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Aquaporin expression and cholesterol content in eel swimbladder tissue

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Abstract

Leakiness of the swimbladder wall of teleost fishes must be prevented to avoid diffusional loss of gases out of the swimbladder. Guanine incrustation as well as high concentrations of cholesterol in swimbladder membranes in midwater and deep-sea fish has been connected to a reduced gas permeability of the swimbladder wall. On the contrary, the swimbladder is filled by diffusion of gases, mainly oxygen and CO_2, from the blood and the gas gland cells into the swimbladder lumen. In swimbladder tissue of the zebrafish and the Japanese eel, aquaporin mRNA has been detected, and the aquaporin protein has been considered important for the diffusion of water, which may accidentally be gulped by physostome fish when taking an air breath. In the present study, the expression of two aquaporin 1 genes (Aqp1aa and Aqp1ab) in the swimbladder tissue of the European eel, a functional physoclist fish, was assessed using immunohistochemistry, and the expression of both genes was detected in endothelial cells of swimbladder capillaries as well as in basolateral membranes of gas gland cells. In addition, Aqp1ab was present in apical membranes of swimbladder gas gland cells. The authors also found high concentrations of cholesterol in these membranes, which were several fold higher than in muscle tissue membranes. In yellow eels the cholesterol concentration exceeded the concentration detected in silver eel swimbladder membranes. The authors suggest that aquaporin 1 in swimbladder gas gland cells and endothelial cells facilitates CO_2 diffusion into the blood, enhancing the switch-on of the Root effect, which is essential for the secretion of oxygen into the swimbladder. It may also facilitate CO_2 diffusion into the swimbladder lumen along the partial gradient established by CO_2 production in gas gland cells. Cholesterol has been shown to reduce the gas permeability of membranes and thus could contribute to the gas tightness of swimbladder membranes, which is essential to avoid diffusional loss of gas out of the swimbladder.

KEYWORDS
carbon dioxide, cholesterol, CO_2 diffusion, European eel, gas gland cells, swimbladder

1 | INTRODUCTION

It was widely accepted for a long time that cell membranes are highly permeable to gases by diffusion, especially to lipid-soluble gases like CO_2 and NH_3 (Itel *et al*., 2012), and the role of channels or pores in gas transport remained neglected. Aquaporins are a family of low-molecular-weight (25–34 kDa), hydrophobic, integral membrane proteins that facilitate rapid and highly selective water movements across the membrane (Cerdà *et al*., 2017; Cerda & Finn, 2010; Finn & Cerdà, 2015). Nonetheless, the expression of aquaporin-1 (Aqp-1) in *Xenopus* oocytes significantly increased CO_2 permeability (Cooper & Boron, 1998; Nakhoul *et al*., 1998), and it has repeatedly been confirmed that some aquaporin members are permeable to CO_2 (Endeward *et al*., 2017; Itel *et al*., 2012). Analysis of the transport characteristics and functional properties of aquaporins resulted in a classification of aquaporins into four subfamilies, namely classical aquaporins, aquaporin-8-type aquaammoniaporins, unorthodox aquaporins and aquaglyceroporins, which have been shown to be permeable to glycerol and urea (Agre, 2004; Finn *et al*., 2014; Finn & Cerdà, 2015).

The importance of aquaporins in mammalian and plant physiology and pathophysiology is well established (Finn *et al*., 2014; Maurel *et al*., 2008; Rojek *et al*., 2008; Verkman, 2009). In aquatic animals, such as fish, the maintenance of body fluid homeostasis is important because they face the problem of direct interaction with their aqueous environment, as the osmolarity of fresh water can differ manifold from their internal body fluids. Fish are known to osmoregulate their body fluids by active ion uptake in fresh water and by ion secretion in saltwater, whereby aquaporins significantly contribute to water homeostasis (Aoki *et al*., 2003; Kwong *et al*., 2013; Madsen *et al*., 2015; Tipsmark *et al*., 2010; Zapater *et al*., 2013). In fish, analysis of aquaporin expression mostly focused on gill, kidney, intestine and gametes (Kwong *et al*., 2013; Madsen *et al*., 2014; Martinez *et al*., 2005a, 2005b; Tipsmark *et al*., 2010). Due to tandem and genomic duplication events in the evolution of fish, they generally possess a higher total number of aquaporin orthologs than mammals. Analysing the aquaporin gene superfamily thus revealed the presence of a larger number of aquaporins in fish than in mammals (Breves, 2020; Finn & Cerdà, 2011, 2015), with teleost species often retaining two or sometimes even three orthologs of human aquaporins (Finn & Cerdà, 2011; Tingaud-Sequeira *et al*., 2010).

Meanwhile, the bifunctional role as water and gas channel has repeatedly been confirmed for fish aquaporin (Chen *et al*., 2010; Perry *et al*., 2010; Talbot *et al*., 2015). Talbot *et al*. showed that a functional knockdown of the aquaporin-1 water channel (Aqp1a1) significantly reduced CO_2 and ammonia excretion in 4 dpf larvae of the zebrafish and that the Aqp1a1 protein could be localized to the yolk-sac epithelium, suggesting that this structure is the main site for CO_2 and ammonia excretion in the larvae (Talbot *et al*., 2015). The expression of Aqp1a in the swimbladder was reported for both larval and adult zebrafish, established by *in situ* hybridization, real-time PCR (RT-PCR) and Western blot analysis, respectively (Chen *et al*., 2010). Zebrafish is a physostome species, and it was speculated that the protein might contribute to the absorption of water, which is accidentally ingested when swallowing air (Chen *et al*., 2010).

