 2013; Engelund and Madsen 2015	qPCR, PhA, WB, IMF, CM, SDS PAGE, PASS, IMH, Oocyte exp, IEM, IMBlott
	Zebrafish	nd	nd	nd	nd	NI	m	nd	nd	NI	nd	NI	nd	nd	Tingaud-Sequeira *et al.,* 2010	*
	Sockeye Salmon	n.d	m	m	NI	NI	nd	nd	NI	nd	NI	NI	NI	NI	Choi *et al.,* 2013	TC, qPCR, WB
Aqp 9a	Zebrafish	m	m	m	m	NI	nd	nd	nd	NI	nd	NI	nd	nd	Hamdi *et al.,* 2009 Tingaud-Sequeira *et al.,* 2010	RT PCR,Cl, Oocyte exp., *
Aqp 9b	Zebrafish	m	nd	nd	m	NI	nd	m	nd	NI	nd	NI	m	nd	Hamdi *et al.,* 2009 Tingaud-Sequeira *et al.*, 2010	RT PCR,Cl, Oocyte exp., *
Aqp 10a	Japanese Medaka	m	m,p	m	NI	NI	m	m	nd	nd	NI	NI	nd	nd	Madsen *et al.,* 2014	WB, IMF, EP
	Zebrafish	m	m	m	nd	NI	nd	m	nd	NI	nd	NI	m	nd	Hamdi *et al.*, 2009 Tingaud-Sequeira *et al.*, 2010	RT PCR,Cl, Oocyte exp., *
Aqp 10b	Atlantic Salmon	n.d	m	m,p	NI	NI	nd	nd	nd	m	NI	NI	NI	NI	Tipsmark *et al.,* 2010b, Engelund and Madsen 2015	qPCR, Ph A, SDS PAGE, BLOT, IMH, IMF, PASS
	European Eel	n.d	m	m	NI	nd	m	nd	nd	nd	NI	nd	NI	NI	MacIver *et al.,* 2009; Martinez *et al.,* 2005a,b	Cl, Seq,WB, NB, IMF, SDS PAGE, TA
	Zebrafish	nd	m	m	nd	NI	nd	nd	nd	NI	nd	NI	m	m	Tingaud-Sequeira *et al.,* 2010	*
	Gilthead Seabream	m	m	m	NI	NI	NI	nd	nd	nd	nd	NI	NI	m,p	Santos *et al.,* 2004; Zilli *et al.*, 2009	RT PCR, WB, cDNA lib cons, oocyte perm. assay, NB, ISH, PhA, PSA
	Silver Seabream	m	m	nd	NI	NI	NI	nd	nd	NI	m	NI	NI	m,p	Santos *et al.,* 2004; Zilli *et al.*, 2009	RT PCR, WB, cDNA lib cons, oocyte perm. assay, NB, ISH, PhA, PSA
	Japanese Eel	m	m	m	NI	NI	m	m	NI	m	NI	NI	NI	NI	Kim *et al.,* 2010	Mol Cl, qPCR, RACE
	Japanese Medaka	m	m	m	NI	NI	m	m	m	m	NI	NI	m	m	Madsen *et al.,* 2014	WB, IMF, EP
	Marine Medaka	m	m	m	m	m	m	m	m	NI	NI	NI	m	m	Kim *et al.,* 2014	RT PCR, PhA

Aquaporin	Fishes	Tissues													References	Techniques employed
		G	I	K	E	H	B	L	M	ST	S	P	O	T		
Aqp 11a	Japanese Medaka	m	m	m	NI	NI	m	m	m	m	NI	NI	m	m	Madsen *et al.,* 2014	WB, IMF, EP
	Marine Medaka	m	m	m	m	m	m	m	m	NI	NI	NI	m	m	Kim *et al.,* 2014	RT PCR, PhA
Aqp 11b	Zebrafish	nd	m	nd	nd	NI	nd	m	nd	NI	nd	NI	m	nd	Tingaud-Sequeira *et al.,* 2010	*
Aqp 12	Marine Medaka	m	m	m	m	m	m	m	m	NI	NI	NI	m	m	Kim *et al.,* 2014	RT PCR, PhA
	Zebrafish	m	m	m	m	NI	m	m	m	NI	m	NI	m	m	Tingaud-Sequeira *et al.,* 2010	*

