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Characterization of aquaporin1b (AQP1b) mRNA in mud loach (*Misgurnus mizolepis*) in response to heavy metal and immunostimulant stimuli

Running title: Characterization of mud loach AQP1b

Sang Yoon Lee[a], Yoon Kwon Nam[b], Yi Kyung Kim[a,c*]

[a]East Coast Life Science Institute, Gangneung-Wonju National University

[b] Department of Marine Bio-Materials and Aquaculture, Pukyong National University

[c]Department of Marine Biotechnology, Gangneung-Wonju National University

*Author for correspondence:

Yi Kyung Kim

Department of Marine Biotechnology

Gangneung-Wonju National University

Gangneung 25457, Korea

E-mail: yikyung1118@gwnu.ac.kr

Tel.: +82-33-640-2409

Fax: +82-33-640-2955

Abstract

Aquaporins (AQPs) facilitate the transport of water or other small solutes into cells in the presence of osmotic gradients. However, the current understanding of piscine AQP gene with cellular stress responses has been still limitedly exemplified. In present study, we characterized the mud loach AQP1b gene at the nucleotide and amino acid levels. We identified three AQP 1b transcript variants (mmAQP1b_tv1, mmAQP1b_tv2, and mmAQP1b_tv3). Then, we examined the AQP1b promoter region and observed several transcription factor binding sites (TFBS) for nuclear factor of activated T-cells (NFAT), SRY-box, c-AMP responsive element binding protein (CREB), GATA binding factor, and hepatic nuclear factor-1. Interestingly, mmAQP1b transcription was differentially modulated by heavy metal or immunostimulant challenge. Further studies to deepen the knowledge of fish AQP-mediated adaptation response potentially relevant to molecular pathogenesis are warranted.

Key words: Aquaporin, heavy metal, immunostimulant, stress, osmoregulation

Summary statement:

We identified mud loach AQP1b transcript variants and consensus sequences involved in stress or innate immunity in promotor region. AQP1b transcription was differentially modulated by heavy metal or immunostimulant challenge.

Introduction

Teleost species have the remarkable ability to withstand acute or long-term fluctuations in environmental salinity. Fish cells monitor the osmolality difference between the intracellular and extracellular spaces, and transport water to recover their volume following cell swelling or shrinkage. Salinity stress is linked to a wide range of biological processes *e.g.* metabolism, mortality, growth, and even immune responses (Baltzegar et al., 2014; Moshtaghi et al., 2016). After being subjected to an imbalance between environments, proteins trigger various complex responses such as changes in structure or function, a consequence of which is altered enzyme activity (Fiol and Kültz, 2007).

Aquaporins (AQPs) facilitate the transport of water or other small solutes into and out of cells in the presence of osmotic gradients. These integral membrane proteins have been identified across phyla, from Archaea (Kozono et al., 2003) to primates (King et al., 2004). Based on amino acid sequence similarity, 13 different AQPs (AQPs 0-12) can be divided into three subfamilies: classical aquaporins that selectively transport water; aquaglyceroporin that transport glycerol and other small molecules in addition to water; and an unorthodox

55 subgroup (Zardoya, 2005; Ishibashi et al., 2011). Genomic and phylogenetic analyses
56 revealed that teleosts possess several AQP isoforms which have undergone lineage-specific
57 changes and divergence via whole-genome duplication events (Tingaud-Sequeira et al., 2010).
58 Zebrafish (*Danio rerio*) shows a much higher diversity of AQPs than tetrapod AQPs.
59 Duplicated or triplicated sub-isoforms have been retained in the zebrafish genome (Tingaud-
60 Sequeira et al., 2010; Finn and Cerdà, 2011; Finn et al., 2014; Madsen et al., 2015). The
61 valuable information regarding the entire aquaporin sequence in water fleas (*Daphnia pulex*)
62 have been recently published in the NCBI non-redundant protein database (Lind et al., 2017).
63 Many studies indicate that AQPs show multiple modes of activation and regulation, enabling
64 them to respond to diverse cellular events such as neutral signal transduction, brain swelling,
65 and cellular migration (Balzegar et al., 2014; Moshtaghi et al. 2016). To date, understanding
66 the physiological role of piscine AQPs has been facilitated by studying the expression of
67 AQPs in response to changes in salinity (Cutler and Cramb, 2002; Watanabe et al., 2005;
68 Giffard-Mena et al., 2011; Kim et al., 2010; Choi et al., 2013; Madsen et al., 2015).
69 Mud loaches (*Misgurnus mizolepis*; Teleostei; Cypriniformes) are currently the most popular
70 freshwater species with growing importance of domestic market in Korea. In addition to its
71 commercial importance, the mud loach has attractive merits as an experimental organism,
72 including small adult size, high fecundity, year-round spawning under controlled conditions,
73 and relatively well-established techniques for genetic manipulation (Nam et al., 2011; Cho et

al., 2012). Given these advantages, mud loaches could be particularly relevant for studying the involvement of aquaporins in physiological processes.

Most studies investigating piscine aquaporin genes focus on the effects of salinity-induced adaptation in piscine AQP genes in response to biological challenges. However, investigating the interaction among genes using molecular genetic approaches has elucidated the molecular mechanisms underlying biological function and regulatory gene expression. Identifying interaction among diverse genes and autonomous adaptation effects are essential for systematically unraveling the cellular or molecular mechanisms of AQP action. Recent reports indicate that AQPs could be involved in inflammasome activation-induced cell volume regulation (Compan et al., 2011; Meli et al., 2018). Previously, we identified a novel AQP1a in mud loaches (mmAQP1a) which is differentially modulated *in vivo* (Lee et al., 2017). In fact, the existence of AQP1a and its duplicate in teleost species is well established, showing specialized expression (Zapater et al., 2011). We found a AQP1a paralog from our next-generation sequencing database for the mud loach (unpublished data). The genetic determinants for the stenohaline freshwater species mud loach AQP1a paralog, called AQP1b have not been extensively explored. In present study, we identified the AQP1a paralog in mud loaches. The AQP1a paralog, AQP1b is a teleost-specific water-selective channel that mediates oocyte hydration, which is based on the coordinated action between osmolyte flux and aquaporin activity (Fabra et al., 2005; Sun et al., 2010). We found three variant AQP1b

93 paralogs that are structurally similar to mmAQP1a. In line with our long-term goal to more
94 deeply understand the involvement of aquaporin molecules in osmoregulation and
95 physiological processes of mud loaches, we aimed to identify the mRNA splice variants of
96 AQP1 in mud loach. We characterized the structural and features of the AQP1 promoter and
97 examined gene expression patterns in response to environmental challenges.
98

99 **Materials and methods**

100 *Isolation of AQP1b cDNA from mud loaches*

101 To isolate the aquaporin cDNA sequence, a mud loach expressed sequence tag (EST)
102 database generated from whole body RNAs was surveyed. Based on the EST survey and a
103 homology search of NCBI GenBank (http://blast.ncbi.nlm.nih.gov/), the partial mud loach
104 AQP sequences were identified. The full-length of mmAQP sequence was obtained from
105 mud loach total RNAs by RT-PCR and/or Vectorette PCR isolation using specific primer
106 pairs (Table 1). At least six clones for each AQP isoform were sequenced to obtain the
107 representative sequence.
108

109 *Cloning the mud loach AQP1b gene and promoter*

Based on the full-length cDNA sequences of the mud loach AQP**,** the gene sequence was isolated using PCR with AQP1-specific primers that bound the untranslated region (UTR) of gene. The oligonucleotide primer pairs and PCR conditions used are shown in Table 1. The amplified PCR products were cloned into pGEM-T easy vector (Promega, Madison, WI, USA). Sequencing for recombinant clones (n = 6) that contained the correct insert size were done by primer walking with gene-specific primers. To isolate the 5'-flanking region, genome walking was performed using a Universal Genome Walker Kit (Clontech Laboratories Inc., USA). The DNA fragments obtained from genome walking were subcloned, sequenced, and assembled into a contig as described. Finally, the AQP genomic DNA spanning the isolated region was PCR amplified. The PCR products were sequenced to obtain the representative genomic sequence of the mud loach AQP gene.

Bioinformatic sequence analysis

The cDNA sequence was analyzed using the Open Reading Frame (ORF) Finder tool (https://www.ncbi.nlm.nih.gov/orffinder/). Using the deduced amino acid sequence, the theoretical molecular weight and isoelectric point (pI) were evaluated with the ExPASy ProtParam online tool (http://web.expasy.org/protparam/). A homology search of the mud loach AQP1 was performed using the NCBI BLASTx algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and/or the Ensembl genome database

(http://asia.ensembl.org/index.html). Multiple sequence alignment of the aquaporin amino acid sequence to representative teleostean and human orthologs (Table 2) was performed using CLUSTAL W (Thompson et al., 1994). Transmembrane domain prediction was performed using the TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/). To gain insight into the transcriptional regulation of AQP, putative transcription factor binding sites (TFBSs) in the proximal promoter region were identified using MatInspecter software (http://www.genomatix.de) This prediction was performed using the default parameters [General Core Promoter Elements (0.9 core / Optimized matrix sim) and Vertebrates (0.9 core / Optimized matrix sim)] based on Matrix Family Library Version 11.0 (September 2017). We then characterized the regulatory regions of AQP1a.2 gene promoter by sequencing 3405 bp from the 5′-flanking region and analyzed this region for *cis*-regulatory elements using MatInspector software.