Assessing the tissue-specific expression of Aqp1 in the Japanese eel using Northern Blot analysis revealed the expression of *aqp1* mRNA in the swimbladder (Aoki *et al*., 2003). The eel is a physostome species as well with a persistent ductus pneumaticus, which functions as the resorbing part of the swimbladder, but the opening to the gut is functionally closed so that water cannot be accidentally ingested. Furthermore, the swimbladder cannot be filled by gulping air on the water surface (Fänge, 1983; Pelster, 1997, 2013). Given this, the eel actually is a functional physoclist, and gas can diffuse into the swimbladder only *via* the activity of gas gland cells. These specialized cells metabolize glucose to produce lactic acid *via* glycolysis and CO_2 mainly in the pentose phosphate pathway (Pelster, 1995a; Pelster *et al*., 1994; Pelster & Scheid, 1993; Walsh & Milligan, 1993). Due to the production of CO_2 within the gas gland cells, the highest CO_2 partial pressure occurs there, and CO_2 is released into the swimbladder, contributing significantly to newly secreted gas, and also into the blood supporting the acidification of the blood (Kobayashi *et al*., 1990). This acidification of the blood is required for the reduction in haemoglobin oxygen-carrying capacity (Root effect), resulting in increasing PO_2 values necessary for the secretion of oxygen (Pelster & Randall, 1998; Pelster & Weber, 1991; Root, 1931). Therefore, CO_2 permeability of gas gland cell membranes and of endothelial cells in the swimbladder is essential for swimbladder function. On the contrary, overall gas permeability of the swimbladder should be low to prevent diffusional loss of gas to the surrounding tissues, which is particularly important during the eels' typical spawning migration to the Sargasso Sea, which might take several months or perhaps more than a year (Righton *et al*., 2016). Eels perform daily vertical migrations, swimming at depths of 100–300 m during night-time and at 600–800 m during daytime (Aarestrup *et al*., 2009; Righton *et al*., 2016; Schabetsberger *et al*., 2016), resulting in hydrostatic pressures ranging from 11 to 80 atm (Righton *et al*., 2012). Because the gas pressure in water is not dependent on the hydrostatic pressure, this results in an extraordinarily high partial pressure gradient between the flexible-walled swimbladder lumen and the surrounding tissues. Indeed, an elevated gas tightness of swimbladder tissue of silver eels, *i.e*., the migrating stage, as compared to yellow eels, the juvenile freshwater stage, was reported (Kleckner, 1980).

The cholesterol content of a membrane has been shown to contribute to its gas tightness (Itel *et al*., 2012). A high cholesterol content in the cell membrane ensures mechanical stability and decreases gas permeability (Endeward *et al*., 2017; Itel *et al*., 2012; Tsiavaliaris *et al*., 2015). Cholesterol may also be an important regulator of the CO_2 permeability of biological membranes, as a high cholesterol content reduces the CO_2 permeability of membranes (Arias-Hidalgo *et al*., 2018; Itel *et al*., 2012; Kai & Kaldenhoff, 2014). In the 1970s, a high cholesterol content was detected in swimbladder tissues of several deep-sea fishes (Phleger, 1971; Phleger *et al*., 1977; Phleger & Benson, 1971). The lipid content of these swimbladders included up

to 49% (dry weight basis) of cholesterol, which was suggested to be related to buoyancy control in some way (Phleger & Benson, 1971) or to facilitate the diffusion of oxygen into the swimbladder (Phleger *et al*., 1977).

Recently, the authors detected mRNAs of several aquaporins, including *aqp1*, in the swimbladder tissue of the European eel in an Illumina RNASeq analysis (Pelster *et al*., 2016a, 2016b; Schneebauer *et al*., 2017, 2020), but the presence and localization of aquaporin 1 protein is still unknown. The authors of this study therefore hypothesized that aquaporins would assure a certain gas permeability for CO_2, whereas a high cholesterol content in the cell membrane would contribute to a decrease in gas permeability of swimbladder membranes. By combining a high cholesterol content of membranes with the presence of gas-permeable aquaporins, CO_2 permeability would be established in gas gland cells supporting acid secretion into the swimbladder capillaries, required for the elevation of oxygen partial pressure in blood and oxygen secretion.

2 | MATERIALS AND METHODS

2.1 | Animals

European eels (*Anguilla anguilla*) were caught by local fishermen either in Lake Constance, Bregenz, Austria, or in the Elbe, Oder or Rhine Rivers in Germany. The European eel is known as a panmictic species (Als *et al*., 2011; Pujolar *et al*., 2014a, 2014b); therefore, the different sampling points should not bias the results of this study. Until sampling, the animals were kept either in an outdoor freshwater basin at the Institute of Zoology at the University of Innsbruck, Austria, or in an outdoor freshwater basin at the Thünen Institute of Fisheries Ecology, Ahrensburg, Germany. Table 1 presents the morphometrics of the silver and yellow eels chosen for the experiments, with the silvering index being calculated according to Durif *et al*. (2005) and the ocular index being calculated according to Pankhurst (1982).

