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RESEARCH ARTICLE

Vasotocin and isotocin regulate aquaporin 1 function in the sea bream

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ABSTRACT

Aquaporins (AQPs) are specific transmembrane water channels with an important function in water homeostasis. In terrestrial vertebrates, AQP2 function is regulated by vasopressin (AVP) to accomplish key functions in osmoregulation. The endocrine control of aquaporin function in teleosts remains little studied. Therefore, in this study we investigated the regulatory role of vasotocin (AVTR) and isotocin (ITR) receptors in *Aqp1* paralog gene function in the teleost gilthead sea bream (*Sparus aurata*). The complete coding regions of *Aqp1a*, *Aqp1b*, *AVTR V1a2-type*, *AVTR V2-type* and *ITR* from sea bream were isolated. A *Xenopus* oocyte-swelling assay was used to functionally characterize AQP1 function and regulation by AVT and IT through their cognate receptors. Microinjection of oocytes with *Aqp1b* mRNA revealed regulation of water transport via PKA (IBMX+forskolin sensitive), whereas *Aqp1a* mRNA injection had the same effect via PKC signaling (PDBU sensitive). In the absence of expressed receptors, AVT and IT (10^{-8} mol l^{-1}) were unable to modify AQP1 function. AVT regulated AQP1a and AQP1b function only when the AVTR V2-type was co-expressed. IT regulated AQP1a function, but not AQP1b, only when ITR was present. Considering that *Aqp1a* and *Aqp1b* gene expression in the sea bream intestine is highly salinity dependent *in vivo*, our results *in ovo* demonstrate a regulatory role for AVT and IT in AQP1 function in the sea bream in the processing of intestinal fluid to achieve osmoregulation.

KEY WORDS: Aquaporin, Water regulation, Isotocin receptor, Vasotocin receptor, Osmoregulation

INTRODUCTION

Fish osmoregulation is under endocrine control via specific effects in the osmoregulatory organs, i.e. gills, kidney and gastrointestinal tract (Avella et al., 1999; Gregorio et al., 2013; Laiz-Carrion et al., 2005; Marshall, 2002; Martos-Sitcha et al., 2014, 2013). In hyperosmotic environmental salinities, high amounts of water ingestion (Fuentes and Eddy, 1997) are necessary to compensate for the ionic imbalance in the plasma/external milieu and the dehydrating effect of seawater (Evans et al., 2005; Grosell, 2010). Water absorption at the intestinal level, which appears to be aquaporin (AQP) mediated (Wood and Grosell, 2012), is driven by simultaneous Cl^- uptake, mediated by an apical $Na^+/K^+/2Cl^-$ (NKCC) co-transporter (Musch et al., 1982) or apical Cl^-/HCO_3^- anion exchangers (Grosell, 2006, 2011).

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The functions of AQPs are related to water balance between the extracellular and intracellular spaces (Agre et al., 1998, 1999; Preston and Agre, 1991; Preston et al., 1992), tissues and different osmoregulatory organs involved in fluid homeostasis in vertebrates (An et al., 2008; Borgnia et al., 1999; Lignot et al., 2002; Martinez et al., 2005a,b; Nielsen et al., 2002). Currently, 13 AQP families have been described in vertebrates (Agre, et al., 2002; King et al., 2004), which are sub-divided into three different groups depending on their genomic structure and specific function: (i) aquaporin group, (ii) aquaglyceroporin group and (iii) superaquaporin group (Echevarria et al., 1996; Ishibashi et al., 1997, 1998, 2000, 1994; Yang and Verkman, 1997). The aquaporin group is composed of AQP 0, 1, 2, 4, 5, 6 and 8 subtypes (Ishibashi et al., 2000). Not all members of the aquaporin group are represented in fish, which lack AQP2 (Cerdá and Finn, 2010; Finn and Cerdá, 2011). In turn, *Aqp1* expression has been shown in water-permeable tissues such as gills, kidney or intestine (Kwong et al., 2013; Madsen et al., 2014; Martinez et al., 2005a,b; Tipsmark et al., 2010; Raldúa et al., 2008).

The vasopressin (AVP) and oxytocin (OXY) families, including the non-mammalian peptides arginine vasotocin (AVT) and isotocin (IT), have been associated with the regulation of blood pressure and antidiuretic functions in osmoregulation (Amer and Brown, 1995; Henderson and Wales, 1974; Maetz et al., 1964; Moon and Mommsen, 1990; Motais and Maetz, 1967). The endocrine effects of AVT and IT rely on the presence of membrane receptors that mediate the hormonal intrinsic information to carry out their physiological action. AVT and IT receptors (AVTR and ITR) have been cloned in some fish such as the Amargosa pupfish (*Cyprinodon variegatus amargosae*; Lema, 2010), the African lungfish (*Protopterus annectens*; Konno et al., 2009) and the gilthead sea bream (*Sparus aurata*, Martos-Sitcha et al., 2014; Martos-Sitcha et al., 2013). In the last of these, two AVTR types (V1a2-type and V2-type) and a single ITR are expressed in a wide range of organs/tissues, especially in those with an osmoregulatory role.

A functional connection between AQP2 and the AVP/AVT system demonstrating the involvement of the latter in water channel regulation has been established in terrestrial vertebrates (Agre et al., 2002; Asahina et al., 1995; Fujita et al., 1995; Funayama et al., 2004). For instance, a role for AVP in the on–off regulation of AQP2 mRNA expression has been shown in the collecting duct of rats (Saito et al., 1997). In addition, AQP2 expression is up-regulated by endogenous and exogenous AVP under various pathophysiological conditions (Asahina et al., 1995; Fujita et al., 1995; Fushimi et al., 1993; Hayashi et al., 1994). In teleosts, AQP2 is absent (Cerdá and Finn, 2010; Finn and Cerdá, 2011) and few reports exist to document a functional link between AVT/IT and AQPs. Amongst these is the co-activation of *Aqp1* and *AVTR* (An et al., 2008) induced by hyperosmotic conditions, which is argued to improve the hypo-osmoregulatory ability in the black porgy (*Acanthopagrus*

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schlegeli). *Aqp1* is consistently expressed in the gastrointestinal tract of several fish and is apparently associated with water movements in the intestine (Cerdá and Finn, 2010; Finn and Cerdá, 2011). Additionally, analysis of the sea bream sequences of *Aqp1a* and *Aqp1b* detected phosphorylation signals of either protein kinase C (PKC) or protein kinase A (PKA) (Tingaud-Sequeira et al., 2008) making them accessible to endocrine control. Furthermore, the potential role of AQP and AVTRs in osmoregulatory function, as well as water absorption processes, has been separately studied in the intestine of sea bream (Gregorio et al., 2013; Raldúa et al., 2008). In a recent study (Martos-Sitcha et al., 2013), we have documented a regulatory role of AVT in the intestine of the sea bream via a bumetanide-sensitive mechanism, likely a NKCC co-transporter, which is a regulator of water movements in fish intestine (Musch et al., 1982).