Tissue sampling for basal expression assays

To investigate the constitutive expression of AQP in various adult mud loach tissues, we collected samples from 13 different tissues (brain, eye, fin, gill, heart, intestine, kidney, liver, muscle, skin, spleen, ovary, and testis) w from six-month-old healthy females (mean body mass = 19 ± 4 g; n = 8 for each group) and males (mean body mass = 12 ± 3 g; n = 8 for each group). The tissues from the sampled individuals were pooled prior to total RNA extraction.

148

149 *Sampling for embryos and early larvae*

150 Fertilized eggs were collected by mating female fish with male fish using the "wet method"
151 (Nam et al., 2004). The fertilized eggs [referred to as zero hours post-fertilization (HPF);
152 hour 0] were incubated and kept at 25 ± 0.5°C and under an ambient photoperiod until
153 hatching. The embryonic developmental stages were determined according to Nam et al.
154 (2004). The embryos were pooled at the 32 cell (2 HPF), early blastula (4 HPF), early
155 gastrula (6 HPF), late gastrula (8 HPF), 3-4 myotome (12 HPF), 16-17 myotome (16 HPF),
156 and 23-24 myotome (20 HPF) stages. Hatching stared at 24 HPF and 100% of the embryos
157 hatched by 28 HPF. After hatching, the larvae were transferred to a 50-L tank held at 24 ±
158 0.5°C. 100 to 150 larvae were collected at 1, 2, 3, 4, 5, 6, 7, and 14 days post-hatching (DPH).
159 Larva rearing was performed as previously described (Nam et al., 2004).

160

161 *In vivo stimulatory treatments*

162 We examined AQP1b mRNA expression following acute exposure to cadmium (Cd),
163 chromium (Cr), copper (Cu), iron (Fe), manganese (Mn), nickel (Ni), or zinc (Zn). Fish (n =
164 12, average body mass = 15.2 ± 4.1 g) were separated into seven experimental groups (one
165 per metal) and one control group and kept in 60 L tanks with the respective treatment. The
166 tanks were filled with tap water and held at 25 ± 0.5°C. Each group was acclimated to the

tanks for one week. The dose for each metal was 5 μM (Cho et al., 2009), and the exposure time was 48 h. All chemicals were of analytical or reagent grade and were obtained from Sigma-Aldrich. No feed was supplied during the exposure period. The tank water was changed every day to maintain the desired metal concentration. The control group was kept in the same condition as the other groups without the addition of any metals. After 48 h of exposure, four tissues (liver, intestine, kidney, and spleen) were collected.

To examine potential immune responses, fish (15.2 ± 4.1 g; n = 3 for each group) were intraperitoneally injected with lipopolysaccharide (LPS; *Escherichia coli* 0111:B4, Sigma-Aldrich, St Louis, MO, USA), polyinosinic:polycytidylic acid [poly(I:C); Sigma-Aldrich], or phosphate buffer saline (PBS, pH 6.8; non-stimulated control). For LPS or poly(I:C) challenge, two doses (5 or 25 μg g^{-1} body weight) were tested. After being injected, each group was transferred to a 60 L tank at 25°C. No feed was supplied during this period. At 24 h post-injection, immune-relevant tissues were surgically removed from each fish and stored at -80°C until analyzed by RT-PCR.

RT-PCR analysis

Total RNA was extracted using TriPure Reagent (Roche Applied Science, Mannheim, Germany) and purified using an RNeasy Mini Plus Kit (Qiagen, Hilden, Germany), including DNase I treatment. For individual experiments, 2 μg total RNA was used for cDNA synthesis

using an Omniscript Reverse Transcription Kit (Qiagen). For the quantitative real-time PCR, the cDNA was diluted 4-fold with sterile distilled water and 2 μL was used as the template for each qPCR reaction. The qRT-PCR analysis was conducted using a LightCycler 480 Real-Time PCR System and LightCycler 480 SYBR Green I Master mix (Roche Applied Science, Germany). 18S RNA (adult tissue and embryonic-early larval samples) and ribosomal protein 7 (RPL7 experimental stimulation analysis) were chosen as the internal control genes. The plasmid DNAs containing the amplified target mRNAs were prepared as standard samples. The qRT-PCR analysis for each experiment included three technical replicates for each biological specimen.

To compare AQP1a.2 transcript levels across tissue types and developmental samples, qPCR data were analyzed using the ΔCt method (Ct of the AQP1b gene subtracted from the Ct of the internal control genes). The relative AQP1a.2 gene expression in the stimulated or challenged groups was analyzed as the fold difference compared to the control group using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008).

Statistical analysis

All data are expressed as means ± standard error. The data were analyzed using one-way ANOVA followed by Duncan's multiple range tests included in SPSS version 10.0 software (SAS Inc., Cary, NC, USA). $P<0.05$ was considered statistically significant.

205

206 **Results**

207

208 *Characteristics of the AQP1b isoform*

209 The AQP1b gene from the mud loach (named mmAQP1b) is 857 bp long with an open
210 reading frame (ORF) encoding 269 amino acids. The calculated molecular mass of this
211 isoform is 28.86 kDa, with a theoretical pI value of 9.35. The nucleotide sequence of the
212 AQP1b cDNA sequence was submitted to GenBank under the accession numbers
213 MT184376-MT184378 (genomic DNA MT184375). The deduced amino acid sequence of
214 mmAQP1b shares the same core architecture as the vertebrate AQPs, including six
215 transmembrane domains and cytoplasmic amino and carboxy termini (Fig. 1). The two
216 hydrophobic loops contain asparagine-proline-alanine (NPA) motifs that regulate selective
217 water conduction and maintain proton gradients across the biological membrane (Gonen and
218 Walz, 2006). The alignment of the putative amino acid sequence of mmAQP1b and
219 orthologous sequences from other teleosts revealed considerable similarity (51-84%) (Table
220 2). In addition, the cysteine residue near the second NPA motif, which is involved in
221 sensitivity to mercury, was identified in mmAQP1b (Preston et al., 1993).

222

223 *Identification of novel splice variants, genomic structure, and organization of mud loach*

224 *aquaporin 1b*

225 While confirming the full-length mmAQP1b cDNA, we identified three different AQP1b

226 transcript variants, which we designated mmAQP1b_tv1, mmAQP1b_tv2, and

227 mmAQP1b_tv3. These variants were 944, 932, and 819 bp long, respectively. Alignment of

228 the AQP transcripts sequences with other teleosts revealed that the variants arose from

229 alternative splicing between exon 4 and exon 5 for AQP1b_tv2 and AQP1b_tv3. All

230 transcript variants had ATG as the start codon and encoded a 269-amino acids peptide with a

231 molecular mass of 28.9 kDa.

232 At the genomic level, the mmAQP1b gene contains five exons (366, 165, 81, 178, and 20 bp

233 in length for exon-I to exon-V, respectively) interrupted by four introns (1367, 185, 454,

234 4036 bp in length for intron-I to intron-IV, respectively). The splice sites contained conserved

235 GT-AG dinucleotides at each junction, and the sequence of each coding exon was clearly

236 matched with its corresponding cDNA sequence.

237

238 *In silico analysis of transcription factor binding sites in the mmAQP1b promoter region*

239 To identify the transcription factor regulating mmAQP1b expression, we sequenced a 3405

240 bp upstream region and analyzed this region for *cis*-regulatory elements using MatInspector

241 software. The transcription start site of AQP1b mRNA was located at -9 bp upstream of the

translational ATG initiation codon. We also detected the presence of consensus sequences for core promoter elements important for basal transcription, such as a TATA box (Table 2). In addition, various elements involved with immune modulation or stress responses were observed, including CCAAT-enhancer binding protein (CEBP) sites, cAMP-responsive element binding protein (CREB) sites, nuclear factor of activated T-cells (NFAT) sites, and signal transducer and activator of transcription (STAT) sites. Interestingly, we identified binding sites for three Sry-related high mobility group [HMG]-box (Sox) family members known to be expressed in teleost oocytes. The binding sites for Sox5, Sox6, and Sox3 were located at -2575 bp, -268 bp, and -1048 bp from the transcription initiation site, respectively. In addition, fork head domain factor (Fox), which are involved in development of lung, brain, thymus, and cardiac tissue, had a predicted binding site at -2572 bp based on the consensus sequence. Binding sites for hepatic nuclear factor 1 (HNF1), a transcription factor that regulates ubiquitous expression in many tissues and cells, were also observed at various locations within the AQP1b promoter.

Tissue distribution and developmental expression of mmAQP1b

mmAQP1b transcript was detected in all examined tissues, although the basal expression level was largely different among these tissues. High AQP1b mRNA expression was detected

in the gill, kidney, and spleen, whereas lower expression was observed in the liver, muscle, and skin (Fig. 2).