Abbreviations: m, mRNA detected by NB and/or RT PCR; p, protein detected by WB and/ or IMC; nd, not detected; NI, not investigated; G, Gills; I, Intestine; K, Kidney; E, Eye; H, Heart; B, Brain; L, Liver; M, Muscle; ST, stomach; S, skin; P, Pancreas; O, Ovary; T, Testis. Cl, Cloning; PCR, Polymerase Chain Reaction, RT PCR, Reverse Transcription Polymerase Chain Reaction; qPCR, Quantitative Real time PCR; RACE, Rapid Amplification of cDNA Ends; sq RT PCR, Semi Quantitative RT PCR; NB, Northern Blotting; WB, Western Blotting; Seq., Sequencing; Mol. Cl., Molecular Cloning; TA, Transport Assay; Seq. An., Sequence Analysis; Ph A, Phylogenetic Analysis; IMF, Immunofluoroscence; IMH, Immunohistochemistry; IMC, Immunocytochemistry; LM, Light Microscopy; CM, Confocal Microscopy; EM, Electron Microscopy; TEM, Transmission Electron Microscopy; IEM, Immuno Electron Microscopy; IGEM, Immuno-Gold Electron Microscopy; CLSM, Confocal Laser Scanning Microscopy; IMFLM, Immuno Fluoroscence Light Microscopy; ImBlott, ImmunoBlotting; RIA, Radio Immunoassay; ISH, In Situ Hybridization; WMIH, Whole Mount Insitu Hybridization; SDS PAGE, Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis; cDNA lib. Cons., cDNA library construction; PSA, Protein Structure Analysis; Hist, Histology; PASS, Periodic Acid Schiff Staining; EP, ElectroPhysiology; TC, Tissue Culture; * (Genome and transcript analysis, cDNA cons., cloning, Swelling assay, Radioactive solute uptake assays, gene expression by RT PCR, Phylogenetic analysis). Data includes studies on fresh water and saltwater acclimated euryhaline fishes combined.

postulated red blood cell water pore (Preston *et al*., 1992). Shortly after establishing the function of CHIP28 as a water pore channel, homologous proteins in mammals, bacteria and plants were also discovered to function as water pores, and the term 'aquaporin' was coined as a descriptive name for members of this protein family (Agre *et al.,* 1993). CHIP28 protein was renamed as aquaporin 1(AQP1) and MIP, which showed strong sequence homology to AQP1 was renamed as aquaporin 0 (AQP0) according to the conventions established by the Human Genome Nomenclature Committee (Agre *et al*., 1993).

Since these early ground-breaking studies, aquaporins have been documented in all kingdoms of life from archaic bacteria to mammals, with several thousand sequences now available in public databases. The largest repertoire is currently found in plants with up to 71 paralogs reported in upland cotton (Park *et al.,* 2010). In vertebrates the highest copy number is found in teleosts with up to 42 paralogs reported in Atlantic Salmon (Finn *et al.,* 2014), while mammals run a close second due to tandem duplication of *AQP7* and *-12* resulting in up to 17 paralogs in humans (King *et al*., 2004; Cerdà and Finn, 2010). Aquaporins in fish were first reported in the year 2000 by Cutler and Cramb, following which there are a rise in copy numbers of aquaporins in fish as new genomes and novel annotations arise. Early studies involved in identifying the Aqp gene and its expression employed techniques like Northern Blotting to identify the Aqp RNA transcripts, Western Blotting, SDS PAGE etc. to identify protein expression, Cloning and *Xenopus* oocyte expression assays to determine the functions of the cloned proteins. Soon the use of Reverse Transcription Polymerase Chain Reaction replaced Northern Blots to study mRNA expression and Real time quantitative PCR has been extensively used to determine the gene expression pattern of the Aquaporin molecules. Localization studies in various tissues are done by techniques like TEM, SEM, IGEM, Immunohistochemistry, Immunocytochemistry, Immunofluoroscence etc. assisted by appropriate staining techniques. Most of the aquaporins discovered so far have been sequenced. Like any other protein structure determination studies, the aquaporin protein structure is determined through *In-Silico* modeling and X-ray Crystallography. Various techniques used by different authors while studying the molecule have been summarized in Table1.