While a functional association between APQs and AVT can be expected in fish, the experimental evidence is mostly circumstantial. Therefore, the present study aimed to explore the putative regulatory role of AVTRs and ITR on *Aqp1* paralog gene function in the sea bream, *S. aurata* L. using a heterologous expression system, the *Xenopus* oocyte.

RESULTS

Aqp1a or *Aqp1b* in the intestine of sea bream

The distribution of all genes analyzed by *in situ* hybridization showed expression in enterocytes of the anterior intestine and rectum of control sea bream (Fig. 1). Intensity wise, *Aqp1a* and *Aqp1b* seemed to show complementary expression, with *Aqp1a* mRNA abundance (Fig. 1A and F) higher in the anterior intestine whereas *Aqp1b* mRNA (Fig. 1B and G) was more abundant in the rectum. The expression of *Aqp1a* and *Aqp1b* was also observed in non-enterocyte cells that resemble fibroblasts (Fig. 1A,B,F,G). In turn, *AVTRV1a2* signal was abundant in both segments of sea bream intestine (Fig. 1C,H). *AVTRV2* expression was present in both intestinal regions (Fig. 1D and I), but its expression was lower in the anterior intestine than in the rectum. *ITR* expression was present at similar levels in enterocytes of both the anterior intestine (Fig. 1E) and rectum (Fig. 1J).

When analyzed by qPCR, *Aqp1a* and *Aqp1b* were expressed in the anterior intestine and the rectum of the sea bream gastrointestinal tract (Fig. 2). A decrease in salinity from 37 to 12 ppt increased *Aqp1a* expression in the anterior intestine of fish. However, this effect was not observed in the rectum, where, regardless of external salinity, *Aqp1a* expression remained unchanged (Fig. 2A). The expression of *Aqp1b* was severalfold higher in the rectum than in the anterior intestine, regardless of the external salinity. In the anterior intestine, *Aqp1b* expression was unaffected by environmental salinity, while higher expression of *Aqp1b* was detected in the rectum of fish adapted to hyperosmotic conditions (35 and 55 ppt, Fig. 2B).

Aqp expression in oocytes

Expression of sea bream *Aqp1* in *Xenopus* oocytes was functionally demonstrated by the linear swelling of oocytes in response to a decrease of external osmolality, which was not observed in water-injected oocytes (Fig. 3). Our results showed that *Aqp1a* and *Aqp1b* mRNA was translated and the proteins were perfectly functional in the oocyte membrane, as shown by the increase of water permeability values mediated by AQP1a and AQP1b in swelling assays (Fig. 3). In addition, no response was observed in oocytes injected with water alone; therefore, the injected *Aqp* mRNA functionally mediates the volume increase observed.

Intracellular signaling of AQP1 function

In oocytes devoid of *AVTR*/*ITR* mRNA and expressing only sea bream *Aqp1a*, PKC stimulation (PDBU treatment) increased Pf by about 25% (Fig. 3A). In contrast, *Aqp1a* expression was insensitive to PKA stimulation by IBMX-FK. The opposite effect was observed in oocytes devoid of *AVTR*/*ITR* mRNA and expressing only *Aqp1b*, where aquaporin function was insensitive to PDBU (PKC stimulation), but responded to IBMX-FK (PKA stimulation, Fig. 3B). P_f in water-injected oocytes was insensitive to PKA stimulation by IBMX-FK or PKC stimulation by PDBU (Fig. 3).

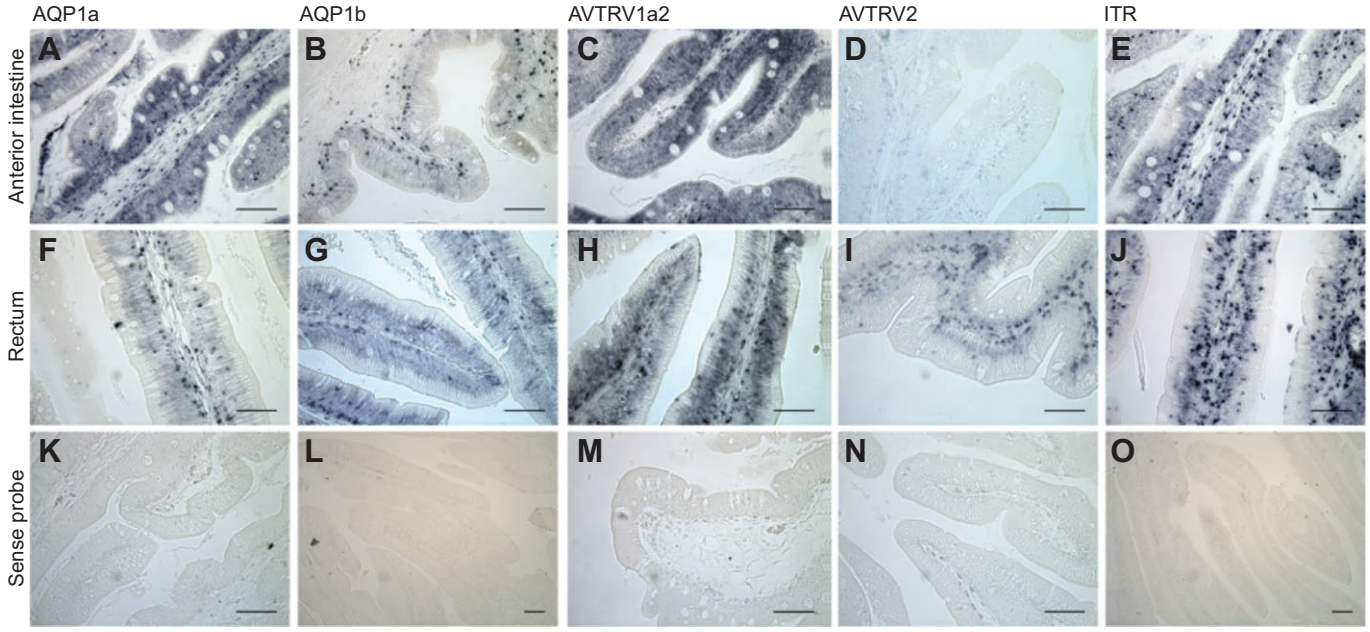

Fig. 1. *In situ* hybridization of aquaporins, arginine vasotocin receptors and isotocin receptor in the sea bream. *In situ* hybridization of *Aqp1a* (A and F), *Aqp1b* (B and G), *AVTRV1a2* (C and H), *AVTRV2* (D and I) and *ITR* (E and J) in the anterior intestine (A–E) and rectum (F–J) of sea bream maintained in 35 ppt seawater. (K–O) Slides hybridized with a sense probe of each gene analyzed (K–M, rectum; N and O, anterior intestine). All scale bars represent 50 μm.