The swimbladder gas gland tissue of *A. anguilla* was isolated following the well-established procedure described by Schneebauer *et al*. (2016, 2017). After the eels were anaesthetized with 2-phenoxyethanol (Carl Roth, Karlsruhe, Germany) according to the AVMA Guidelines for the Euthanasia of Animals (Leary *et al*., 2020), they were subsequently decerebrated and spinally pithed. The abdominal wall was opened ventrally, and the swimbladder was carefully dissected and freed from connective tissue to reveal the actual gas gland tissue. For comparison, muscle tissue, which served for comparison for several studies on swimbladder tissue (Pelster *et al*., 2016a, 2016b; Schneebauer *et al*., 2016), was also dissected. The tissue was immediately shock-frozen in liquid nitrogen and stored at −80°C until further analysis. The tissue was sampled in compliance with the Austrian and German law and approved by the Tierversuchskommission of the University of Innsbruck, Austria.

In addition, for Western blot analysis and RT-PCR American yellow eels (*Anguilla rostrata*), with a weight range between 100 and 300 g and a silver index of 1–2, were sourced locally from Mount Desert Island, ME, USA, and kept in freshwater basins. Before sampling they were adapted to sea water for at least 7 days. Tissue sampling and the processing of protein samples were performed at the Mount Desert Island Biological Laboratory (MDIBL) in accordance with the IACUC regulations of both MDIBL and Georgia Southern University, USA, and per the procedures basically described in previous studies (Cutler *et al*., 2012).

2.2 | Cholesterol quantification

Cholesterol was measured using a Cholesterol Quantification Kit (Sigma-Aldrich, St. Louis, Missouri, USA) according to the manufacturer's instructions; 30 mg of swimbladder or muscle tissue was homogenized in 600 µl of chloroform:isopropanol:IGEPAL (7:11:0.1) (Sigma-Aldrich) solution in Precellys Tubes CKMix (Bertin Technologies, Montigny-le-Bretonneux, France) with a Precellys 24 homogenizer (Bertin Technologies) for 3 × 30 s at 6000 rpm with 120 s breaks on ice. The obtained homogenates were centrifuged at 13,000*g* for 10 min, and 300 µl of the organic phase was transferred to a new Eppendorf tube. Any insoluble and organic material was removed by air-drying at 50°C and centrifuged again using the Savant

TABLE 1 Morphometrics of European eel *Anguilla anguilla* presented as mean ± S.D., silvering index according to Durif *et al*. (2005) and ocular index according to Pankhurst (1982)

	Yellow eels ($n = 7$)	Silver eels ($n = 8$)
Body mass (g)	328.14 ± 66.99	962.63 ± 339.68
Body length (cm)	60.00 ± 2.07	81.50 ± 7.68
Pectoral fin length (mm)	25.63 ± 2.97	39.68 ± 3.55
Horizontal eye diameter (mm)	6.27 ± 0.32	10.41 ± 1.17
Vertical eye diameter (mm)	5.73 ± 0.36	10.10 ± 0.80
Silver index	2.14 ± 0.35	4.50 ± 0.50
Ocular index	4.68 ± 0.39	10.18 ± 1.17

TABLE 2 Primer sequences used for the amplification of aqp1aa and aqp1ab as well as the primer sequences for *gapdh* and *beta 1 actin*, used as housekeeping genes

Aqp1aa	Sense 5′-GCGCTGGGGTTAAACAGCCTAAACAAAAT-3′
	Antisense 5′-CAGTGAAGCTGATGGCTGTTAGGTGTC-3′
Aqp1ab	Sense 5′-GGAGTTAACAAGCTAAATGGTGTTGCTGTGG-3′
	Antisense 5′-CATCCGGTGTAGCGCATTGCTG-3′
GAPDH	Sense 5′-GACTCATGAGCACAGTACACGCCA-3′
	Antisense 5′-CCATACCGGTCAGCTTGCCGTTAAG-3′
Beta 1 actin	Sense 5′-ACATCAGGGTGTGATGGTTGGCATG-3′
	Antisense 5′- GGTGTTGAAGGTCTCAAACATGATCTGGGT-3′

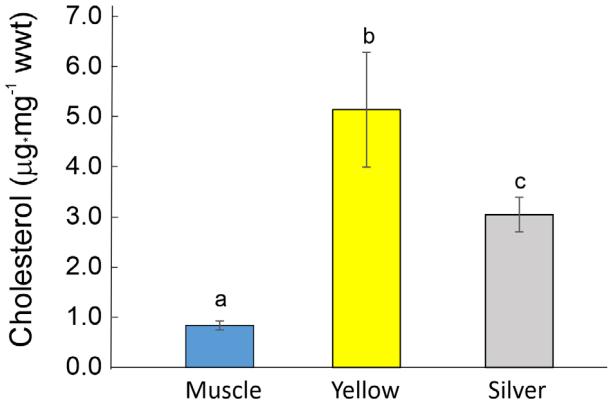

FIGURE 1 Total amount of cholesterol in European yellow and silver eel swimbladder gas gland tissue (µg mg^{-1}). For comparison, yellow eel muscle tissue has also been analysed. $n = 5$ for muscle tissue and yellow eel swimbladder; $n = 6$ for silver eel swimbladder tissue; mean ± S.D.; different small letters indicate significant differences between samples ($P < 0.05$)