Diversification of aquaporins

Aquaporins are a diverse superfamily comprising of a number of isoforms in the vertebrate group which is the consequence of a series of whole genome duplication events and other genetic alterations in the course of evolution. WGD accompanied by horizontal gene transfer and gene duplications lead to the addition of newer AQPs whose functions became diverse due to neo and sub-functionalization of the novel genes (Semon and Wolfe, 2007). Some or all of the duplicated genes may get lost (Wolfe, 2001) or even chromosomes (diploidization) after a duplication event. Whole genome duplication is believed to have occurred twice near the origin of the vertebrate group, a third time in fish (Brunet *et al.,* 2006) and a fourth time in salmonid ancestors (Davidson *et al.,* 2010).

Since the discovery of AQP1 and AQP0, number of labs around the world started studying on this group of proteins and as a result numerous homologous and allied proteins were reported in diverse organisms including unicellular microorganisms, plants, invertebrates and vertebrates, turning aquaporins into a large family of related proteins.

Aquaporins are basically classified according to their permeation preference for water, glycerol, or other small solutes and gases, molecular structure and phylogeny (Stahlberg *et al.*, 2001; King *et al.,* 2004; Takata *et al.,* 2004; Zardoya, 2005; Tingaud-Sequeira *et al.*, 2010). Therefore, based on the sequence around the highly conserved pore forming NPA motif, Aquaporins are Grossly divided into: Classical water selective aquaporins that include subfamilies AQP 0, -1, -2, -4, -5, -14, and -15; Aquaglyceroporins that include AQP 3, -7, -9, -10, -3 that transport glycerol and urea additionally and Unorthodox Aquaporins or Superaquaporins that include AQP 6, -8, -11, -12, -16 (Dong *et al.,* 2016) due to their different characteristics such as NH3 permeability for AQP8 (Saparov *et al.*, 2007), ability to transport anions (AQP6) (Yasui *et al.*, 1999), or intracellular locations and modified NPA motifs (AQP11 and -12) (Itoh *et al.*, 2005; Gorelick *et al.*, 2006). An extensive phylogenetic analysis of deuterostome genomic data has revealed that the vertebrate superfamily contains 17 classes of aquaporins (Aqp0 - Aqp16; Finn *et al.*, 2014). E. coli has one member from each of classical aquaporin, (AqpZ) and aquaglyceroporin (GlpF). Another subfamily of aquaporins named AQPxlo, closely related to aquaglyceroporins was found to exist in Western-clawed frog and prototheria (Virkki *et al.,* 2002). Mammals are reported to have 13 aquaporin isoforms (AQPs 0-12). Zebrafish (*Danio rerio)* contains 18 AQP paralogs; Pufferfish (*Fugu rubripes)* contains 17 isoforms (Tingaud-Sequeira *et al.*, 2010). Due to a recent Whole Genome duplication event in the ancestors of the salmonid fishes, the Atlantic salmon has been found to express the highest number of AQP paralogs, 42 paralogs distributed in 12 AQP classes (Finn *et al.,* 2014).

Structure of the aquaporin

The first aquaporin structure to be determined was the human aquaporin AQP1 shortly after its discovery by Peter Agre and colleagues. Initially, by the use of techniques like mutagenesis, epitope tagging, and spectroscopic and freeze-fracture electron microscopy methods, the basic features of the aquaporin structure were defined. Then protein structure modeling with the primary sequence of AQP1 using constraints established from high resolution 3D electron micrographs studies of recombinants developed by reconstitution of purified AQPs in lipid bilayers yielded the first atomic model for AQP1 structure (Murata *et al.,* 2000).