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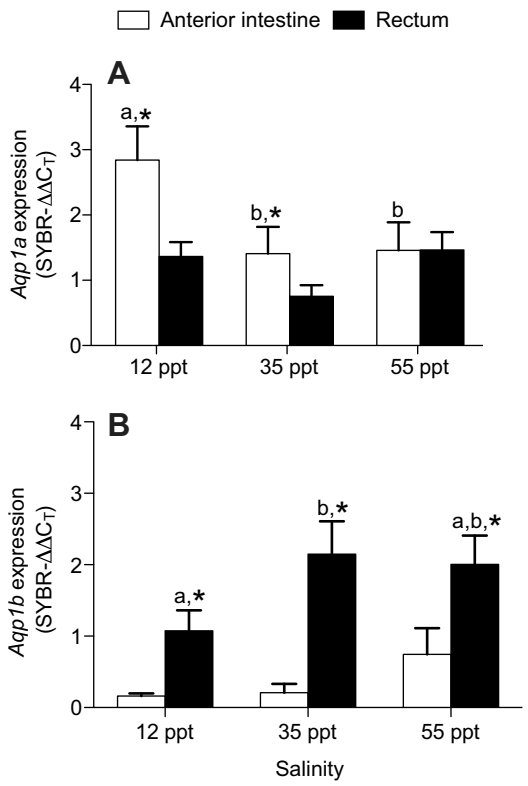

Fig. 2. Relative expression of *Aqp1a* and *Aqp1b* in the anterior intestine and the rectum of the sea bream after long-term acclimation to 12, 35 and 55 ppt seawater. (A) *Aqp1a*, (B) *Aqp1b*. Results are shown in arbitrary units (SYBR-ΔΔC$_T$) determined by qPCR. Each column represents the mean± s.e.m. (*N*=7). Within a particular intestinal region, bars displaying different letters are significantly different (*P*<0.05, one-way ANOVA followed by Bonferroni *post hoc* test). Asterisks represent significant differences between intestinal regions at the same salinity (*P*<0.05, Student's *t*-test).

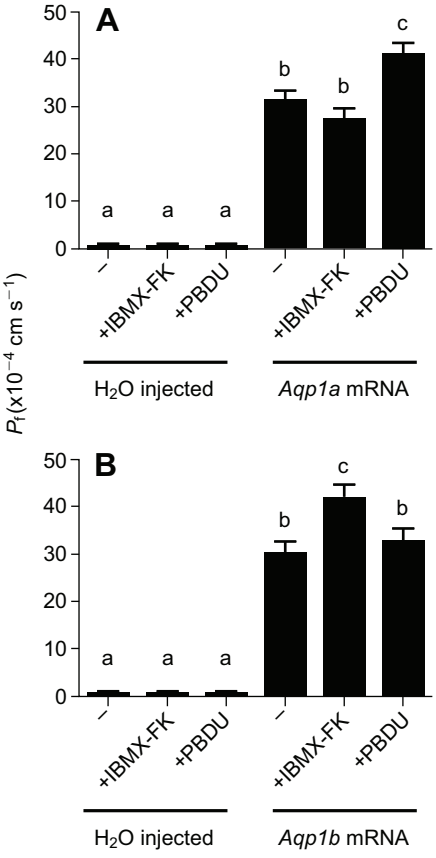

Fig. 3. Osmotic water permeability (P_f) in *Xenopus laevis* oocytes expressing *Aqp1a* or *Aqp1b* in response to IBMX-FK or PDBU. (A) *Aqp1a*, (B) *Aqp1b*. +IBMX-FK indicates the presence of 100 μmol l^{-1} IBMX+ 10 μmol l^{-1} FK; +PDBU indicates the presence of 2 μmol l^{-1} PDBU; –, no treatment. Each bar represents the mean±s.e.m. of 20–25 oocytes from three different animals. Groups displaying different letters are significantly different (*P*<0.05, one-way ANOVA followed by Bonferroni *post hoc* test).

AVT and IT in *Aqp1* paralog gene function

In oocytes devoid of *AVTR*/*ITR* mRNA, but expressing either *Aqp1a* (Fig. 4A) or *Aqp1b* alone (Fig. 4B), incubation with AVT or IT (10^{-8} mol l^{-1}) was without effect on water permeability. This lack of effect to exogenous AVT and IT (10^{-8} mol l^{-1}) was also observed in water-injected oocytes.

In the absence of hormonal treatment with AVT or IT, co-expression of *Aqp1a* or *Aqp1b* with *AVTRV1a2*, *AVTRV2* or *ITR* was without affect on basal P_f when compared with oocytes injected with either *Aqp1a* or *Aqp1b* alone (Figs 4, 5, 6). To test whether AVT or IT modified P_f via AQP1a or AQP1b, a typical dose–response with 100-fold increments between 10^{-12} and 10^{-6} mol l^{-1} was tested for each AQP–receptor combination (Figs 5 and 6). AQP1a function (expressed as changes in P_f) was unaffected by AVT treatment when its mRNA was co-expressed with *AVTRV1a2* mRNA (Fig. 5A,B). In contrast, co-expression of *Aqp1a* mRNA with either *AVTRV2* or *ITR* mRNA produced a typical dose–response increase of P_f following stimulation with AVT or IT, respectively (Fig. 5). In both cases, the calculated EC_{50} was $10^{-9.8}$ mol l^{-1} (Fig. 5). The AVT/IT effect was robust even after 300 s and the response to the hormone was immediate (Fig. 5).

In the case of AQP1b, incubation with AVT significantly enhanced water permeability only when co-expressed with the AVTR V2-type (Fig. 6D,E,F). The effect conformed to a typical dose–response curve with a calculated EC_{50} of $10^{-9.9}$ mol l^{-1} (Fig. 6F). Additionally, co-expression of *Aqp1b* with either *AVTRV1a2* or *ITR* mRNA was without effect on oocyte swelling when the receptors were stimulated with their corresponding hormones (Fig. 6).

DISCUSSION

In the present study, we established the functional regulation of AQP1 homologs by AVT and IT via specific receptors in the sea bream. These findings reinforce the importance of the AVT/AVP system in AQP-mediated water balance in vertebrates (Mahlmann et al., 1994; Mordasini et al., 2005; Schafer et al., 1990; Warne, 2001; Warne et al., 2002).

mRNA expression of *Aqp1* paralogs, i.e. *Aqp1a* and *Aqp1b*, was identified in different portions of the sea bream intestine (Raldúa et al., 2008). *In vivo* effects of external salinity on aquaporin expression in the intestine and in particular the anterior intestine and the rectum further highlight the clear functional specialization in intestinal fluid processing (ion and water transport) previously demonstrated in the sea bream (Gregorio et al., 2013; Martos-Sitcha et al., 2013). This is in agreement with *Aqp1a* mRNA levels in the anterior intestine, as well as higher *Aqp1b* expression in the rectum, previously reported for the sea bream (Raldúa et al., 2008).