The mRNA expression level of mmAQP1b was regulated during embryonic and larval development (Fig. 3). mmAQP1b expression was low at fertilization (0 hpt) and further decreased until 6 hpt (early gastrula stage). Expression gradually begun to increase with developmental stage until 28 hpt. Afterward, mmAQP1b expression in embryos was remarkably increased until hatching, and dynamically increased even more until 2 dph. Then, mmAQP1b mRNA decreased to the level observed at 28 hpt, although the rate of decrease slowed 4 to 7 dph.

Modulation of mmAQP1b in response to heavy metal exposure

The response of mmAQP1b to acute waterborne metal exposure was variable according to the metal and tissue type. In the intestine, AQP1b transcription was upregulated by most heavy metals. Cu and Fe caused 3.6- and 2.0-fold decreases of AQP1b transcription, respectively. Ni exposure did not significantly alter mRNA transcription. In the kidney, transcriptional suppression occurred in the groups exposed to Cu (7.3-fold decrease), Fe (3.2-fold), and Mn (2.0-fold). The maximum induction of AQP1b transcript in the kidney was observed with Cr (2.12-fold) treatment. Meanwhile, hepatic AQP1b transcription was induced by all tested metals. Three metals, Cd (25.2-fold increase relative the non-exposed

279 control), Cr (15.1-fold), and Ni (9.18-fold) induced more AQP1b transcription in the liver
280 than the other heavy metals: Cu (5.1-fold) and Zn (2.59-fold). In the spleen, four metal-
281 treated groups did not show increased AQP1b mRNA. Of the four groups, one group (Cu)
282 displayed significantly reduced AQP1b mRNA after challenge. Maximum inducibility in the
283 spleen was 2-fold (the Cu-exposed group), while the other treatments induced only
284 moderately increased AQP1b transcription from 1.19-1.28 folds.

285

286 *AQP expression after immune challenge*

287

288 Experimental challenge with LPS and poly(I:C) altered AQP1b gene expression in many
289 groups, and the patterns were variable among tissues types (Fig. 5). In the intestine,
290 mmAQP1b mRNA was reduced by LPS or poly(I:C) challenge (each 1.27-fold lower than the
291 relative PBS-injected group). In contrast, renal AQP1b expression was significantly
292 upregulated in all challenge groups, with 2.21-fold or 3.34-fold increases relative to PBS-
293 injected controls. A similar pattern was observed in the spleen. AQP1b mRNA expression in
294 the spleen was induced by LPS (2.1-fold) and poly(I:C) (1.93-fold). In the liver, LPS
295 downregulated AQP1b mRNA by 2.32-fold. Other challenges did not show significant
296 differences.

297

298

299 **Discussion**

300 We determined that mmAQP1b is similar in sequence and predicted topology to previously
301 identified AQPs. The mud loach AQP1b has traditional structural features of aquaporins,
302 such as six transmembrane domains. These are vital characteristics that appear in the major
303 intrinsic protein (MIP) family and in aquaporins (Borgnia et al., 1999). The tandem repeat
304 structures with two NPA sequences have been proposed to form tight turn structures that
305 interact in the membrane to form the pathway for water to move through the protein (Nielsen
306 et al., 1999). At the genome level, the mmAQP1b gene has a somewhat different
307 organizational structure (*i.e.* 5 exons interrupted by 4 introns) compared to most other
308 teleostean AQP1 orthologues, which have 4 exons (Tingaud-Sequeira et al. 2008, 2010; Kim
309 et al., 2014). We also isolated the complete mRNA of two AQP1b transcript variants.
310 Analysis of mmAQP1b cDNA using available information on the genome organization of the
311 AQP gene in teleost suggest that each isoform is generated by alternative splicing. This leads
312 to a splicing event where the 5' splice site of the intron is different, which leads to mRNAs
313 with different C-termini. However, the rest of the AQP gene sequence is highly conserved
314 between the two variants.
315 *In silico* analysis of mmAQP1b promotor identified various putative cis-regulatory elements
316 that may serve targets for sequence-specific transcription factors. We found numerous

317 consensus sequences that may be bound by transcription factors involved in stress and/or
318 innate immunity in teleosts such as STAT, CEBP, CREB, and NFAT5. In particular, NFAT5,
319 a member of the nuclear factor of activated T cell family, plays crucial roles in detecting
320 environmental salinity and immune responses under pathophysiological conditions associated
321 with hyperosmotic stress in teleosts and mammals (Küper et al., 2015; Lorgn et al., 2017). In
322 addition, the canonical motifs for STAT, a key factor in the JAK/STAT pathway were
323 identified, suggesting that AQP1b is involved in inflammation-mediated modulation upon
324 pathogen infection. This observation is consistent with the expression profiles of AQP1a and
325 3a in kidney, intestine, liver, and spleen of mud loaches after immune challenge (Lee et al.,
326 2017). The mmAQP1b promoter possesses potential CREB sites responsive to cAMP, similar
327 to those found in mammalian and teleost AQP genes (Zapater et al., 2013; Wang and Zheng,
328 2011). We also observed a potentially conserved site for glucocorticoid-responsive and
329 related elements, which are induced by glucocorticoid or progesterone receptors in mammals
330 and teleosts (Zapater et al., 2013; Lieberman et al., 1993; Moon et al., 1997). Additionally,
331 HNF-1 is a major regulator of glucose homeostasis in the liver, kidney, and pancreas in
332 mammals (Pontoglio, 2000). This evidence indicates that AQP-mediated cellular pathways
333 could be directly or indirectly associated with carbohydrate metabolism in the teleost liver for
334 energy supply during saline challenge.
335 Interestingly, the *in silico* analysis showed putative binding sites for SOX transcription

336 factors in the mmAQP1b promoter region, similar to several fish species (Cerdà et al., 2013;
337 Zapater, et al., 2013; Wei et al., 2016). Recently, SOX transcription factors were reported to
338 be involved in diverse physiological processes such as the formation of the nervous system
339 (Overton et al., 2002), gonadogenesis (Weiss et al., 2003), or sex determining factor.
340 During embryonic development, mmAQP1b expression was weakly expressed during early
341 embryogenesis, followed by a considerable increase until 2 dph and a subsequent decline.
342 Additionally, AQP1b expression is firstly detected from the onset of fertilization to the 32-
343 cell stage, indicating that AQP1b is maternally inherited, as reported for the common
344 mummicho (*Fundulus heteroclitus*) and zebrafish (Tingaud-Sequeira et al., 2009; Chen et al.,
345 2010). Maternal molecules such as transcripts and protein are provided as a source as of
346 cellular energy, structural components, and defense responses during embryonic and larval
347 development in fish. In addition, there was a decrease in mmAQP1b transcript levels from 4-
348 6 HPF (early blastula-gastrula stage).
349 In the present study, the expression of mmAQP1b mRNA was detectable in various tissues,
350 including the gill, kidney, and spleen. Some tissues also showed difference in transcript levels.
351 The freshwater teleost kidney is unable to produce hyperosmotic urine, in contrast to
352 seawater-adapted piscine kidneys, which switch to water saving function via expression of
353 divalent ions. Therefore, the function of piscine renal AQP is regulated by environmental
354 salinity (Tipsmark et al., 2010). The expression of AQP1a.2 transcript is not limited to

355 osmoregulatory tissues, but may also occur in non-osmoregulatory tissues (eye, spleen, and
356 testis), as suggested in several teleost species (An et al., 2008; Tingaud-Sequeira, 2010; Kim
357 et al., 2010, 2014; Madsen et al., 2014).

358 AQP1b transcription showed the significant responses to the immunostimulants LPS and
359 poly(I:C). In particular, renal and splenic AQP1b transcripts were significantly higher than in
360 other tissues. As evidenced by Lehmann et al. (2008), intraperitoneal LPS injection allows
361 LPS immediate access to the circulation. Therefore, LPS induces systemic immune responses.
362 The kidney is the primary excretory organ critical for the maintenance of homeostasis in fish.
363 A recent study suggested that intraperitoneal LPS injection decreases blood flow in the spleen
364 and kidney via vasoconstriction, thereby increasing the volume of interstitial fluid and
365 redistributing blood flow (Wang et al., 2018). Thus, LPS challenge may ultimately impair the
366 transient osmotic gradient, indicating an improper balance of the stable internal water
367 environment and dissolved ion concentrations in the kidney. A previous study reported that
368 upregulated AQP1 transcripts serves a protective role by reversing LPS-induced damage in
369 human renal proximal tubule epithelial cells (Wang et al., 2018). Thus, altered AQP1a
370 protein expression is associated with altered renal physiology.

371 AQPs have been proposed as molecular osmosensors that maintain water homeostasis.
372 Further, AQPs are a possible regulator of innate host defenses at the level of the plasma
373 membrane (Meli et al., 2018). In the present investigation, we characterized AQP variant

374 transcript levels and investigated AQP1b modulation in response to heavy metal exposure
375 and immune challenge. This study provides a comprehensive basis and strengthens the
376 knowledge of the underlying mechanisms of AQP in physiological and pathological
377 processes. Further studies to deepen the knowledge of fish AQP-mediated mechanisms
378 potentially relevant to molecular pathogenesis are required.