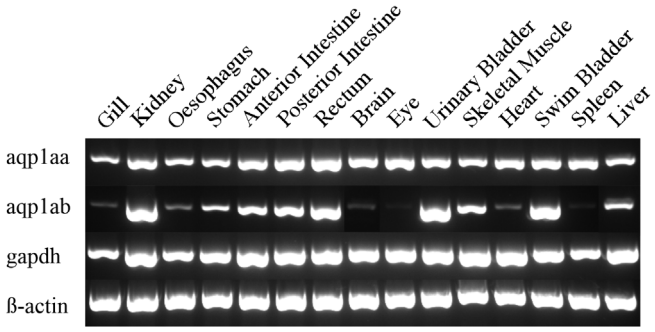

FIGURE 2 Agarose gel electrophoresis (2%) of RT-PCR (real-time PCR) reactions of cDNAs produced from total RNA samples from various tissues of a yellow American eel (*Anguilla rostrata*). Genes amplified were Aquaporin 1 (*aqp1aa*, *aqp1ab*) and two housekeeping genes, glyceraldehyde 3-phosphate dehydrogenase (*gapdh*; accession no. AB07521) and *beta 1 actin* (accession no. GU001950)

Speedvac Sc110 (ThermoFisher Scientific, Waltham, Massachusetts, USA). The remaining dried lipids were dissolved in 150 µl of the provided cholesterol assay buffer. Appropriate dilutions of the samples were filled up to 50 µl, and 50 µl of the provided reaction mix was added in a 96-well plate (Sarstedt, Nümbrecht, Germany). After 60 min incubation at 37°C protected from light, the total amount of cholesterol was detected in a colorimetric assay measuring the absorbance at 570 nm on a spectrophotometer (EnSpire Multimode Plate Reader, Perkin Elmer, Rodgau, Germany). The amount of cholesterol present in the sample (µg mg^{-1} tissue; shown as mean ± S.D.) was determined from the standard curve. Statistical analyses were performed using the *t*-test ($P < 0.05$) in SigmaPlot 14.0.

2.3 | RT-PCR

To analyse the transcription of the two aquaporin 1 genes (*aqp1aa* and *aqp1ab*), as well as the two housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) and *beta 1 actin* (*ß1 actin*), PCR was performed. Reverse transcription was performed using Superscript IV reverse transcriptase (Life Technologies, Carlsbad, California, USA) on cDNA made from 1 µg total RNA from various tissues of yellow American eel (*A. rostrata*) using an Oligo dT_{26} primer and Superase in thermostable RNase Inhibitor (Life Technologies) according to the manufacturer's instructions. The cDNA was then diluted to 200 µl. This was followed by PCR on 1 µl of cDNA per reaction, using Phusion Flex Hot Start DNA Polymerase (New England Biolabs, Frankfurt, Germany) and employing 35 cycles of heating to 98°C for 10 s, 69°C for 15 s and 72°C for 20 s. The primers were designed at intron–exon junctions to eliminate the amplification of genomic DNA; the primer sequences are presented in Table 2. The PCRs were then screened on a 2% agarose electrophoresis gel.

2.4 | Western blot

After sacrifice and dissection, *A. rostrata* tissues were homogenized in Protease Inhibitor Cocktail I Animal free (Research Products International Corp., Mt Prospect, IL, USA), and Western blot was processed as described in Cutler *et al.* (2012). The antibodies used were custom affinity–purified polyclonal antibodies (Genscript, Piscataway Township, New Jersey, USA), raised in rabbits against the peptide NH2-CGD YDVNGPDDVPAVEMSSK-COOH (Aqp1) and NH2-HPKLGDLTERLKVL C-COOH (Aqp1ab), which were cross-linked *via* the terminal cysteine by the company to Keyhole Limpet Hemocyanin and injected with Freund's complete and incomplete adjuvant. The resulting plasma was affinity purified using each peptide attached to a sepharose column. The sequences of the Aqp peptides used were from parts of the derived amino acid sequences of Aqp1aa (previously named Aqp1) and Aqp1ab (previously named Aqp1dub) that are common to the three *Anguilla* species, *A. anguilla*, *A. rostrata and Anguilla japonica*. Protein concentration was determined according to Bradford (1976).