The complementary pattern of *Aqp* mRNA expression in the intestine, also described in Atlantic salmon (Tipsmark et al., 2010),

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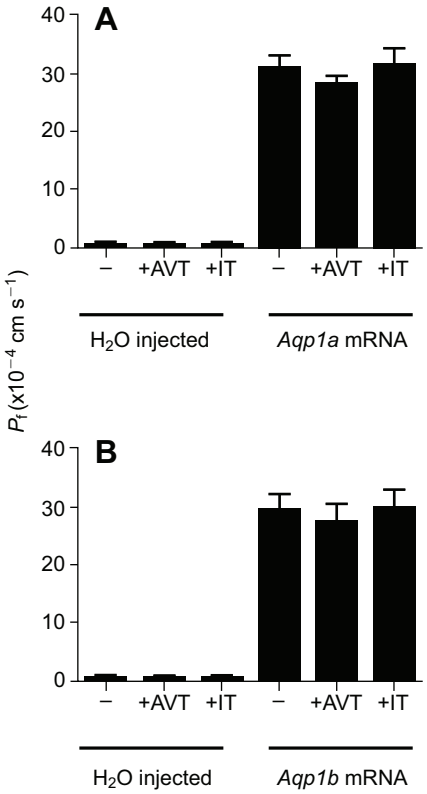

Fig. 4. Osmotic water permeability (P_f) in *X. laevis* oocytes expressing *Aqp1a* or *Aqp1b* in response to AVT or IT. +AVT, 10^{-8} mol l^{-1} AVT; +IT, 10^{-8} mol l^{-1} IT; –, no ligand. Each bar represents the mean±s.e.m. of 20–25 oocytes from three different animals. No significant differences due to the presence of hormones were observed in water-injected or *Aqp*-injected oocytes (P<0.05, one-way ANOVA followed by Bonferroni *post hoc* test).

may reveal a functional overlap/complementation between the two *Aqp1* paralog genes in the anterior and posterior intestine, at least in the sea bream. These observations, based on mRNA expression alone, are in good agreement with the minor absolute effect of salinity on water absorption in the anterior intestine of salinity-challenged sea bream (Gregorio et al., 2013), whereas increasing water absorption in the rectum of sea bream challenged with 55 ppt (Gregorio et al., 2013) may be mostly mediated by AQP1b action. Taking into account the presence of AVTR V2-type and ITR in both intestinal regions (Martos-Sitcha et al., 2013, 2014), two main factors could determine the functional importance of the regulatory action of AVT and IT on AQP1 function in sea bream: firstly, the relative distribution/abundance of *Aqp1* homologs in the intestinal tract; and secondly, the availability of receptor ligand(s) in the plasma, which is also salinity dependent (Martos-Sitcha et al., 2013).

To understand how AVT and IT systems may regulate AQP1 function in sea bream, we employed a swelling assay in *Xenopus* oocytes. This assay has been used to characterize different AQPs because of their large size and the lack of endogenous water channels in the membrane (Agre et al., 1999). In fact, the expression of wild-type *Aqp* genes has previously been demonstrated to increase water P_f values in *Xenopus* oocytes (Fushimi et al., 1997, 1994, 1993; Kuwahara et al., 1995; Yang and Verkman, 1997). AVT and IT receptors have recently been cloned in different teleost species, including the sea bream, and are considered to be relevant elements in osmoregulation (Konno et al., 2009; Lema, 2010; Martos-Sitcha et al., 2013). These receptors achieve the biological actions of the ligands via intracellular second-messenger pathways. The regulation of AQP function can be achieved via different signaling pathways (Dibas et al., 1998). AVTR/AVPR V1a-type and ITR were shown to act via PKC, which uses phospholipase C (PLC), inositol-1,4,5-trisphosphate (IP3) or Ca^{2+} intracellular signaling pathways (Wargent et al., 1999). In contrast, PKA, whose activity is dependent on cellular levels of cyclic AMP (cAMP), is preferentially used by AVT/AVP V2-type receptors (Wargent et al., 1999; Warne, 2001). Although direct phosphorylation of AQPs was not demonstrated in this study, we have shown that sea bream *Aqp1a*-mediated water transport is positively regulated by PKC but not PKA signaling, whereas the opposite holds true for *Aqp1b* (Fig. 3). This shows that AQP1 function is regulated by phosphorylation, which might constitute a rapid response mechanism to osmoregulatory challenges. This feature of sea bream AQP1 contrasts with human AQP1, which is insensitive to direct stimulation with cAMP, but instead resembles human AQP2 (Tsunoda et al., 2004). Strikingly, the fact that sea bream *Aqp1a* and *Aqp1b* are specifically stimulated via different intracellular signaling pathways further highlights sub-functionalization of these paralog genes.

The *Xenopus* oocyte heterologous system has already been used for neuropeptide receptor characterization (Akhundova et al., 1996; Hausmann et al., 1995; Kimura et al., 1992; Mahlmann et al., 1994; Sugimoto et al., 1994). However, to our knowledge, characterization of the interaction between AVTR and ITR with AQP function has not been carried out, in spite of the conceivable co-regulation suggested by other authors in *in vivo* studies (An et al., 2008; Hatakeyama et al., 2001; Konno et al., 2010). Here, we suggest a selective and physiologically specific effect of this approach to study AQP function and regulation. AVT and IT alone were unable to stimulate function in *Aqp* mRNA-only injected oocytes. Such stimulation was only possible when *Aqp* mRNA was co-injected with specific *AVTR* or *ITR* mRNA and in the presence of their cognate hormone, demonstrating a non-genomic correlation of AVT/IT with aquaporin function. These results show that in the assay there are no endogenous AVT or IT receptors and that the effects observed in our assay are highly specific for each AQP–receptor–hormone combination. However, AVTR V1a2-type did not regulate either AQP1a or AQP1b function, even in the presence of AVT (Figs 5 and 6). This result is unexpected, considering that the intracellular pathway preferentially used by this receptor, i.e. PKC, is active in oocytes and enhanced P_f in AQP1a alone (Fig. 3). This suggests that PKC signaling might regulate AQP1a function via other hormones/receptors or independently of hormonal regulation. In contrast, AVT stimulation of oocyte swelling, mediated by AQP1a or AQP1b, occurs only when the AVTR V2-type receptor is present (Figs 5 and 6). More importantly, the significant hormonal effects on swelling were achieved at 10^{-8} mol l^{-1} AVT, which is within the physiological range of circulating AVT in sea bream plasma (Kleszczynska et al., 2006; Mancera et al., 2008) and at the level observed for AVT actions in ion transport in *ex vivo* experiments in sea bream intestine (Martos-Sitcha et al., 2013). Additionally, based on AQP sensitivity (Fig. 3), the effect mediated by AVT V2-type receptors on both AQP1a and AQP1b function would involve stimulation of PKA and perhaps PKC. It is unsurprising that AQP1a is regulated by AVTRV2 given that PKA is the preferential signaling pathway associated with this receptor, and PKC signaling seems to play a minor role (Wargent et al., 1999; Warne, 2001). However, previous studies in frog demonstrated the V2-type receptor could also function via stimulation of PLC pathways, one of the alternative pathways for PKC (Acharjee et al., 2004; Zhu et al., 1994). Further studies will be required to establish whether