379

380 **Competing interests**

381 The authors declare that they have no conflict of interest.

382

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386

387 **Authors' contributions**

388 Sang Yoon Lee contributed to the management of mu loach, gene-cloning, gene-
389 expression analyses and data analysis and; Yi Kyung Kim and Yoon Kwon Nam
390 developed and supervised the experiment, and preparation of the manuscript draft, and
391 modified the manuscript.

392

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References

An, K. W., Kim, N. N. and Choi, C. Y. (2008). Cloning and expression of aquaporin 1 and arginine vasotocin receptor mRNA from the black porgy, *Acanthopagrus schlegli*: effect of freshwater acclimation. *Fish Physiol. Biochem.* 34, 185-194.

Baltzegar, D. A., Reading, B. J., Douros, J. D. and Borski, R. J. (2013). Role for leptin in promoting glucose mobilization during acute hyperosmotic stress in teleost fishes. *J. Endocrinol.* doi: 10.1530/JOE-13-0292.

Borgnia, M., Nielsen, S., Engel, A. and Agre, P. (1999). Cellular and molecular biology of the aquaporin. *Annu. Rev. Biochem.* 68, 425-458.

Compan, V., Baroja-Mazo, A., López-Castejón, G., Gomez, A. I., Martínez, C. M., Angosto, D., Montero, M. T., Herranz, A. S., Bazán, E., Reimers, D., Mulero, V. and Pelegrín, P. (2012). Cell volume regulation modulates NLRP3 inflammasome activation. Immunity 37, 487–500.

Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T. J., Higgins, D. G. and Thompson, T. D. (2003). Multiple sequence alignment with the clustal series of programs. *Nucleic Acids Res.* 31, 3497–3500.

410 **Chen, L. M., Zhao, J., Musa-Aziz, R., Pelletier, M. F., Drummond, I. A., and Boron, W.**
411 **F.** (2010). Cloning and characterization of a zebrafish homologue of human AQP1: a
412 bifunctional water and gas channel. *Am. J. Physiol.* 299, R1163–R1174.

413 **Cho, Y. S., Lee, S. Y., Kim, K. Y. and Nam, Y. K.** (2009). Two metallothionein genes from
414 mud loach *Misgurnus mizolepis* (Teleostei; Cypriniformes): Gene structure, genomic
415 organization, and mRNA expression analysis. *Comp. Biochem. Physiol.* 153B, 317-326.

416 **Cho, Y. S., Kim, B. S., Kim, D. S. and Nam, Y. K.** (2012). Modulation of warm-
417 temperature-acclimation-associated 65-kDa protein genes (*Wap65-1* and *Wap65-2*) in
418 mud loach (*Misgurnus mizolepis*, Cypriniformes) liver in response to different
419 stimulatory treatments. *Fish Shellfish Immunol.* 32, 662-669.

420 **Cerdà, J., Zapater, C., Chauvigné, F. and Finn, R. N.** (2013). Water homeostasis in the
421 fish oocyte: new insights into the role and molecular regulation of a teleost-specific
422 aquaporin. *Fish Physiol. Biochem.* 39, 19-27.

423 **Cutler, C. P. and Cramb, G.** (2000). Water transport and aquaporin expression in fish. In
424 *Molecular biology and physiology of water and solute transport*, pp. 433-441. Springer,
425 Boston, MA.

426 **Fabra, M., Raldúa, D., Power, D. M., Deen, P. M. and Cerda, J.** (2005). Marine fish egg
427 hydration is aquaporin-mediated. *Science*, 307, 545-545.

428 **Fiol D. F. and Kültz D.** (2007). Osmotic stress sensing and signaling in fishes. FEBS J. 274,
429 5790-5798.

430 **Finn, R. N. and Cerdà, J.** (2011). Aquaporin evolution in fishes. *Front. Physiol.* 26, 2:44.
431 doi: 10.3389/fphys.2011.00044.

432 **Finn, R. N., Chauvigné, F., Hlidberg, J. B., Cutler, C. P. and Cerdà, J.** (2014). The
433 lineage-specific evolution of aquaporin gene clusters facilitated tetrapod terrestrial
434 adaptation. *PloS one*, 9, e113686.

435 **Giffard-Mena, I., Boulo, V., Abed, C., Cramb, G. and Charmantier, G.** (2011).
436 Expression and localization of Aquaporin 1a in the sea-bass (*Dicentrarchus labrax*)
437 during ontogeny. *Front. Physiol.* 2, 34.

438 **Ishibashi, K., Kondo, S., Hara, S. and Morishita, Y.** (2011). The evolutionary aspects of
439 aquaporin family. *Am. J. Physiol.* 300, R566-576.

440 **Kim, Y. K., Watanabe, S., Kaneko, T., Huh, M. D. and Park, S. l.** (2010). Expression of
441 aquaporins 3, 8 and 10 in the intestines of freshwater- and seawater-acclimated Japanese
442 eels *Anguilla japonica*. Fish. Sci. 76, 695-702.

443 **Kim, Y. K., Lee, S. Y., Kim, B. S., Kim, D. S. and Nam, Y. K.** (2014). Isolation and
444 mRNA expression analysis of aquaporin isoforms in marine medaka *Oryzias dancena*, a
445 euryhaline teleost. *Comp. Biochem. Physiol.* 171, A1-8.

King, L.S., Kozono, D., Agre, P., 2004. From structure to disease: the evolving tale of aquaporin biology. Nat. Rev. Mol. Cell Biol. 24, 224-234.

Kozono, D., Ding, X., Iwasaki, I., Meng, X., Kamagata, Y., Agre, P., Kitagawa, Y., 2003. Functional expression and characterization of an archaeal aquaporin AqpM from *Methanothermobacter marburgenesis*. J. Biol. Chem. 278, 10649-10656.

Küper, C., Beck, F. X., & Neuhofer, W. (2015). Generation of a conditional knockout allele for the NFAT5 gene in mice. *Frontiers in physiology*, *5*, 507.

Lee, S. Y., Nam, Y. K., & Kim, Y. K. (2017). Characterization and expression profiles of aquaporins (AQPs) 1a and 3a in mud loach *Misgurnus mizolepis* after experimental challenges. Fisheries and Aquatic Sciences, 20(1), 23.

Lieberman, B. A., Bona, B. J., Edwards, D. P., & Nordeen, S. K. (1993). The constitution of a progesterone response element. *Molecular Endocrinology*, *7*(4), 515-527.

Lehmann, G.L., Carreras, F.I., Soria, L.R., Gradilone, S.A., Marinelli, R.A., 2008. LPS induces the TNF-α-mediated downregulation of rat liver aquaporin-8: role in sepsis-associated cholestasis. Am. J. Physiol. Gastrointest. Liver Physiol. 294, G567-G575.

Lind, U., Järvå, M., Rosenblad, M. A., Pingitore, P., Karlsson, E., Wrange, A. L., Kamdal E, Sundell K, André C, Jonsson PR, Havenhand, J., Eriksson LA, Hedfalk K, Blomberg A.

463 (2017). Analysis of aquaporins from the euryhaline barnacle Balanus improvisus reveals
464 differential expression in response to changes in salinity. PloS one, 12(7), e0181192.

465 Lorgen, M., Jorgensen, E. H., Jordan, W. C., Martin, S. A., Hazlerigg, D. G. (2017). NFAT5
466 genes are part of the osmotic regulatory system in Atlantic salmon (Salmo salar). Marine
467 genomics, *31*, 25-31.

468 Madsen, S.S., Bujak, J., Tipsmark, C.K., 2014. Aquaporin expression in the Japanese medaka
469 (*Oryzias latipes*) in freshwater and seawater: challenging the paradigm of intestinal water
470 transport? J. Exp. Biol. 217, 3108-3121.

471 Madsen, S. S., Engelund, M. B., & Cutler, C. P. (2015). Water transport and functional
472 dynamics of aquaporins in osmoregulatory organs of fishes. *The Biological*
473 *Bulletin*, *229*(1), 70-92.

474 Meli, R., Pirozzi, C., & Pelagalli, A. (2018). New perspectives on the potential role of
475 aquaporins (AQPs) in the physiology of inflammation. *Frontiers in physiology*, *9*, 101.

476 Moon, C., King, L. S., & Agre, P. (1997). Aqp1 expression in erythroleukemia cells: genetic
477 regulation of glucocorticoid and chemical induction. *American Journal of Physiology-*
478 *Cell Physiology*, *273*(5), C1562-C1570.

479 Moshtaghi, A., Rahi, M. L., Nguyen, V. T., Mather, P. B., & Hurwood, D. A. (2016). A
480 transcriptomic scan for potential candidate genes involved in osmoregulation in an
481 obligate freshwater palaemonid prawn (*Macrobrachium australiense*). *PeerJ*, *4*, e2520.

482 Nam, Y. K., Choi, G. C., Kim, D. S. (2004). An efficient method for blocking the 1st mitotic
483 cleavage of fish zygote using combined thermal treatment, exemplified by mud loach
484 (*Misgurnus mizolepis*). Theriogenology, 61(5), 933-945.