2.5 | Immunohistochemistry

For immunohistochemical localization of aquaporin 1, the eel-specific antibodies Aqp1aa and Aqp1ab were used basically following the protocol described by Boesch *et al.* (2003). Briefly, swimbladder tissue of two silver and two yellow European eels was washed twice with phosphate-buffered saline (PBS, pH 7.4) before fixation in paraformaldehyde overnight at 4°C. After the tissues were washed thrice in PBS, they were dehydrated in a series of ethanol (30 min in 70% ethanol, 30 min in 80% ethanol, 30 min in 90% ethanol, 3 × 1 h in 100% ethanol). Sequential incubations were performed in methyl benzoate, benzene, benzene/paraffin and paraffin before the tissue was finally embedded in paraffin. Paraffin sections (5 µm) were cut using an Autocut 2040 (Reichert-Jung, R. Jung GmbH Germany, Vienna, Austria) and mounted on glass slides (Marienfeld, Lauda-Königshofen, Germany). Blocking of non-specific binding was performed with 1% bovine serum albumin

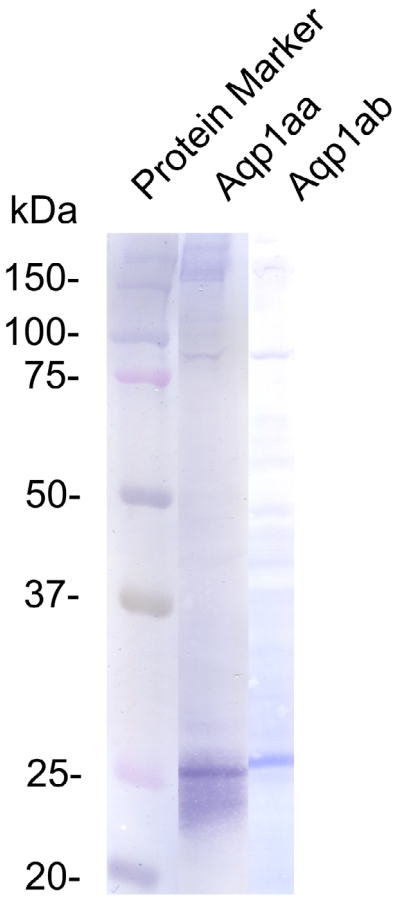

FIGURE 3 Western blot of crude membrane extract proteins from the rectum of yellow American eels (*Anguilla rostrata*) using the custom affinity–purified polyclonal eel Aqp1aa and Aqp1ab antibody; 300 μg of protein was used. Sizes of the precision plus kaleidoscope prestained protein size marker (Biorad, Hercules, CA, USA) are as indicated in kilodaltons (kDa)

in tris-buffered saline with Tween (TBS-T). Incubation with the primary antibodies (1:100) was performed overnight at 4°C. After washing thrice with TBS-T, the Dako EnVision System HRP (DAB) kit (Santa Clara, CA, Agilent, USA) was used according to the manufacturer's instructions for visualization of the target protein using bright-field light microscopy (Leica CTR 5000, Wetzlar, Germany). Control sections were incubated with the secondary antibody omitting the primary antibody.

3 | RESULTS

3.1 | Cholesterol quantification

Cholesterol concentration in swimbladder gas gland cells of yellow and silver European eels determined in a colorimetric assay was at least thrice higher than in yellow eel muscle tissue ($P < 0.01$; Figure 1). Interestingly, in yellow eel gas gland tissue the cholesterol concentration was *c.* 5 μg mg^{-1} and thus significantly higher than that in silver eel gas gland tissue with *c.* 3 μg mg^{-1} ($P < 0.05$).