The aquaporin protein is a trans-membrane water channel. It exists as a homotetramer, i.e. the quaternary structure of the AQP consists of 4 identical subunits (monomers). Each AQP monomer consists of six membrane spanning alpha-helices that have a central water-transporting pore (Sui *et al.,* 2001, Murata *et al.,* 2000). Therefore, unlike ion channels where four subunits surround a single pore, each monomer in an AQP tetramer has its own pore. Both C and N termini of the AQP protein face the cytosol. Two types of loops are found in an AQP subunit: Cytosolic and Extracytosolic. Cytosolic loop lies between second and third transmembrane domain (B) and extracytosolic domain lies between the fifth and sixth transmembrane domain (E). These two loops form short hydrophobic helices that clip halfway into the membrane from opposite sides. These two loops generally contain the conserved

NPA (Asn-Pro-Ala) motif or the signature motif (Fig1). Asparagine residues are key to the formation of water selective filter (Krane *et. al.,* 2003). This NPA motif is responsible for forming the pore of diameter 0.3 nm midway between the leaflets of the bilayers and is the characteristic and identifying feature of AQP protein, indicating an "Hour Glass Model" (Jung *et. al.,* 1994). Water molecule flows through an AQP channel in a single file, one at a time at the rate of several billion per second. Structural and molecular dynamic studies have established the atomic phenomenon responsible for the process of membrane water transport. The ability to block large or charged solutes and protons while conferring minimal resistance to water permeation is determined by the structural features of AQPs with conserved positions for different amino acid residues to best suit their function. The narrowest point of the water pore (3 Å diameter) at midway between the leaflets of the bilayers have the walls formed from hydrophobic transmembrane domains TM1, 2, 4, and 5. The two highly conserved Asn-76 and Asn-192 in the NPA motifs are juxtaposed, providing the polar residues for hydrogen bonding. Moreover, the terminal parts of the pore-forming loops B and E each contain short ~ -helices which create a partial positive charge in the centre of the membrane. This structure lies just below a 2.8 Å constriction surrounded by residues Phe-56, His-180, Cys-189 (the mercury inhibition site), and Arg-195 (de Groot *et al.,* 2001). Arg 195 is highly conserved among all the members of the aquaporin super family and it provides a functionally important positive charge at the narrowest segment of the channel. His-180 is uncharged at neutral pH, but becomes protonated at lower pH, providing a second positive charge. Together, Arg-195, His-180 and the positive dipoles from the pore helices provide strong repelling charges resisting passage of protons (hydronium ions) during water permeation.

Recently, structural study was done via homology modeling and Molecular Dynamic Simulations on AQP8 of seven vertebrates including man, cattle, rat, falcon, turtle, frog and salmon based on the solved structure of the plant aquaporin A*f*TIP2;1 and was speculated to have an almost similar selectivity filter (Kirscht *et al.,* 2018).

Aquaporins in fish

Maintenance of body fluid composition, cell volume regulation and osmoregulation are some of the most essential survival tasks for metazoan animals. Living in a world of water, aquatic animals face the particular problem of directly interacting with their aqueous environment that differs markedly in osmolality from their internal body fluids. In freshwater (FW) fish, homeostasis is maintained by osmotic water uptake across the exposed epithelial surfaces of the gill and the gut. They compensate by drinking little and producing large volumes of dilute urine. Conversely, to maintain internal milieu, marine fish experience osmotic water loss to the sea and compensate by drinking more seawater (SW) and excreting the excess salts ingested via the gills and the kidney. Just like in mammals, aquaporins play distinct roles for water transport at both cellular and organ level in fishes. But due to the vast number of fish species (almost 32,000) inhabiting almost every aquatic environment, from small mountain streams, rivers, extreme salty desert lakes to the oceans, the challenge to obtain consensus as well as specific knowledge about aquaporin physiology in this vertebrate group is greatly overwhelming. Now, aquaporin has been established as the main candidate of transcellular pathway of water transport through apical and basolateral cellular membranes in the epithelia. Paracellular pathway is another means of water transport across hydrophobic epithelia that occur through intercellular junction complexes. Aquaporin is the molecular plumbing system of the cells (Agre *et al*., 1998).