485 Nam, Y.K., Cho, Y.S., Lee, S.Y., Kim, B.S., Kim, D.S., 2011. Molecular characterization of
486 hepcidin gene from mud loach (*Misgurnus mizolepis*; Cypriniformes). Fish Shellfish
487 Immunol. 31, 1251-1258.

488 Nielsen, S., Kwon, T.H., Christensen, B.M., Promeneur, D., Frøkiaer, J., Marples, D., 1999.
489 Physiology and pathophysiology of renal aquaporins. J. Am. Soc. Nephrol. 10, 647-663.

490 Overton PM, Meadows LA, Urban J, Russell S (2002) Evidence for differential and
491 redundant function of the Sox genes Dichaete and Sox N during CNS development in
492 Drosophila. Development 129: 4219–4228

493 Pontoglio M (2000). Hepatocyte nuclear factor 1, a transcription factor at the crossroads of
494 glucose homeostasis. J Am Soc Nephrol, 11(suppl 2), S140-S143.

495 Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative C(T)
496 method. Nat. Protoc. 3, 1101-1108.

497 Sun, Y., Zhang, Q., Qi, J., Chen, Y., Zhong, Q., Li, C., ... & Wang, Z. (2010). Identification
498 of differential genes in the ovary relative to the testis and their expression patterns in half-
499 smooth tongue sole (Cynoglossus semilaevis). *Journal of Genetics and Genomics*, *37*(2),
500 137-145.

501 Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. Clustal W: improving the sensitivity of
502 progressive multiple sequence alignment through sequence weighting, position specific
503 gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673-4680.

504 Tingaud-Sequeira, A., Chauvigné, F., Fabra, M., Lozano, J., Raldúa, D., & Cerdà, J. (2008).
505 Structural and functional divergence of two fish aquaporin-1 water channels following
506 teleost-specific gene duplication. BMC evolutionary biology, *8*(1), 259.

507 Tingaud-Sequeira,A.,Zapater,C., Chauvigné,F.,Otero,D.,and Cerdà,J.(2009).Adaptive plas-
508 ticity of killifish (*Fundulushet- eroclitus*) embryos: dehydration- stimulated development
509 and differential aquaporin-3expression. *Am. J. Physiol.Regul.Integr.Comp.Phys- iol.* 296,
510 R1041–R1052.

511 Tingaud-Sequeira, A., Calusinska, M., Finn, R.N., Chauvigné, F., Lozano, J., Cerdà, J., 2010.
512 The zebrafish genome encodes the largest vertebrate repertoire of functional aquaporins
513 with dual paralogy and substrate specificities similar to mammals. BMC Evol. Biol. 10, 38.

514 Tipsmark,C.K.,Sørensen,K.J.,and Madsen,S.S.(2010).Aquaporin expression dynamics in
515 osmoregulatory tissues of Atlantic salmon during smoltification and seawater acclimation.
516 *J. Exp.Biol*. 213, 368–379.

517 Wang, Y., Cui, H., Niu, F., Liu, S. L., Li, Y., Zhang, L. M., Du HB, Zhao ZG, Niu, C. Y.
518 (2018). Effect of resveratrol on blood rheological properties in LPS-challenged
519 rats. *Frontiers in physiology*, *9*, 1202.

520 Wang, W., Zheng, M.,2011.Role of cAMP-PKA/CREB pathway in regulation of AQP 5
521 production in rat nasal epithelium. Rhinology49,464–469.

522 Wei, L., Yang, C., Tao, W., Wang, D. 2016. Genome-wide identification and transcriptome-
523 based expression profiling of the sox gene family in the nile tilapia (*Oreochromis*
524 *niloticus*). *International journal of molecular sciences*, *17*(3), 270.

525 Weiss J, Meeks JJ, Hurley L, Raverot G, Frassetto A, et al. (2003) Sox3 is required for
526 gonadal function, but not sex determination, in males and females. Molecular and
527 Cellular Biology 23: 8084–8091

528 Wong, K.F., Luk, J.M., Cheng, R.H., Klickstein, L.B., Fan, S.T., 2007. Characterization of
529 two novel LPS-binding sites in leukocyte integrin βA domain. FASEB J. 21, 3231-3239.

530 Wong, M.K., Ozaki, H., Suzuki, Y., Iwasaki, W., Takei, Y. 2014. Discovery of osmotic
531 sensitive transcription factors in fish intestine via a transcriptomic approach. BMC
532 Genomics 15:1134. doi: 10.1186/1471-2164-15-1134.

533 Wu, X.M., Wang, H.Y., Li, G.F., Zang, B., Chen, W.M., 2009. Dobutamine enhances
534 alveolar fluid clearance in a rat model of acute lung injury. Lung 187, 225–231.

535 Yokoi, H., Kobayashi, T., Tanaka, M., Nagahama, Y., Wakamatsu, Y., Takeda, H., ... &
536 Ozato, K. 2002. Sox9 in a teleost fish, medaka (*Oryzias latipes*): evidence for diversified
537 function of Sox9 in gonad differentiation. *Molecular Reproduction and Development: Incorporating Gamete Research*, *63*(1), 5-16

539 Zapater, C., Chauvigné, F., Norberg, B., Finn, R. N., Cerdà, J. 2011. Dual
540 neofunctionalization of a rapidly evolving aquaporin-1 paralog resulted in constrained
541 and relaxed traits controlling channel function during meiosis resumption in teleosts.
542 *Molecular biology and evolution*, 28(11), 3151-3169.

543 Zapater, C., Chauvigné, F., Tingaud-Sequeira, A., Finn, R. N., Cerdà, J. 2013. Primary
544 oocyte transcriptional activation of aqp1ab by the nuclear progestin receptor determines
545 the pelagic egg phenotype of marine teleosts. *Developmental biology*, 377(2), 345-362.

546 Zardoya, R., 2005. Phylogeny and evolution of the major intrinsic protein family. Biol. Cell
547 97, 397-414.

548

549 **Figure legends**

550

551 Figure 1. Nucleotide alignment of AQP1b variant tv1, tv2, and tv3 cDNA sequences.

552 Nucleotide gaps are represented by dashes (-).

553

554 Figure 2. Multiple amino acid sequence alignments and transmembrane topology prediction

555 of AQP1b transcript variants. Asterisks and hyphens indicate identical residues and gaps

556 introduced for optimum alignments, respectively. Two NPA motifs are shown in bold light

557 blue. The locations of 6 putative membrane–spanning domains are shown by navy boxes

558 above the alignment. *A. japonica*: Japanese eel *Anguilla japonica*; *A. anguilla*: European eel

559 *Anguilla anguilla*; *M. mizolepis* tv1, tv2, tv3: mud loach *Misgurnus mizolepis*; *D. rerio*:

560 zebrafish *Danio rerio*; *S. salar*: Atlantic salmon *Salmo salar*; *S. aurata*: gilt-head sea bream

561 *Sparus aurata*; *S. senegalensis*: Senegalese sole *Solea senegalensis*; *I. punctatus*: channel

562 catfish *Ictalurus punctatus*; *H. fossilis*: stinging catfish *Heteropneustes fossilis*; *O. mordax*:

563 rainbow smelt *Osmerus mordax*; *A. schlegelii*: blackhead seabream *Acanthopagrus schlegelii*;

564 *D. labrax*: European bass *Dicentrarchus labrax*; *T. nigroviridis*: green pufferfish *Tetraodon*

565 *nigroviridis*; *F. heteroclitus*: mummichog *Fundulus heteroclitus*; *H. hippoglossus*: Atlantic

566 halibut *Hippoglossus hippoglossus*; *O. dancena*: marine medaka *Oryzias dancena*; *E. Lucius*:

567 northern pike *Esox Lucius*.

568 Figure 3. Gene organization and mRNA variants of the aquaporin 1ab gene in mud loaches.
569 mmAQP1b genes contain 5 exons. Each exon is represented by a different color and by
570 Roman numerals. The mRNA transcript length is listed on each exon.

571

572 Figure 4. Tissue distribution of mmAQP1b in adult tissues. Abbreviations: brain (Br), eye
573 (Ey), fin (Fi), gill (Gi), heart (He), intestine (In), kidney (Ki), liver (Li), muscle (Mu), spleen
574 (Sp), ovary (Ov), and testis (Te).

575

576 Figure 5. Expression of mmAQP1b mRNA during embryogenesis and larval development.
577 Variant transcripts in different tissues under abiotic stress. Data are represented as means ±
578 SDs. Letters indicate significant difference (one-way ANOVA).

579

580 Figure 6. Transcriptional responses of mmAQP1b to acute metal exposure in different tissues.
581 AQP1b expression in metal-exposed groups are expressed as fold changes relative to the non-
582 exposed control group. Data represent means ± SDs. Different letters indicate significant
583 differences, as analyzed by ANOVA followed by Duncan's multiple range tests.

