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

4 **Running title:** Characterization of mud loach AQP1b

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20

21 **Abstract**

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

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

35 **Summary statement:**

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

39

40 **Introduction**

41 Teleost species have the remarkable ability to withstand acute or long-term fluctuations in
42 environmental salinity. Fish cells monitor the osmolality difference between the intracellular
43 and extracellular spaces, and transport water to recover their volume following cell swelling
44 or shrinkage. Salinity stress is linked to a wide range of biological processes *e.g.* metabolism,
45 mortality, growth, and even immune responses (Baltzegar et al., 2014; Moshtaghi et al.,
46 2016). After being subjected to an imbalance between environments, proteins trigger various
47 complex responses such as changes in structure or function, a consequence of which is
48 altered enzyme activity (Fiol and Kültz, 2007).

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

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

74 [al., 2012](#)). Given these advantages, mud loaches could be particularly relevant for studying
75 the involvement of aquaporins in physiological processes.

76 Most studies investigating piscine aquaporin genes focus on the effects of salinity-induced
77 adaptation in piscine AQP genes in response to biological challenges. However, investigating
78 the interaction among genes using molecular genetic approaches has elucidated the molecular
79 mechanisms underlying biological function and regulatory gene expression. Identifying
80 interaction among diverse genes and autonomous adaptation effects are essential for
81 systematically unraveling the cellular or molecular mechanisms of AQP action. Recent
82 reports indicate that AQPs could be involved in inflammasome activation-induced cell
83 volume regulation (Compan et al., 2011; Meli et al., 2018). Previously, we identified a novel
84 AQP1a in mud loaches (mmAQP1a) which is differentially modulated *in vivo* ([Lee et al.,](#)
85 [2017](#)). In fact, the existence of AQP1a and its duplicate in teleost species is well established,
86 showing specialized expression ([Zapater et al., 2011](#)). We found a AQP1a paralog from our
87 next-generation sequencing database for the mud loach (unpublished data). The genetic
88 determinants for the stenohaline freshwater species mud loach AQP1a paralog, called AQP1b
89 have not been extensively explored. In present study, we identified the AQP1a paralog in
90 mud loaches. The AQP1a paralog, AQP1b is a teleost-specific water-selective channel that
91 mediates oocyte hydration, which is based on the coordinated action between osmolyte flux
92 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 Vectorsite 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 MatInspector 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 $25 \pm 0.5^{\circ}\text{C}$ 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 started 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 $24 \pm$
 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 $25 \pm 0.5^{\circ}\text{C}$. Each group was acclimated to the

tanks for one week. The dose for each metal was 5 μ M (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 μ g 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 μ g 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 μ L 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 Δ Ct 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 $\pm$ 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 began 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

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

AQP expression after immune challenge

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

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 mummichog (*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

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

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

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

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

Competing interests

The authors declare that they have no conflict of interest.

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Authors' contributions

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

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Figure legends

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

Nucleotide gaps are represented by dashes (-).

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

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

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

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

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

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

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

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

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

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

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

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

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

northern pike *Esox Lucius*.

Figure 3. Gene organization and mRNA variants of the aquaporin 1ab gene in mud loaches.

mmAQP1b genes contain 5 exons. Each exon is represented by a different color and by

Roman numerals. The mRNA transcript length is listed on each exon.

Figure 4. Tissue distribution of mmAQP1b in adult tissues. Abbreviations: brain (Br), eye

(Ey), fin (Fi), gill (Gi), heart (He), intestine (In), kidney (Ki), liver (Li), muscle (Mu), spleen

(Sp), ovary (Ov), and testis (Te).

Figure 5. Expression of mmAQP1b mRNA during embryogenesis and larval development.

Variant transcripts in different tissues under abiotic stress. Data are represented as means $\pm$

SDs. Letters indicate significant difference (one-way ANOVA).

Figure 6. Transcriptional responses of mmAQP1b to acute metal exposure in different tissues.

AQP1b expression in metal-exposed groups are expressed as fold changes relative to the non-

exposed control group. Data represent means $\pm$ SDs. Different letters indicate significant

differences, as analyzed by ANOVA followed by Duncan's multiple range tests.

585 Figure 7. Differential modulation of mmAQP isoforms by immunostimulant exposure. Data
586 represent means $\pm$ SDs, with letters indicating significant differences, as analyzed by
587 ANOVA followed by Duncan's multiple range tests.