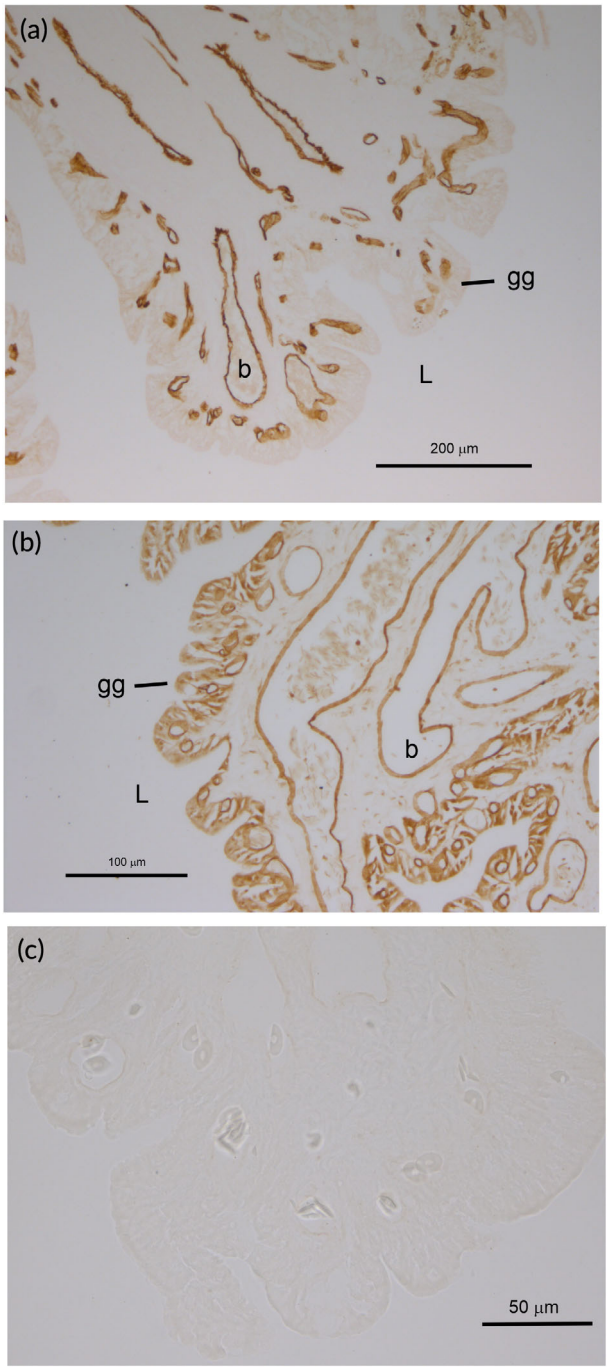

FIGURE 4 Immunohistochemical staining of (a) Aqp1aa and (b) Aqp1ab in swimbladder gas gland tissue from European eel *Anguilla anguilla*. Both antibodies revealed positive staining, whereas (c) no background staining was observed in the negative control. Scale bars are 50, 100 or 200 μm, respectively. b: blood vessel; gg: gas gland cell; L: swimbladder lumen

3.2 | RT-PCR

The RT-PCR gel (Figure 2) revealed that both *aqp1aa* and *aqp1ab* were expressed in most yellow American eel tissues studied, including the swimbladder. There was some variability in the level of expression, particularly for *aqp1ab*, which was hardly detectable in the eye and

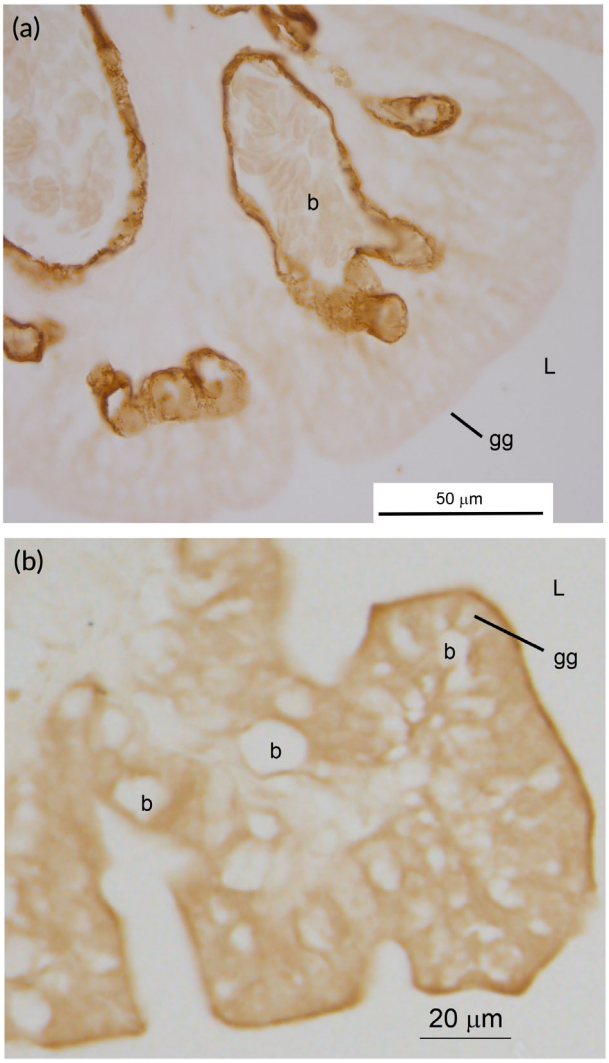

FIGURE 5 Immunohistochemical staining of (a) Aqp1aa and (b) Aqp1ab in swimbladder gas gland tissue from *Anguilla anguilla* at higher magnification. Positive staining of (a) Aqp1aa was detected in endothelial cells, whereas (b) Aqp1ab staining was prominent in apical regions of gas gland cells. Scale bars are 50 or 20 μm, respectively. b: blood vessel; gg: gas gland cell; L: swimbladder lumen

spleen. The control housekeeping genes *gapdh* and *ß1 actin* were amplified in all tissue cDNA samples as expected. The full-size RT-PCR gel is shown in Supporting Information Figure S1.

3.3 | Western blot

Specificity of the generated antibodies was assessed in a Western blot using eel rectum. Similar to Aqp3 (Lignot *et al*., 2002), Western blots with the Aqp1aa antibody showed a smaller-than-expected size of 25 kDa (Figure 3). The faint bands detectable at high molecular weight (100–150 kDa) with the Aqp1aa antibody probably represent multi-meric forms of the AQPs which are known to exist in other species (Smith & Agre, 1991). The Western blot with the Aqp1ab antibody revealed a single band at the expected size of 27.5 kDa (Figure 3).

3.4 | Immunohistochemistry

Immunohistochemical staining with Aqp1aa and Aqp1ab revealed the presence of aquaporin 1 in European eel swimbladder gas gland cells and in endothelial cells of the swimbladder vasculature (Figure 4). The antibody Aqp1aa resulted in a positive signal in endothelial cells of swimbladder capillaries. Some staining was also observed in basolateral membranes of gas gland cells but not in apical membranes (Figure 4a). The antibody Aqp1ab revealed an additional positive staining in the apical region of gas gland cells (Figure 4b). At higher magnification the staining of the antibody directed against Aqp1aa was most prominent in endothelial cells, whereas no staining was detected in blood cells (Figure 5a). Aqp1ab antibody staining was prominent in the apical region of gas gland cells and in endothelial cells (Figure 5b). The basolateral membranes of gas gland cells often are in intimate contact with blood capillaries, and in these cells staining was most prominent at the apical membranes (Figure 5b). Some staining was also observed in basolateral membranes of gas gland cells. For both antibodies no difference was detected between yellow and silver eel swimbladder gas gland samples.