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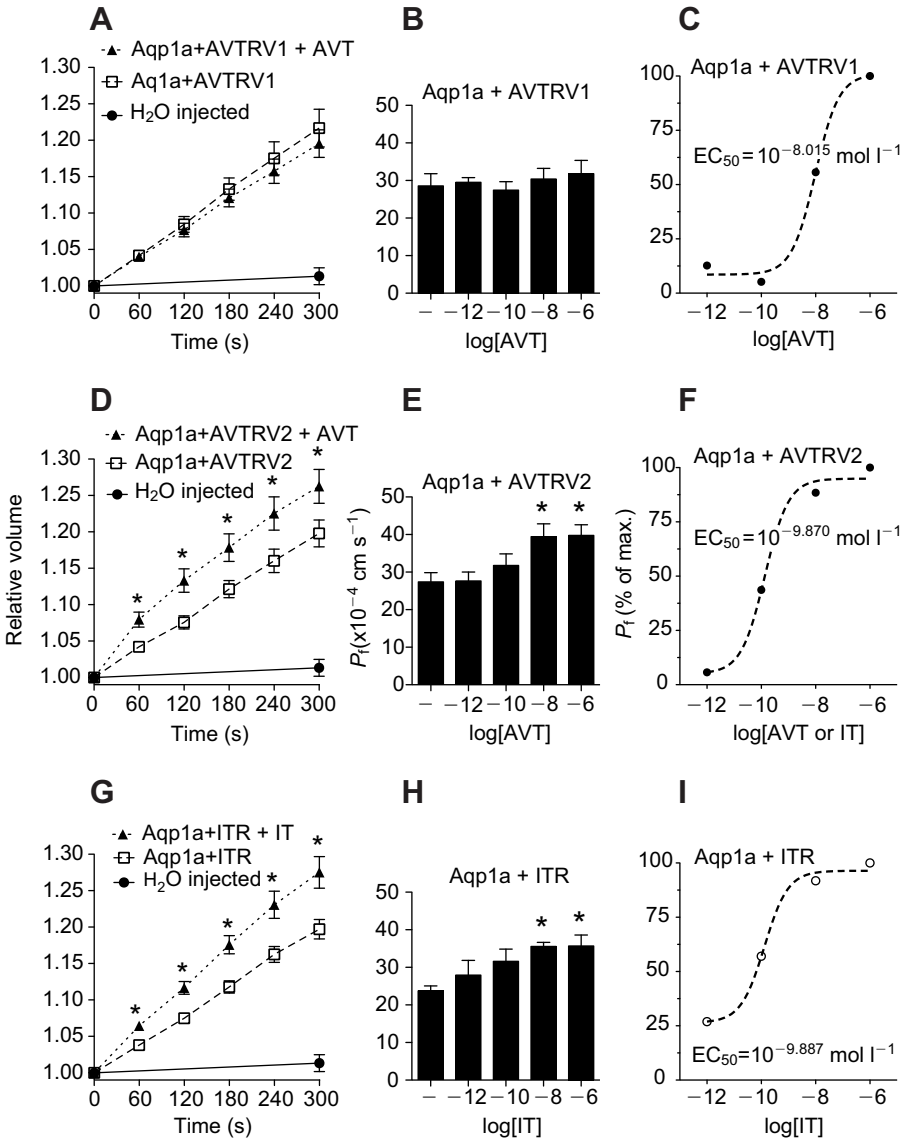

Fig. 5. Relative volume increase and P_f of *X. laevis* oocytes co-injected with *Aqp1a* and *AVTRV1*, *Aqp1a* and *AVTRV2*, or *Aqp1a* and *ITR* mRNA, with or without stimulation with AVT or IT. (A,D,G) Relative volume increase following co-injection with *Aqp1a* and *AVTRV1* (A), *Aqp1a* and *AVTRV2* (D) or *Aqp1a* and *ITR* (G) mRNA with or without stimulation with 10^{-8} mol l^{-1} AVT (A,D) or 10^{-8} mol l^{-1} IT (G). Water-injected oocytes were used as controls. In A, D and G, asterisks represent a significantly different increase in relative volume in the presence or absence of hormone (P<0.05, ANCOVA). (B,E,H) The corresponding dose–response relationships of osmotic water permeability (P_f) in response to hormonal stimulation in the range 10^{-12} to 10^{-6} mol l^{-1}. In B, E and H, asterisks represent significant differences from basal P_f (P<0.05, one-way ANOVA followed by Bonferroni *post hoc* test). (C,F,I) The adjusted sigmoidal dose–response curves (from B, E and H, respectively) for calculation of individual EC_{50} values depicted in each graph.

sea bream AVT V2-type receptors could also function via PKC signaling.

The isotocinergic system has been proposed to play endocrine roles related to reproductive stage, social status and behavior in teleosts (Almeida et al., 2012; Kleszczynska et al., 2012). The role of IT in the osmoregulatory process is not well established, and sometimes seems dubious because of the lack of predictable plasma levels of the hormone (Hyodo and Urano, 1991; Kleszczynska et al., 2006; Warne et al., 2000). In osmoregulatory tissues, IT receptor expression responds to osmotic challenges in several species of teleosts, including the sea bream (Martos-Sitcha et al., 2013). However, other studies *in vitro* suggest an indirect osmoregulatory role of copeptin, a peptide derived from an IT precursor, via pituitary stimulation of prolactin expression (Flores et al., 2007). Our present results also suggest a function for IT in water balance via functional regulation of AQP1a, but not AQP1b, in sea bream (Figs 5 and 6). Significant up-regulation of sea bream AQP1a function by IT was achieved at concentrations of 10^{-8} mol l^{-1}, with a calculated EC_{50} of $10^{-9.8}$ mol l^{-1} (Fig. 5). These values give physiological meaning to the regulation of AQP1a by IT, considering that circulating plasma levels of the hormone in the sea bream are in the range 1–180 nmol l^{-1} (Kleszczynska et al., 2006; Mancera et al., 2008). Additionally, AQP1a is sensitive to PKC stimulation (Fig. 3), but not to PKA stimulation, as proposed for the ITR in teleosts (Hausmann et al., 1995).