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MM_AQP1b_x1 ATGGCACGGGAGTTTAAGAGTTGGTCTTTTTGGCGGGCAGTATTGGCCGAGTTTGTGCGGATGACCCTTTTTATTTTCATCGGTATAGCCTCCGCTATCGGTAACAAGCATAACAAATTTCTGACCAA
MM_AQP1b_x2 ATGGCACGGGAGTTTAAGAGTTGGTCTTTTTGGCGGGCAGTATTGGCCGAGTTTGTGCGGATGACCCTTTTTATTTTCATCGGTATAGCCTCCGCTATCGGTAACAAGCATAACAAATTTCTGACCAA
MM_AQP1b_x3 ATGGCACGGGAGTTTAAGAGTTGGTCTTTTTGGCGGGCAGTATTGGCCGAGTTTGTGCGGATGACCCTTTTTATTTTCATCGGTATAGCCTCCGCTATCGGTAACAAGCATAACAAATTTCTGACCAA
*****
MM_AQP1b_x1 GAGGTAAAGTAGCTTTAGCTTTTGGTCTGGCCATTGCAACACTCGCTCAGAGTTTGGGGCATATCAGTGGAGCCCACCTGAACCCAGCGGTTACCGTAGGAGTGTTAGTTAGCTGTCAGATCAGCTTC
MM_AQP1b_x2 GAGGTAAAGTAGCTTTAGCTTTTGGTCTGGCCATTGCAACACTCGCTCAGAGTTTGGGGCATATCAGTGGAGCCCACCTGAACCCAGCGGTTACCGTAGGAGTGTTAGTTAGCTGTCAGATCAGCTTC
MM_AQP1b_x3 GAGGTAAAGTAGCTTTAGCTTTTGGTCTGGCCATTGCAACACTCGCTCAGAGTTTGGGGCATATCAGTGGAGCCCACCTGAACCCAGCGGTTACCGTAGGAGTGTTAGTTAGCTGTCAGATCAGCTTC
*****
MM_AQP1b_x1 TTCAGGGCCATCATGTATATTGTTGCTCAGATGTTAGGGGCTGTTGTGGCAAGTGGCATCATGTTCAAAGTTAGCCCGGACCCTGAATCAACACTGGGGCTTAATATGCTGAGTCGCGGTGTAAAAACA
MM_AQP1b_x2 TTCAGGGCCATCATGTATATTGTTGCTCAGATGTTAGGGGCTGTTGTGGCAAGTGGCATCATGTTCAAAGTTAGCCCGGACCCTGAATCAACACTGGGGCTTAATATGCTGAGTCGCGGTGTAAAAACA
MM_AQP1b_x3 TTCAGGGCCATCATGTATATTGTTGCTCAGATGTTAGGGGCTGTTGTGGCAAGTGGCATCATGTTCAAAGTTAGCCCGGACCCTGAATCAACACTGGGGCTTAATATGCTGAGTCGCGGTGTAAAAACA
*****
MM_AQP1b_x1 GGTGAGGCGTTTGCCATTGAGCTTTTTGCAACATTTAGCTGGTCCTCTGTGTGTTGGCCACAACAGATAAGCGTCGAACCGATGTTATGGGCTCTGCACCTCTTGCCATCGGGCTATCGGTTGGTTTG
MM_AQP1b_x2 GGTGAGGCGTTTGCCATTGAGCTTTTTGCAACATTTAGCTGGTCCTCTGTGTGTTGGCCACAACAGATAAGCGTCGAACCGATGTTATGGGCTCTGCACCTCTTGCCATCGGGCTATCGGTTGGTTTG
MM_AQP1b_x3 GGTGAGGCGTTTGCCATTGAGCTTTTTGCAACATTTAGCTGGTCCTCTGTGTGTTGGCCACAACAGATAAGCGTCGAACCGATGTTATGGGCTCTGCACCTCTTGCCATCGGGCTATCGGTTGGTTTG
*****
MM_AQP1ab_x1 GGACACCTGGTAGCTATCAGTTACACCGGGTGCGGTATCAATCCTGCTCGATCTTTCGGACCAGCTGTTGTTCTTGAAGCATTCAAAAACCAAGTGGATATACTGGATTGCGCCCTTGACCGGAGGGGTG
MM_AQP1ab_x2 GGACACCTGGTAGCTATCAGTTACACCGGGTGCGGTATCAATCCTGCTCGATCTTTCGGACCAGCTGTTGTTCTTGAAGCATTCAAAAACCAAGTGGATATACTGGATTGCGCCCTTGACCGGAGGGGTG
MM_AQP1ab_x3 GGACACCTGGTAGCTATCAGTTACACCGGGTGCGGTATCAATCCTGCTCGATCTTTCGGACCAGCTGTTGTTCTTGAAGCATTCAAAAACCAAGTGGATATACTGGATTGCGCCCTTGACCGGAGGGGTG
*****
MM_AQP1ab_x1 GCCGCTGCCCTTGTGTACGACTTCTTGCTGTACCCAAAGAAGGAAGGGTTTGGCAGGCGCATGAATGTTCTGAAAAGTGGCGAAGAACCGGAATCGTCTGCGACTGAACCCCTAATCGAACCAAGAACCC
MM_AQP1ab_x2 GCCGCTGCCCTTGTGTACGACTTCTTGCTGTACCCAAAGAAGGAAGGGTTTGGCAGGCGCATGAATGTTCTGAAAAGTGGCGAAGAACCGGAATCGTCTGCGACTGAACCCCTAATCGAACCAAGAACCC
MM_AQP1ab_x3 GCCGCTGCCCTTGTGTACGACTTCTTGCTGTACCCAAAGAAGGAAGGGTTTGGCAGGCGCATGAATGTTCTGAAAAGTGGCGAAGAACCGGAATCGTCTGCGACTGAACCCCTAATCGAACCAAGAACCC
*****
MM_AQP1ab_x1 CCCAGATCTGTTCTGgtcagtggtccaggccatgagatgtgatgagtcttGAGGTCTAGGAGATGAGTGA
MM_AQP1ab_x2 CCCAGATCTGTTCTGgtcagtggtccaggccatgagat-----GAGGTCTAGGAGATGAGTGA
MM_AQP1ab_x3 CCCAGATCTGTTCTG-----GAGGTCTAGGAGATGAGTGA
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Lee et al., Fig. 2