4 | DISCUSSION

Experiments were performed using European and American eels, but due to availability and logistics not all experiments could be performed on both species. The two species belong to the same genus and can hardly be separated by visual inspection, and they are even known to hybridize (Pujolar *et al*., 2014a, 2014b). *A. anguilla* and *A. rostrata* spawn in the Sargasso Sea and show a similar vertical migration pattern during their spawning migration (Aarestrup *et al*., 2009; Righton *et al*., 2012; Schabetsberger *et al*., 2016). Previous experimental studies on swimbladder function used American and European eels successfully, and no difference in function was observed (Pelster *et al*., 1992, 1994; Pelster & Scheid, 1992a, 1992b). The antigens used for the production of polyclonal antibodies were based on a protein sequence identical in *A. anguilla*, *A. rostrata* and *A. japonica*, and the obtained antibodies used in this study worked in American and European eels.

Searching the transcriptome of swimbladder gas gland tissue of European yellow and silver eels published by Pelster *et al*. (2016a, 2016b) revealed the expression of aquaporin 1, 3, 4, 8, 10 and 11, and aquaporin 1 was by far the most prominent isoform. The relative expression value of *aqp1* exceeded the value of *aqp3* more than 200-fold, and the others were even lower in their relative expression value (Pelster *et al*., 2016a, 2016b). The presence of Aqp1 transcripts in European eel gas gland tissue has meanwhile been confirmed by Schneebauer *et al*. (2020), but there appears to be no significant difference in the expression level between yellow and silver eels. The

present RT-PCR experiments also confirmed the mRNA expression of *aqp1aa* and *aqp1ab* for swimbladder tissue of the American eel. *Aqp1aa* was in fact detected in every tissue examined. In European eels high levels of *aqp1ab* mRNA abundance were observed only in the oesophagus and kidney (Martinez *et al*., 2005b), but the authors detected *aqp1ab* mRNA in most tissues of American eels, except for the brain, eye and spleen, where it was hardly detectable. These data clearly confirm the expression of aquaporin *aqp1aa and aqp1ab* mRNA in eel swimbladder tissue, but mRNA expression of aquaporin 1 genes in other tissues is not yet clear.

Using Western blots of rectum tissue the specificity of the Aqp1aa and Aqp1ab antibodies was confirmed. As shown in Figure 3 for the Aqp1aa and Aqp1ab antibodies, only a single band was obtained, and the band obtained for Aqp1aa was around the expected size of 27 kDa. The smaller 25 kDa size of the Aqp1aa protein was surprising because the antigen used to make the antibody was essentially very similar to that of Martinez *et al*. (2005a, 2005b), only owning four extra amino acids at the NH_2- end of the peptide. Therefore, the size difference may have been due to a different-sized marker being employed in the two studies. The size of the main Aqp1ab protein bands (27 kDa) was very close to those expected (27.8 kDa). Kagawa *et al*. (2011) published a Western blot using an Aqp1b antibody raised against the C-terminal end of the protein (NH_2-AQEPLLEGCSAAQWTKG-COOH), but the size for Aqp1b was not indicated. Measuring the size from their Western blot figure yields a size of 25 kDa. Some variability in the apparent size of proteins may depend on the different-sized markers used; it is also possible that the movement of the proteins was not exactly proportional to the theoretical weight.

RT-PCR, Western blot and immunohistochemical analysis revealed the presence of two copies of aquaporin 1 in swimbladder tissue. Aqp1ab is an Aqp1 duplicate, as described by Martinez *et al*. (2005b). The protein isolated from eel kidney contains 263 amino acids (Martinez *et al*., 2005a, 2005b), like Aqp1aa, with an amino acid sequence identity of 69%. *Aqp1aa* and *aqp1ab* mRNA were detected in the oesophagus (Martinez *et al*., 2005a, 2005b), which is in line with the presence of both isoforms in swimbladder tissue, because the swimbladder is an evolutionary derivative of the oesophagus.