In conclusion, the present study provides an indication of a functional involvement of AVT and IT in the regulation of water transport via AQP1 paralogs in the sea bream. The (likely) most important target of this regulation relates to regional salinity-dependent fluid processing in the gastrointestinal system, and maybe other epithelia. The levels at which exogenous AVT and IT regulate AQP-dependent water transport were in the normal physiological range for sea bream plasma (Kleszczynska et al., 2006; Mancera et al., 2008), pointing to a physiological role of both hormones *in vivo*. Additionally, only AVTRV2 and ITR were able to modulate AQP1 function in oocyte swelling assays. However, it will be important to establish how (or whether) this putative regulatory correlation in replicated in a sea bream intestinal cell *in vivo*.

MATERIALS AND METHODS

Peptides and chemicals

Arginine vasotocin ([Arg^8]-vasotocin acetate), isotocin ([Ser^4,Ile^8]-oxytocin), forskolin (FK), 3-isobutyl-1-methylxanthine (IBMX) and

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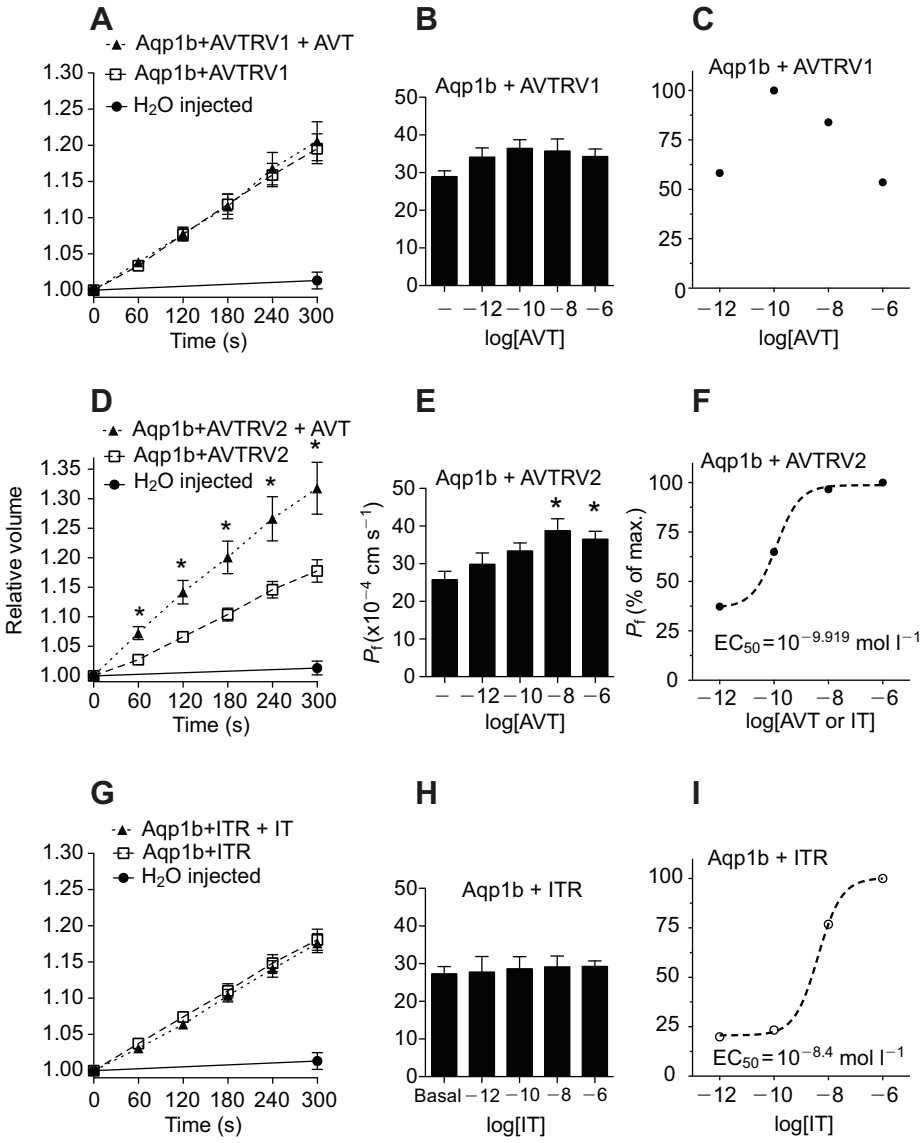

Fig. 6. Relative volume increase and P_f of *X. laevis* oocytes co-injected with *Aqp1b* and *AVTRV1*, *Aqp1b* and *AVTRV2*, or *Aqp1b* and *ITR* mRNA, with or without stimulation with AVT or IT. (A,D,G) Relative volume increase following co-injection with *Aqp1b* and *AVTRV1* (A), *Aqp1b* and *AVTRV2* (D) or *Aqp1b* and *ITR* (G) mRNA with or without stimulation with 10^{-8} mol l^{-1} AVT (A,D) or 10^{-8} mol l^{-1} IT (G). Water-injected oocytes were used as controls. In A, D and G, asterisks represent a significantly different increase in relative volume in the presence or absence of hormone (P<0.05, ANCOVA). (B,E,H) The corresponding dose–response relationships of osmotic water permeability (P_f) in response to hormonal stimulation in the range 10^{-12} to 10^{-6} mol l^{-1}. In B, E and H, asterisks represent significant differences from basal P_f (P<0.05, one-way ANOVA followed by Bonferroni *post hoc* test). (C,F,I) The adjusted sigmoidal dose–response curves (from B, E and H, respectively) whenever possible for calculation of individual EC_{50} values depicted in each graph.

phorbol 12,13-dibutyrate (PDBU) were of the highest grade and were supplied by Sigma-Aldrich (Madrid, Spain).

Animals

Sea bream (*S. aurata*) juveniles were obtained from commercial sources (CUPIMAR SA, Cádiz, Spain) and maintained in open-seawater circuits under natural conditions of water temperature (18–20°C), photoperiod and salinity (37 ppt) at a density of <5 kg m^{-3}. For maintenance, fish were fed twice daily to a final ration of 2% of body mass, with a commercial sea bream diet (Trow España SA, Cojóbar, Burgos, Spain). All fish were fasted for 24 h before experimental manipulations.