584

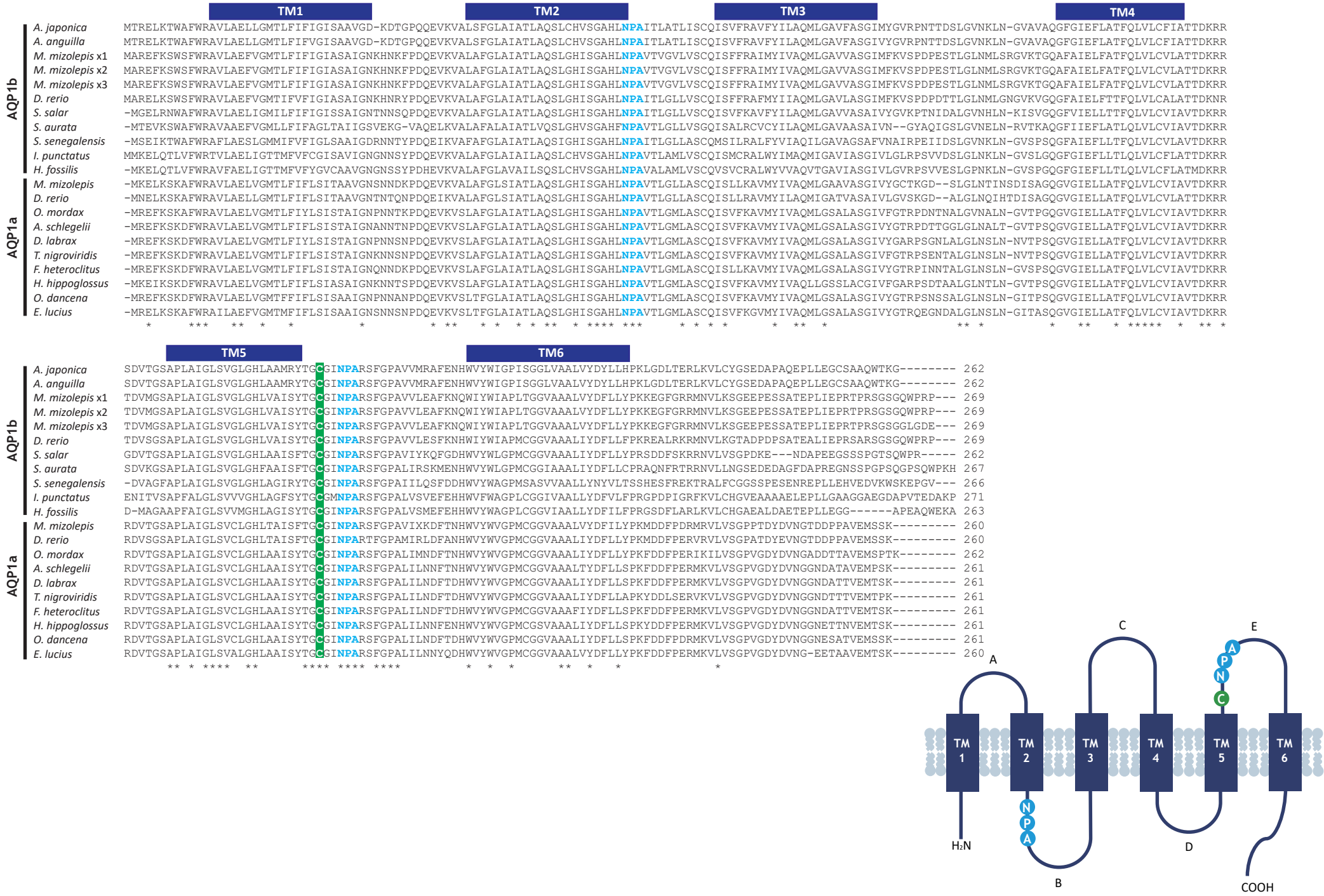
585 Figure 7. Differential modulation of mmAQP isoforms by immunostimulant exposure. Data

586 represent means ± SDs, with letters indicating significant differences, as analyzed by

587 ANOVA followed by Duncan's multiple range tests.

588


MM_AQP1b_x1 ATGGCACGGGAGTTTAAGAGTTGGTCTTTTTGGCGGGCAGTATTGGCCGAGTTTGTCGGGATGACCCTTTTTATTTTCATCGGTATAGCCTCCGCTATCGGTAACAAGCATAACAAATTTCCTGACCAA
MM_AQP1b_x2 ATGGCACGGGAGTTTAAGAGTTGGTCTTTTTGGCGGGCAGTATTGGCCGAGTTTGTCGGGATGACCCTTTTTATTTTCATCGGTATAGCCTCCGCTATCGGTAACAAGCATAACAAATTTCCTGACCAA
MM_AQP1b_x3 ATGGCACGGGAGTTTAAGAGTTGGTCTTTTTGGCGGGCAGTATTGGCCGAGTTTGTCGGGATGACCCTTTTTATTTTCATCGGTATAGCCTCCGCTATCGGTAACAAGCATAACAAATTTCCTGACCAA
MM_AQP1b_x1 GAGGTTAAAGTAGCTTTAGCTTTTGGTCTGGCCATTGCAACACTCGCTCAGAGTTTGGGGCATATCAGTGGAGCCCACCTGAACCCAGCGGTTACCGTAGGAGTGTTAGTTAGCTGTCAGATCAGCTTC
MM_AQP1b_x2 GAGGTTAAAGTAGCTTTAGCTTTTGGTCTGGCCATTGCAACACTCGCTCAGAGTTTGGGGCATATCAGTGGAGCCCACCTGAACCCAGCGGTTACCGTAGGAGTGTTAGTTAGCTGTCAGATCAGCTTC
MM_AQP1b_x3 GAGGTTAAAGTAGCTTTAGCTTTTGGTCTGGCCATTGCAACACTCGCTCAGAGTTTGGGGCATATCAGTGGAGCCCACCTGAACCCAGCGGTTACCGTAGGAGTGTTAGTTAGCTGTCAGATCAGCTTC
MM_AQP1b_x1 TTCAGGGCCATCATGTATATTGTTGCTCAGATGTTAGGGGCTGTTGTGGCAAGTGGCATCATGTTCAAAGTTAGCCCGGACCCTGAATCAACACTGGGGCTTAATATGCTGAGTCGCGGTGTAAAAACA
MM_AQP1b_x2 TTCAGGGCCATCATGTATATTGTTGCTCAGATGTTAGGGGCTGTTGTGGCAAGTGGCATCATGTTCAAAGTTAGCCCGGACCCTGAATCAACACTGGGGCTTAATATGCTGAGTCGCGGTGTAAAAACA
MM_AQP1b_x3 TTCAGGGCCATCATGTATATTGTTGCTCAGATGTTAGGGGCTGTTGTGGCAAGTGGCATCATGTTCAAAGTTAGCCCGGACCCTGAATCAACACTGGGGCTTAATATGCTGAGTCGCGGTGTAAAAACA
MM_AQP1b_x1 GGTCAGGCGTTTGCCATTGAGCTTTTTGCAACATTTCAGCTGGTCCTCTGTGTGTTGGCCACAACAGATAAGCGTCGAACCGATGTTATGGGCTCTGCACCTCTTGCCATCGGGCTATCGGTTGGTTTG
MM_AQP1b_x2 GGTCAGGCGTTTGCCATTGAGCTTTTTGCAACATTTCAGCTGGTCCTCTGTGTGTTGGCCACAACAGATAAGCGTCGAACCGATGTTATGGGCTCTGCACCTCTTGCCATCGGGCTATCGGTTGGTTTG
MM_AQP1b_x3 GGTCAGGCGTTTGCCATTGAGCTTTTTGCAACATTTCAGCTGGTCCTCTGTGTGTTGGCCACAACAGATAAGCGTCGAACCGATGTTATGGGCTCTGCACCTCTTGCCATCGGGCTATCGGTTGGTTTG
MM_AQP1ab_x1 GGACACCTGGTAGCTATCAGTTACACCGGGTGCGGTATCAATCCTGCTCGATCTTTCGGACCAGCTGTTGTTCTTGAAGCATTCAAAAACCAGTGGATATACTGGATTGCGCCCTTGACCGGAGGGGTG
MM_AQP1ab_x2 GGACACCTGGTAGCTATCAGTTACACCGGGTGCGGTATCAATCCTGCTCGATCTTTCGGACCAGCTGTTGTTCTTGAAGCATTCAAAAACCAGTGGATATACTGGATTGCGCCCTTGACCGGAGGGGTG
MM_AQP1ab_x3 GGACACCTGGTAGCTATCAGTTACACCGGGTGCGGTATCAATCCTGCTCGATCTTTCGGACCAGCTGTTGTTCTTGAAGCATTCAAAAACCAGTGGATATACTGGATTGCGCCCTTGACCGGAGGGGTG
MM_AQP1ab_x1 GCCGCTGCCCTTGTGTACGACTTCTTGCTGTACCCAAAGAAGGAAGGGTTTGGCAGGCGCATGAATGTTCTGAAAAGTGGCGAAGAACCGGAATCGTCTGCGACTGAACCCCTAATCGAACCAAGAACC
MM_AQP1ab_x2 GCCGCTGCCCTTGTGTACGACTTCTTGCTGTACCCAAAGAAGGAAGGGTTTGGCAGGCGCATGAATGTTCTGAAAAGTGGCGAAGAACCGGAATCGTCTGCGACTGAACCCCTAATCGAACCAAGAACC
MM_AQP1ab_x3 GCCGCTGCCCTTGTGTACGACTTCTTGCTGTACCCAAAGAAGGAAGGGTTTGGCAGGCGCATGAATGTTCTGAAAAGTGGCGAAGAACCGGAATCGTCTGCGACTGAACCCCTAATCGAACCAAGAACC
MM_AQP1ab_x1 CCCAGATCTGGTTCTGgtcagtggcccaggccatgagatgtgatgagtcttGAGGTCTAGGAGATGAGTGA
MM_AQP1ab_x2 CCCAGATCTGGTTCTGgtcagtggcccaggccatgagat----------GAGGTCTAGGAGATGAGTGA
MM_AQP1ab_x3 CCCAGATCTGGTTCTG----------GAGGTCTAGGAGATGAGTGA