The first report on aquaporins in fishes was made by Cutler and Cramb in the year 2000 who cloned an AQP3b paralog from the gill of European eel *Anguilla anguilla* Linnaeus, 1758. Using the European Eel as a model, initial molecular identification and characterization of AQP transmembrane proteins in fish were done. Initial studies focused on the cloning and identification of aquaporin homologues in osmoregulatory tissues such as the gill, intestine and kidney, using degenerate RT-PCR. Though the number of papers addressing to aquaporins in fish were comparatively lesser than in mammals, the increasing studies on them have contributed to a rise in copy number of aquaporins in this vertebrate group.

Tingaud-Sequeira *et al*. (2010) established that zebrafish *Danio rerio* (Hamilton, 1822) and other teleosts retain up to 18 aquaporin genes with homologies to all of the mammalian orthologs except Aqp 2, -5, and -6. Published genomes of the Atlantic salmon *Salmo salar* Linnaeus, 1758 (Davidson *et al*., 2010) and rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) (Berthelot *et al*., 2014) have added even more paralogs to this list. One of the major challenges is to describe and understand the differentiated functionality of such extreme diversity. A number of studies addressing salinity-dependent expression of aquaporins in euryhaline fishes were done where the primary role of aquaporins in osmoregulation was verified. To maintain homeostasis, different aquaporin isoforms either get upregulated or downregulated in different parts of the body according to the environmental condition. Fish germ cells and gametes express aquaporins which have evolved to play vital adaptive roles in the survival of the gametes when they are released into the external aquatic environment. Piscine aquaporins allows sperms, oocytes and embryos to endure tremendous osmotic challenges and oxidative stresses in the outside (Cerdá *et al.,* 2017).

Dong *et al.* (2016) has identified 37 *aqp* genes from the Common Carp (*Cyprinus carpio*) genome. Lee *et al.* (2017) studied the expression profile and characterization of the AQPs 1a and 3a in mud loach *Misgurnus mizolepis* in multiple tissues including immune responsive tissue and osmoregulatory tissues. There are only limited published data on Catfish aquaporins. Chaube *et al.,* 2011 reported the Aquaporin 1b in the catfish *Heteropneustes fossilis* and studied the molecular and functional aspects of the isoform. Acharjee *et al*., 2011 have reported the hormonal regulation of aquaporin 1ab in the oocytes of *H. fossilis*. That aquaporins are involved in water homeostasis in reproductive organs and gametes of teleosts in addition to osmoregulatory mechanisms have also been reported by several other authors (Chauvigné *et al*., 2011).

The authors have investigated the expression patterns of 5 aquaporins in different organs of *H. fossilis* and verified the occurrence of aqp 1, -3, -8, -11, -12 in the gills, liver, kidney, intestine, stomach, muscle and brain of the fish (unpublished data). All of them showed different expression patterns in different organs when subjected to hyperosmotic environment.

Aquaporins in the gills

As the most important osmoregulatory organs in fish the gills play important roles in ion regulation in both salt water and fresh water fishes. In fresh water fishes, surplus water accumulate across the large surface area of the gill and is excreted as hypotonic urine by the kidney; in marine fishes, osmotic loss of water occurs across the gill which is compensated for by oral ingestion and intestinal absorption of saltwater and net secretion of excess salts in the gills. The gill epithelium is largely composed of pavement cells (PC), mucus cells (MC), and various types of ion-transporting, mitochondrion-rich cells (MRC). Aquaporins present in the gills are mostly associated with cell volume regulation and CO_2 elimination. The most elaborately studied gill aquaporins included aqp3 in *Anguilla Anguilla* (Cutler and Cramb, 2000) who reported a downregulation of the paralog aqp3b in the gills of saltwater acclimated fishes; followed by a plethora of reports supporting them by other authors in different fishes. Paralogs of aqp1 have been found in many teleosts with different cellular localization. For instance, chloride cells in rainbow wrasse (Brunelli *et al*., 2010), surface of gill lamellae in gilthead Seabream (Cerdá and Finn, 2010) etc. Several other aquaporins have also been detected in the gill; like *aqp10b*, -*11a*, -*8ab,* and -*12* in two medaka species (*Oryzias dancena*: Kim *et al*., 2014; *O. latipes*: Madsen *et al*., 2014).