Sexually mature female *Xenopus laevis* were obtained from commercial sources (Xenopus Express, France), maintained at 18°C in 60 l tanks and fed daily.

All animal manipulations were carried out in compliance with the Guidelines of the European Union Council (86/609/EU) and Portuguese legislation for the use of laboratory animals. All animal protocols were performed under a Group-1 license from the Direcção-Geral de Veterinária, Ministerio da Agricultura, do Desenvolvimento Rural e das Pescas, Portugal.

Aqp1 and *AVTR*/*ITR* constructs

For tissue collection, sea bream (body mass 250 g, immature males) were anesthetized (2-phenoxyethanol 1:10,000; Sigma-Aldrich). After decapitation, samples from the anterior intestine, which corresponds to a section of 2–2.5 cm in length caudal to the point of insertion of the pyloric caeca, were collected from individual fish, kept overnight in RNA*Later* (Ambion, Austin, TX, USA) at 4°C and stored at −20°C until utilized for RNA extraction (within 2 weeks). Total RNA was isolated using the E.Z.N.A. Total RNA Kit (OMEGA Bio-tek, Norcross, GA, USA) following the manufacturer's instructions, and the quantity and quality assessed (Nanodrop 1000, Thermo Scientific, Barrington, IL, USA). RNA was treated with DNase using a DNA-free kit (Ambion, Life Technologies, Paisley, UK); 500 ng total RNA were reverse transcribed (RevertAid™ First Strand cDNA Synthesis Kit, no. K1622, Fermentas, Thermo Scientific) and PCR amplifications were carried out with 200 ng of cDNA with iProof™ High-Fidelity DNA Polymerase (Bio-Rad, Hercules, CA, USA) with the following protocols: 98°C, 4 min; [98°C, 15 s; 60°C, 15 s; 72°C, 1 min]×35 cycles; 72°C, 10 min) for *Aqp1a* (GenBank accession number: AY626939), *Aqp1b* (accession no. AY626938), *AVTRV1a2* (accession no. KC195974), *AVTRV2* (accession no. KC960488) and ITR (accession no. KC195973). Primers used for amplification are shown in Table 1. For identity confirmation, PCR products were ligated to pGEM T-easy vector (PROMEGA, Madison, WI, USA) and sequenced in both directions (CCMar, Faro, Portugal).

Aqp1a and Aqp1b distribution in the sea bream intestine

In situ hybridization

Sea bream (body mass 200 g, immature males, N=3) kept at 35 ppt were anesthetized (2-phenoxyethanol 1:10,000) and killed by decapitation. The

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Table 1. Primers used for molecular identification of open reading frames (ORFs) of the *Aqp1a*, *Aqp1b*, *AVTRV1a2*, *AVTRV2* and *ITR* sequences

	Nucleotide sequence (5′–3′)	Size (bp)
UTR primers		
Aqp1a-Fw	CACAGCCAGACAGCAACACTGC	822
Aqp1a-Rv	TTAAGTCTGTGTGGGACTATTTTG	
Aqp1b-Fw	GGTTTGACACACCGAACACATTCG	851
Aqp1b-Rv	GCTTAAGTCTGTGTGGGATCAGTGC	
AVTR V1-type Fw	GGTCTCTGGTTTTCAGTCC	1266
AVTR V1-type Rv	CTGTGAGCTGTGTACCTTGATG	
AVTR V2-type Fw	AGGACACGCGTGAGAAAGCTTACC	1596
AVTR V2-type Rv	ACTGTGTGTCATTCTCTGTCGC	
ITR Fw	GACTCTTGTGTTGTGATGTGACCG	1295
ITR Rv	ATTGCCAGGTTACTCAACTACAGG	
ORF primers		
*Eco*RI-Aqp1a-Fw	CCG*GAATTC*ATGAGAGAGTTCAAGA	777
*Xba*I-Aqp1a-Rv	GCAAGCTAG*TCTAGA*CTATTTTGACGTC ATCTCTACAGC	
*Eco*RI-Aqp1b-Fw	CCG*GAATTC*ATGACAGAAGTAAAAA	804
*Xba*I-Aqp1b-Rv	GCTGGCTAG*TCTAGA*TCAGTGCTTTGGC CACTGACTTGG	
A-V1-type Fw	***A***ATGGGAACCCCTGGAAACGAC	1167
*Xba*I-V1-type Rv	CTAG*TCTAGA*CTAGCTGTGATTTTCCTCC GGCTG	
*Eco*RI-V2-type Fw	CCG*GAATTC*ATGGAAAGCATCAGTTTGG	1521
*Xba*I-V2-type Rv	CTAG*TCTAGA*TCAGTACAGGCTGTCCTTGG	
*Eco*RI-ITR Fw	CCG*GAATTC*ATGGAGGACTTTTTACGCGA	1177
*Xba*I-ITR Rv	GCTAG*TCTAGA*TCAGTGTCCAAGTCCCCCC GTGCT	

Restrictions sites are in italics and underlined. Additional nucleotides added for maintaining the ORF are in bold italics and underlined.
UTR, untranslated region.

anterior intestine and rectum were collected from individual fish, washed and fixed in 4%PFA/1× PBS pH 7 overnight at 4°C. The following day the tissues were washed in 1× PBS+0.1% Tween-20 (Sigma; PBT) and stored at −20°C in methanol until use (within 3 days). Tissues were embedded in paraffin as described elsewhere (Campinho et al., 2012) and serial 5 µm sections were cut and set on glass slides.

In situ hybridization sense and anti-sense cRNA DIG-labeled probes for *Aqp1a*, *Aqp1b*, *AVTRV1a2*, *AVTRV2* and *ITR* were generated by *in vitro* transcription using SP6 or T7 (Fermentas), respectively, according to the manufacturer's instructions. *In situ* hybridization analysis was carried out as described elsewhere (Campinho et al., 2012). All slides for the same gene analyzed were processed in parallel. Images were taken in a Leica DM2000 light microscope coupled to a Leica DFC480 digital camera. Scale bars were added to images in ImageJ (Schindelin et al., 2012) and figures assembled in Photoshop (Adobe).

qPCR

For *Aqp1a* and *Aqp1b* expression analysis, the same experimental protocol previously described (Gregorio et al., 2013; Martos-Sitcha et al., 2013) was used. In short, juvenile sea bream (N=21; 20–30 g body mass) were kept at different salinities (12, 35 or 55 ppt) for 2 months before tissue collection and were considered fully adapted (Laiz-Carrion et al., 2005). Fish were fed normally during the trial and no mortality was registered.