Both antibodies indicated an extensive presence of Aqp1aa in endothelial cells next to gas gland cells, as well as in membranes of gas gland cells. In zebrafish swimbladder, Aqp1aa protein has been detected by Western blot, and it has been proposed that it may act as a water channel playing a role in angiogenesis, whereas in red blood cells it was supposed to act as a gas channel (Chen *et al*., 2010). Aoki *et al*. (2003) also detected *aqp1* mRNA in the intestine and the swimbladder of *A. japonica*, and the presence of the protein was connected to water movements. In the eel, the ductus pneumaticus is functionally closed so that the eel cannot gulp air and the swimbladder can be filled only by gas secretion as in physoclist fish (Fänge, 1983; Pelster, 2013, 2021). Therefore, they cannot accidentally swallow water as proposed for air-breathing fish (Pelster & Wood, 2018), making water movements in the swimbladder tissue not meaningful. In the swimbladder, blood must be acidified to switch on the Root effect, which is achieved by the production of lactic acid and CO_2 in gas gland cells (Pelster, 1995a; Pelster *et al*., 1994; Pelster & Scheid, 1993; Walsh & Milligan, 1993). Protons are secreted *via* V-type proton ATPase and sodium–proton exchange (Pelster, 1995b, 2004), and CO_2 leaves the cell by diffusion (Kobayashi *et al*., 1990). Equilibrium of the CO_2/HCO_3^- reaction in the interstitial fluid is achieved *via* membrane-bound carbonic anhydrase (Boesch *et al*., 2003; Würtz *et al*., 1999). Facilitation of CO_2 diffusion by CO_2-permeable Aqp1aa and Aqp1ab, present in basolateral gas gland cell membranes as well as in endothelial cells, would speed up CO_2 movements and blood acidification. This would enhance the Root effect and the resulting increase in PO_2, required for the secretion of oxygen as the main swimbladder gas (Kobayashi *et al*., 1990). The authors therefore suggest that Aqp1aa and Aqp1ab channels in swimbladder tissue mainly contribute to CO_2 movements and are not primarily responsible for water movements. In swimbladder tissue, partial pressure gradients as a driving force for CO_2 movements are much smaller than oxygen partial pressure gradients (Kobayashi *et al*., 1990), so that facilitation of CO_2 diffusion through aquaporin channels may significantly speed up CO_2 movements.

Aqp1ab has also been detected in the apical region of gas gland cells. Gas gland cells have a thickness of *c*. 20 μm, and the basolateral folding of these cells is in immediate contact to swimbladder capillaries (Prem *et al*., 2000). In these cells Aqp1ab staining was localized to the apical membrane region; therefore, the authors assume it is located in the apical membranes of gas gland cells. This assumption is also supported by the detection of Aqp1 protein in the membrane fraction of European eel gas gland cells (Sialana *et al*., 2018). Because CO_2 is produced in gas gland cell metabolism (Pelster *et al*., 1994; Walsh & Milligan, 1993), the highest PCO_2 is expected in gas gland cells, providing a diffusion gradient not only into the blood but also into the swimbladder lumen. The presence of Aqp1ab could facilitate the diffusion of CO_2 into the swimbladder lumen, and it has been shown that newly secreted gas in the eel swimbladder contains about 25% CO_2 (Kobayashi *et al*., 1990; Pelster & Scheid, 1992a, 1992b).

Lipid accumulation has been documented repeatedly for mid-water fish that perform extended vertical migrations and for deep-sea fish as well (Morris & Culkin, 1989; Phleger, 1991). Those lipids typically consist of cholesterol and phospholipids and, in some species, may have a bilayer membrane configuration (Phleger *et al*., 1978; Phleger & Benson, 1971; Phleger & Holtz, 1973). The cholesterol content of cell membranes varies with species, tissue and environmental temperature (Crockett, 1998; Farkas *et al*., 2001). The authors used muscle tissue as a comparison, which has been used for comparative studies on swimbladder tissue before (Pelster *et al*., 2016a, 2016b; Schneebauer *et al*., 2016), and their measurements revealed a much-higher cholesterol content (five times) in swimbladder tissue as compared to muscle tissue of yellow eels. Already in the 1980s it has been shown that the addition of cholesterol to phospholipid membranes reduces the gas permeability *c*. 10-fold (Finkelstein, 1976; Wittenberg *et al*., 1980). Recent studies focusing on the effect of cholesterol on CO_2 permeability of membranes confirmed that an increasing cholesterol content of membranes results in a decrease in CO_2 permeability (Itel *et al*., 2012; Tsiavaliaris *et al*., 2015). The much-higher cholesterol

content in swimbladder membranes therefore could contribute to a lower CO_2 permeability and an increased gas tightness of these membranes, supporting the effect of guanine incrustation. The swimbladder wall of eels has a silvery appearance due to the incrustation of the submucosa with guanine crystals, which significantly reduce the gas permeability (Denton *et al*., 1972; Kleckner, 1980; Lapennas & Schmidt-Nielsen, 1977). A high cholesterol content in swimbladder membranes therefore would also be beneficial for the reduction in the diffusional loss of gas molecules out of the swimbladder along the diffusion gradient from the hyperbaric swimbladder lumen encountered at depth to the normobaric gas partial pressure in the surrounding tissues and water. Somewhat unexpected was the reduction in membrane cholesterol content observed in silver eels. This may be the result of a redistribution of cholesterol from other tissues to the gonads previously observed in silver eels (Lewander *et al*., 1974). A decrease in cholesterol may, however, impair the contribution of cholesterol to gas tightness of the membranes to some degree, but it will also facilitate CO_2 diffusion. This observation supported the conclusion that in silver eels acidification of the blood is very important to switch on the Root effect, because oxygen, with a contribution of *c*. 60%–65%, is the most important gas in gas secretion in European eels (Kobayashi *et al*., 1990; Pelster & Scheid, 1992a, 1992b). In American silver eels oxygen contributed even more than 80% to newly secreted gas (Kleckner, 1980).

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SUPPORTING INFORMATION

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