Aquaporins in the Gastro-intestinal tract

The gastro-intestinal tract of fish is very essential for water homeostasis in salt water. Salt water ingested by SW-acclimated fishes to compensate for osmotic loss of water is absorbed by the intestine in combination with salts and the salts are then excreted from the body. The fluid is initially desalinated by removal of salts in the esophagus (Parmelee and Renfro, 1983; Grosell, 2006, 2011). Some efflux of water may also occur here. Past the stomach, the bulk absorption of fluid in the intestine is believed to be driven by active uptake of salts into the lateral-intercellular space (Diamond and Bossert, 1967). Fluid

absorption takes place through/between the principal enterocytes of the intestinal epithelium along the entire length of the intestine, including its pyloric appendices (in salmonids). Several ion transport proteins aid in establishing the osmotic gradient. Aquaporin expression pattern often varies according to intestinal segments and cellular localization which includes apical brush border and basolateral sites. The first studies were reported in eels by Lignot *et al.* (2002) and Aoki *et al.* (2003), who found that SW acclimation stimulated intestinal aquaporin mRNA and protein expression. Salinity dependent regulation of aquaporins in the intestinal segments were reported for *aqp1aa* in European eel (Martinez *et al.*, 2005b); sea bass: Giffard-Mena *et al.*, 2007, 2012; Zebrafish (Chen *et al.*, 2010); gilthead seabream: Raldua *et al.*, 2008; Atlantic salmon (Tipsmark *et al.*, 2010); Climbing perch, (Ip *et al.*, 2013). Aqp1aa protein is localized in the apical brush-border and lateral membrane in the intestinal epithelium of fishes. The tandem duplicated paralog *aqp1ab* is found mostly in the rectum of SW-acclimated fish (Raldua *et al.*, 2008) and localized in the brush border membrane of enterocytes. Similarly, occurrence of Aqp1a reported in Black porgy (An *et al.*, 2008) and Japanese Medaka (Madsen *et al.*, 2014) is localized in the brush border membrane.

Lignot *et al.* (2002) reported expression of Aqp3b in the European eel which is localized in macrophage-like cells deep within the intestinal epithelium and in goblet cells of the eel rectum, suggesting that this aquaglyceroporin was not a primary water channel in fluid absorption in the eel (Cutler and Cramb, 2002) but helped in mucus production (Lignot *et al.*, 2002). Occurrence of aqp3a is reported in the esophagus of Atlantic salmon (Tipsmark *et al.*, 2010), and in the intestine of Sockeye Salmon (Choi *et al.*, 2013).

Expression of Aquaporin 7 (Aqp7) is reported in the intestine of zebrafish (Tingaud-Sequeira *et al.*, 2010) and Japanese medaka (Madsen *et al.*, 2014). The paralog Aqp10a was reported in zebrafish intestine, (Tingaud-Sequeira *et al.*, 2010) Japanese and marine medaka (Kim *et al.*, 2014; Madsen *et al.*, 2014). *Aqp10b* is present in zebrafish intestine (Tingaud-Sequeira *et al.*, 2010), European silver eels (Martinez *et al.*, 2005); Atlantic salmon (Tipsmark *et al.*, 2010) and Japanese eels (Kim *et al.*, 2010).

The unorthodox aquaporin 8 and its paralogs have been reported in the intestine of eels and salmon (Cutler *et al.*, 2009; Kim *et al.*, 2010; Tipsmark*et al.*, 2010; Choi *et al.*, 2013), Japanese and Marine Medaka (Kim *et al.*, 2014; Madsen *et al.*, 2014) with cellular localization mostly in brush border membrane of enterocytes. In Atlantic salmon, eight paralogs of aqp8 has been identified (Aqp8aa1, -aa2, -ab1, -ab2, -bb1, -bb2, -ba1, and -ba2; Finn *et al.*, 2014). Paralogs of aquaporin 11 and 12 also have been reported in the intestine of zebrafish and medaka species (Tingaud- Sequeira *et al.*, 2010; Kim *et al.*, 2014; Madsen *et al.*, 2014).