TM1
TM2
TM3
TM4
AQP1b
A. japonica MTRELKTWAFWRAVLAELLGMTLFIFIGISAAVGD-KDTGPQQEVKVALSFGLAIATLAQSLCHVSGAHLNPAITLATLISCQISVFRAVFYILAQMLGAVFASGIMYGVRPNTTDSLGVNKLN-GVAVAQGFGIEFLATFQLVLCFIATTDKRR
A. anguilla MTRELKTWAFWRAVLAELLGMTLFIFVGISAAVGD-KDTGPQQEVKVALSFGLAIATLAQSLCHVSGAHLNPAITLATLISCQISVFRAVFYILAQMLGAVFASGIVYGVRPNTTDSLGVNKLN-GVAVAQGFGIEFLATFQLVLCFIATTDKRR
M. mizolepis x1 MAREFKSWSFWRAVLAEFVGMTLFIFIGIASAIGNKHNKFPDQEVKVALAFGLAIATLAQSLGHISGAHLNPAVTVGVLVSCQISFFRAIMYIVAQMLGAVVASGIMFKVSPDPESTLGLNMLSRGVKTGQAFAIELFATFQLVLCVLATTDKRR
M. mizolepis x2 MAREFKSWSFWRAVLAEFVGMTLFIFIGIASAIGNKHNKFPDQEVKVALAFGLAIATLAQSLGHISGAHLNPAVTVGVLVSCQISFFRAIMYIVAQMLGAVVASGIMFKVSPDPESTLGLNMLSRGVKTGQAFAIELFATFQLVLCVLATTDKRR
M. mizolepis x3 MAREFKSWSFWRAVLAEFVGMTLFIFIGIASAIGNKHNKFPDQEVKVALAFGLAIATLAQSLGHISGAHLNPAVTVGVLVSCQISFFRAIMYIVAQMLGAVVASGIMFKVSPDPESTLGLNMLSRGVKTGQAFAIELFATFQLVLCVLATTDKRR
D. rerio MARELKSWSFWRAVLAEFVGMTIFVFIGIASAIGNKHNRYPDQEVKVALAFGLAIATLAQSLGHISGAHLNPAITLGLLVSCQISFFRAFMYIIAQMLGAVLASGIMFKVSPDPDTTLGLNMLGNGVKVGQGFAIELFTTFQLVLCALATTDKNR
S. salar -MGELRNWAFWRAVLAELIGMILFIFIGISSAIGNTNNSQPDQEVKVALAFGLAIATLAQSLGHISGAHLNPAITLGLLVSCQISVFKAVFYILAQMLGAVVASAIVYGVKPTNIDALGVNHLN-KISVGQGFVIELLTTFQLVLCVIAVTDKRR
S. aurata -MTEVKSWAFWRAVAAEFVGMLLFIFAGLTAIIGSVEKG-VAQELKVALAFALAIATLVQSLGHVSGAHFNPAVTLGLLVSGQISALRCVCYILAQMLGAVAASAIVN--GYAQIGSLGVNELN-RVTKAQGFIIEFLATLQLVLCVIAVTDKRR
S. senegalensis -MSEIKTWAFWRAFLAESLGMMIFVFIGLSAAIGDRNNTYPDQEIKVAFAFGLAIATLAQSIGHISGAHLNPAITLGLLASCQMSILRALFYVIAQILGAVAGSAFVNAIRPEIIDSLGVNKLN-GVSPSQGFAIEFLLTFQLVLCVLAVTDKRR
I. punctatus MMKELQTLVFWRTVLAELIGTTMFVFCGISAVIGNGNNSYPDQEVKVALAFGLAIAILAQSLCHVSGAHLNPAVTLAMLVSCQISMCRALWYIMAQMIGAVIASGIVLGLRPSVVDSLGLNKLN-GVSLGQGFGIEFLLTLQLVLCFLATTDKRR
H. fossilis -MKELQTLVFWRAVFAELIGTTMFVFYGVCAAVGNGNSSYPDHEVKVALAFGLAVAILSQSLCHVSGAHLNPAVALAMLVSCQVSVCRALWYVVAQVTGAVIASGIVLGVRPSVVESLGPNKLN-GVSPGQGFGIEFLLTLQLVLCFLATMDKRR
AQP1a
M. mizolepis -MKELKSKAFWRAVLAELVGMTLFIFLSITAAVGNSNNDKPDQEVKVALAFGLSIATLAQSLGHISGAHLNPAVTLGLLASCQISLLKAVMYIVAQMLGAAVASGIVYGCTKGD--SLGLNTINSDISAGQGVGIELLATFQLVLCVIAVTDKRR
D. rerio -MNELKSKAFWRAVLAELLGMTLFIFLSITAAVGNTNTQNPDQEIKVALAFGLSIATLAQSLGHISGAHLNPAVTLGLLASCQISLLRAVMYILAQMIGATVASAIVLGVSKGD--ALGLNQIHTDISAGQGVGIELLATFQLVLCVLATTDKRR
O. mordax -MREFKSKAFWRAVLAELVGMTLFIYLSISTAIGNPNNTKPDQEVKVSLAFGLAIATLAQSLGHISGAHLNPAVTLGMLASCQISVFKAVMYIVAQMLGSALASGIVFGTRPDNTNALGVNALN-GVTPGQGVGIELLATFQLVLCVIAVTDKRR
A. schlegelii -MREFKSKDFWRAVLAELVGMTLFIFLSISTAIGNANNTNPDQEVKVSLAFGLAIATLAQSLGHISGAHLNPAVTLGMLASCQISVFKAVMYIVAQMLGSALASGIVYGTRPDTTGGLGLNALT-GVTPSQGVGIELLATFQLVLCVIAVTDKRR
D. labrax -MREFKSKDFWRAVLAELVGMTLFIYLSISTAIGNPNNSNPDQEVKVSLAFGLAIATLAQSLGHISGAHLNPAVTLGMLASCQISVFKAVMYIVAQMLGSALASGIVYGARPSGNLALGLNSLN-NVTPSQGVGIELLATFQLVLCVIAVTDKRR
T. nigroviridis -MREFKSKDFWRAVLAELVGMTLFIFLSISTAIGNKNNSNPDQEVKVSLAFGLAIATLAQSLGHISGAHLNPAVTLGMLASCQISVFKAVMYIVAQMLGSALASGIVFGTRPSENTALGLNSLN-NVTPSQGVGIELLATFQLVLCVIAVTDKRR
F. heteroclitus -MREFKSKDFWRAVLAELVGMTLFIFLSISTAIGNQNNDKPDQEVKVSLAFGLAIATLAQSLGHISGAHLNPAVTLGMLASCQISLLKAVMYIVAQMLGSALASGIVYGTRPINNTALGLNSLN-GVSPSQGVGIELLATFQLVLCVIAVTDKRR
H. hippoglossus -MKEIKSKDFWRAVLAELVGMTLFIFLSIASAIGNKNNTSPDQEVKVSLAFGLAIATLAQSLGHISGAHLNPAVTLGMLASCQISVFKAVMYIVAQLLGSSLACGIVFGARPSDTAALGLNTLN-GVTPSQGVGIELLATFQLVLCVIAVTDKRR