Aquaporins in the kidney

Teleost kidney is mesonephric which lacks the discrete cortical and medullary zonation and the loop of Henle of the metanephric kidney of birds and mammals to produce hypertonic urine (Hickman and Trump, 1969). In fresh water fish, for the production of hypotonic urine, reabsorption of salts and water from filtrate occurs in the proximal tubules, important salts like NaCl are reabsorbed in the relatively water-impermeable distal tubules, collecting duct and in the urinary bladder (Beyenbach, 2004). In salt water fishes, the kidney produces hypertonic urine with much reduced volume (Beyenbach, 2004). Salts like NaCl, Mg^{2+}, SO_4^{2-}, are secreted directly into the proximal tubules in both glomerular and aglomerular marine teleosts (Schmidt-Nielsen and Renfro, 1975; Beyenbach, 2004).

The first aquaporin to be reported in fish kidney was an AQP10-like paralog in gilthead seabream (Santos *et al.*, 2004). Aqp10b is present in eel and zebrafish kidney (MacIver *et al.*, 2009; Tingaud Sequeira *et al.*, 2010) where it functions both as a transporter of water and other small organic solutes such as glycerol and urea.

The paralogs of aquaporin 1 are present in the kidney of many teleosts including marine and freshwater fishes. Paralog *aqp1a* is found in kidney of sea bass and marine Medaka (Giffard-Mena *et al.*, 2007, 2012; Kim *et al.*, 2014); European eels (Martinez *et al.*, 2005a) and gilthead seabream (Cerdá and Finn, 2010). *Aqp1aa* and *aqp1ab* are present in the kidney of

Atlantic salmon and European eel (Martinez *et al*., 2005a; Tipsmark *et al*., 2010). *Aqp1aa* is also reported in black porgy (An *et al*., 2008) and climbing perch (Ip *et al*., 2013).

Aqp3 paralogs have been reported in the kidney of European eel (*aqp3b,* Martinez *et al.,* 2005a); *aqp3a* in Atlantic salmon (Tipsmark *et al*., 2010; Engelund and Madsen, 2015), sea bass (Giffard-Mena *et al*., 2007), and *Mozambique tilapia* (Watanabe *et al*., 2005). Two paralogs of aquaporin 8 (*aqp8aa* and *aqp8ab*) are expressed in the stenohaline zebrafish kidney. Paralog *aqp8ab* is present in Japanese Medaka and marine medaka (Kim *et al*., 2014; Madsen *et al*., 2014). Abundant occurrence of Aqp8a in the basolateral membrane of proximal tubule cells in rainbow trout and Atlantic salmon was reported by Engelund and Madsen (2011, 2015). In medaka, *aqp11a* and *aqp12* are both expressed in the kidney (Kim *et al*., 2014; Madsen *et al*., 2014). *Aqp 12* is also found in the zebrafish kidney at a high level (Tingaud-Sequeira *et al*., 2010).

Conclusion

This review concludes that aquaporins play a vital role in the normal physiology of an organism by maintaining osmotic balance in the body. Different aquaporin isoforms have different functions and yet their expression changes in response to changing osmotic gradient in the environment. Even though several breakthrough findings have been accomplished in the study of this diverse group of protein in animals, there is still a huge future prospective in this field as complete characterization of all the isoforms is hitherto not explored. Moreover, increasing studies have only added to the long list of paralogs in teleosts; all of them with different functional properties and localization in the body. It is therefore desirable to investigate every possible occurrence of aquaporins in fish as they are always in direct contact with water. The authors have investigated the occurrence of aquaporins in different tissues of the singhi catfish *Heteropneustes fossilis* and found aqp 1, 3, 8, 11, and 12 in the gills, intestine, stomach, liver, kidney, brain and muscle tissues by the use of RT PCR. Complete structure modeling studies and functional characterization of these aquaporins are to be done in future investigations.

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