O. dancena -MREFKSKDFWRAVLAELVGMTFFIFLSISAAIGNPNNANPDQEVKVSLTFGLAIATLAQSLGHISGAHLNPAVTLGMLASCQISVFKAVMYIVAQMLGSALASGIVYGTRPSNSSALGLNSLN-GITPSQGVGIELLATFQLVLCVIAVTDKRR
E. lucius -MRELKSKAFWRAILAEFVGMTMFIFLSISAAIGNSNNSNPDQEVKVSLTFGLAIATLAQSLGHISGAHLNPAVTLGMLASCQISVFKGVMYIVAQMLGSALASGIVYGTRQEGNDALGLNSLN-GITASQGVGIELLATFQLVLCVIAVTDKRR
TM5
TM6
AQP1b
A. japonica SDVTGSAPLAIGLSVGLGHLAAMRYTGCGINPARSFGPAVVMRAFENHWVYWIGPISGGLVAALVYDYLLHPKLGDLTERLKVLCYGSEDAPAQEPLLEGCSAAQWTKG-------- 262
A. anguilla SDVTGSAPLAIGLSVGLGHLAAMRYTGCGINPARSFGPAVVMRAFENHWVYWIGPISGGLVAALVYDYLLHPKLGDLTERLKVLCYGSEDAPAQEPLLEGCSAAQWTKG-------- 262
M. mizolepis x1 TDVMGSAPLAIGLSVGLGHLVAISYTGCGINPARSFGPAVVLEAFKNQWIYWIAPLTGGVAAALVYDFLLYPKKEGFGRRMNVLKSGEEPESSATEPLIEPRTPRSGSGQWPRP--- 269
M. mizolepis x2 TDVMGSAPLAIGLSVGLGHLVAISYTGCGINPARSFGPAVVLEAFKNQWIYWIAPLTGGVAAALVYDFLLYPKKEGFGRRMNVLKSGEEPESSATEPLIEPRTPRSGSGQWPRP--- 269
M. mizolepis x3 TDVMGSAPLAIGLSVGLGHLVAISYTGCGINPARSFGPAVVLEAFKNQWIYWIAPLTGGVAAALVYDFLLYPKKEGFGRRMNVLKSGEEPESSATEPLIEPRTPRSGSGGLGDE--- 269
D. rerio TDVSGSAPLAIGLSVGLGHLVAISYTGCGINPARSFGPAVVLESFKNHWIYWIAPMCGGVAAALIYDFLLFPKREALRKRMNVLKGTADPDPSATEALIEPRSARSGSGQWPRP--- 269
S. salar GDVTGSAPLAIGLSVGLGHLAAISFTGCGINPARSFGPAVIYKQFGDHWVYWLGPMCGGVAAALIYDFLLYPRSDDFSKRRNVLVSGPDKE---NDAPEEGSSSPGTSQWPR----- 262
S. aurata SDVKGSAPLAIGLSVGLGHFAAISFTGCGINPARSFGPALIRSKMENHWVYWLGPMCGGIAAALIYDFLLCPRAQNFRTRRNVLLNGSEDEDAGFDAPREGNSSPGPSQGPSQWPKH 267
S. senegalensis -DVAGFAPLAIGLSVGLGHLAGIRYTGCGINPARSFGPAIILQSFDDHWVYWAGPMSASVVAALLYNYVLTSSHESFREKTRALFCGGSSPESENREPLLEHVEDVKWSKEPGV--- 266
I. punctatus ENITVSAPFALGLSVVVGHLAGFSYTGCGMNPARSFGPALVSVEFEHHWVFWAGPLCGGIVAALLYDFVLFPRGPDPIGRFKVLCHGVEAAAAELEPLLGAAGGAEGDAPVTEDAKP 271
H. fossilis D-MAGAAPFAIGLSVVMGHLAGISYTGCGINPARSFGPALVSMEFEHHWVYWAGPLCGGVIAALLYDFILFPRGSDFLARLKVLCHGAEALDAETEPLLEGG------APEAQWEKA 263
AQP1a
M. mizolepis RDVTGSAPLAIGLSVCLGHLTAISFTGCGINPARSFGPAVIXKDFTNHWVYWVGPMCGGVAAALVYDFILYPKMDDFPDRMRVLVSGPPTDYDVNGTDDPPAVEMSSK--------- 260
D. rerio RDVSGSAPLAIGLSVCLGHLTAISFTGCGINPARTFGPAMIRLDFANHWVYWVGPMCGGVAAALIYDFLLYPKMDDFPERVRVLVSGPATDYEVNGTDDPPAVEMSSK--------- 260
O. mordax RDVTGSAPLAIGLSVCLGHLAAISYTGCGINPARSFGPALIMNDFTNHWVYWVGPMCGGVAAALIYDFLLYPKFDDFPERIKILVSGPVGDYDVNGADDTTAVEMSPTK-------- 262
A. schlegelii RDVTGSAPLAIGLSVCLGHLAAISYTGCGINPARSFGPALILNNFTNHWVYWVGPMCGGVAAALTYDFLLSPKFDDFPERMKVLVSGPVGDYDVNGGNDATAVEMPSK--------- 261
D. labrax RDVTGSAPLAIGLSVCLGHLAAISYTGCGINPARSFGPALILNDFTDHWVYWVGPMCGGVAAALIYDFLLSPKFDDFPERMKVLVSGPVGDYDVNGGNDATTVEMTSK--------- 261
T. nigroviridis RDVTGSAPLAIGLSVCLGHLAAISYTGCGINPARSFGPALILNDFTDHWVYWVGPMCGGVAAALIYDFLLAPKYDDLSERVKVLVSGPVGDYDVNGGNDTTTVEMTPK--------- 261
F. heteroclitus RDVTGSAPLAIGLSVCLGHLAAISYTGCGINPARSFGPALILNDFTDHWVYWVGPMCGGVAAAFIYDFLLSPKFDDFPERMKVLVSGPVGDYDVNGGNDATTVEMTSK--------- 261
H. hippoglossus RDVTGSAPLAIGLSVCLGHLAAISYTGCGINPARSFGPALILNNFENHWVYWVGPMCGSVAAALIYDFLLSPKYDDFPERMKVLVSGPVGDYDVNGGNETTNVEMTSK--------- 261
O. dancena RDVTGSAPLAIGLSVCLGHLAAISYTGCGINPARSFGPALILNDFTDHWVYWVGPMCGGVAAALIYDFLLSPKFDDFPDRMKVLVSGPVGDYDVNGGNESATVEMSSK--------- 261
E. lucius RDVTGSAPLAIGLSVALGHLAAISYTGCGINPARSFGPALILNNYQDHWVYWVGPMCGGVAAALVYDFLLYPKFDDFPERMKVLVSGPVGDYDVNG-EETAAVEMTSK--------- 260
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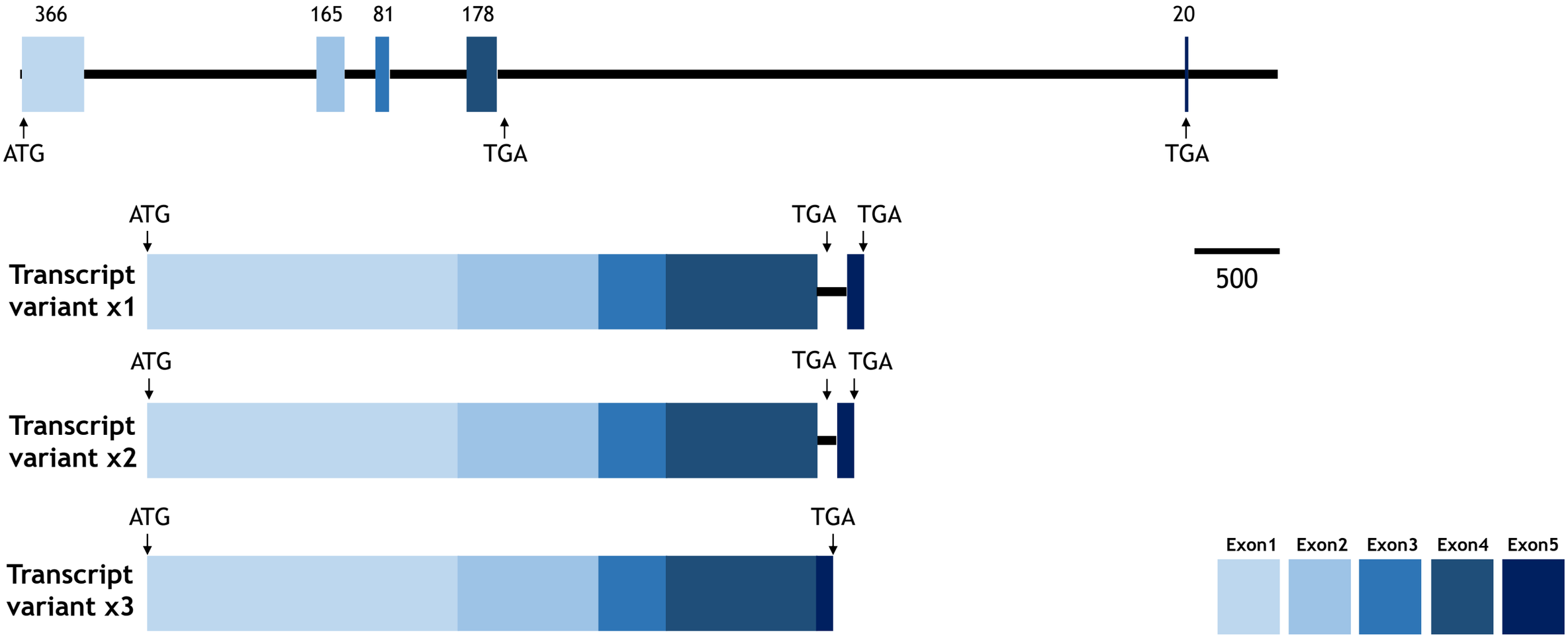
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