Total RNA was isolated and cDNA was produced as described above. Real-time PCR (qPCR) amplifications were performed in duplicate in a final volume of 10 µl with 5 µl PerfeCTa SYBR® Green FastMix™ (Quanta BioSciences), 0.5 pmol µl^{-1} of each forward and reverse primer, and 200 ng cDNA. qPCR was performed with the following cycling conditions: 95°C, 10 min; [95°C, 20 s; 60°C, 35 s] for 45 cycles. Melting curves for all analyzed genes were performed from 60°C to 95°C with stepping intervals of 15 s. 18S ribosomal RNA (*18S*) was used as an internal standard. All calibration curves exhibited correlation coefficients R^2>0.98, and the corresponding real-time PCR efficiencies were >99%.

Table 2. Primer sequences (5′ to 3′), PCR amplicon size (bp) and corresponding NCBI accession numbers used for qPCR expression analysis of *Aqp1a*, *Aqp1b* and *18S*

Primer	Sequence	Amplicon	Accession no.
qAqp1a-Fw	GGCTCTCACGTACGATTTCC	153	AY626939
qAqp1a-Rv	TCTGTGTGGGACTATTTTGACG		
qAqp1b-Fw	GCGACGGAGTGATGTCAAAGG	203	AY626938
qAqp1b-Rv	AGATAAGAGCCGCCGCTATGC		
18S Fw	AACCAGACAAATCGCTCCAC	139	AY993930
18S Rv	CCTGCGGCTTAATTTGACTC		

Relative gene quantification was performed using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). Primers used for qPCR are shown in Table 2.

Expression vectors and capped mRNA production

A PCR was run using the pGEM T-easy vectors containing the specific sequences as templates, and the same PCR program as described above. Specific restriction enzyme digestion sites were included in the primer sequences used and the open reading frames of the fusion proteins were maintained (Table 1). Amplified PCR products, as well as pCS2+ were double-digested for 2 h at 37°C with *Xba*I and *Eco*RI (FastDigest, Fermentas, Thermo Scientific) and fragments of interest were separated in agarose gel and purified (GeneJET Gel extraction kit, Fermentas, Thermo Scientific). Digested products were cloned into pCS2+, amplified, purified and sequenced to confirm correct assembly of the constructs.

For capped mRNA production, 5 µg of each expression vector was linearized with *Not*I (FastDigest, Fermentas, Thermo Scientific) for 2 h at 37°C and precipitated overnight at −20°C using 0.1 volumes of sodium acetate (3 mol l^{-1}) and 2.5 volumes of 100% ethanol. The linearized DNA template was centrifuged and cleaned twice with 70% ethanol then resuspended in nuclease-free water. DNA purity and concentration were obtained spectrophotometrically (Nanodrop 1000, Thermo Scientific). *In vitro* capped messenger RNA (cRNA) synthesis was carried out from 1 µg of linearized vector using the mMESSAGE mMACHINE® SP6 kit (Ambion, Life Technologies) for 2 h at 37°C. At the end of the *in vitro* transcription reaction, the remaining DNA was digested with TURBO™ DNase. Transcribed mRNA was phenol–chloroform purified and precipitated as described above then resuspended in H_2O.The integrity and expected size of each mRNA reaction was examined by electrophoresis, the quantity was assessed (Nanodrop 1000), and it was aliquoted and stored at −80°C until use.

Oocyte isolation, microinjection and swelling assay

For ovary collection, sexually mature female *X. laevis* were anesthetized with neutralized benzocaine (250 mg l^{-1}) and killed by decapitation. Both ovaries were removed and kept in sterile MBS solution (88 mmol l^{-1} NaCl, 1 mmol l^{-1} KCl, 2.5 mmol l^{-1} $NaHCO_3$, 5 mmol l^{-1} Hepes, pH 7.6). For oocyte defolliculation, the ovary was manually disaggregated in small lumps and transferred to sterile Ca^{2+}-free ND96 solution (96 mmol l^{-1} NaCl, 2 mmol l^{-1} KCl, 1 mmol l^{-1} $MgCl_2$, 5 mmol l^{-1} Hepes, pH 7.6) containing type Ia collagenase (1 mg ml^{-1}, Sigma-Aldrich) for 1.5–2 h with gentle agitation. After defolliculation, oocytes were individualized, and washed three times in Ca^{2+}-free ND96 solution, three times in ND96 solution containing 1.8 mmol l^{-1} $CaCl_2$ and three times with NDE (ND96 containing 2.5 mmol l^{-1} pyruvate, 10 µg ml^{-1} streptomycin, 100 U ml^{-1} penicillin, pH 7.6). Oocytes in stages V and VI were identified under the stereomicroscope and maintained in NDE at 18°C until use (within 3 days of isolation) with daily medium changes.

Twenty-four hours after defolliculation, oocytes received a single microinjection (Nanoliter 2000, World Precision Instruments, Sarasota, FL, USA) of 50 nl of water with 10 ng of each cRNA in the following combinations: (1) no cRNA (H_2O injected control); (2) *Aqp1a*; (3) *Aqp1b*; (4) *Aqp1a+AVTRV1a2*; (5) *Aqp1a+AVTRV2*; (5) *Aqp1a+ITR*; (7) *Aqp1b+AVTRV1a2*; (8) *Aqp1b+AVTRV2*; and (9) *Aqp1b+ITR*.

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Swelling assays were carried out 3 days post-injection in all the tested combinations. Swelling was produced by a change in osmolality (ΔOsm) of 180 mOsm; standard NDE=201.00±1.73 mOsm kg^{-1}; $NDE_{swelling}$=21.66±1.20 mOsm kg^{-1} (Vapro 5520 osmometer Wescor, USA). The osmotic water permeability coefficient (P_f, cm s^{-1}) of each oocyte was calculated from the oocyte surface area (S, cm^2), initial oocyte volume (V_0, cm^3), molar volume of water (υ_w=18 cm^3 mol^{-1}) and the initial rate of oocyte swelling [$d(V/V_0)/dt$, s^{-1}], according to the expression (Agre et al., 1999):

$$P_f = V_0 \cdot [d([V/V_0])/dt]/(S \cdot \upsilon_w \cdot \Delta Osm), \quad (1)$$

where P_f is 10^{-4} cm s^{-1}. Oocyte volume (assuming the shape of a perfect sphere) and surface area were calculated from images captured at timed intervals up to 5 min with a digital camera (Visicam 10.0, VWR, Portugal) attached to a stereomicroscope (SZ-60 Olympus). According to this general method, the following sub-assays were performed: (1) sensitivity of AQP1a and AQP1b to PKA (with a combination of 100 µmol l^{-1} IBMX and 10 µmol l^{-1} FK) or PKC (2 µmol l^{-1} PDBU); (2) effect of AVT or IT (10^{-8} mol l^{-1}) on AQP1a and AQP1b functionality in oocytes devoid of sea bream receptors; and (3) water permeability in oocytes co-expressing *Aqp1a* or *Aqp1b* together with *AVTRV1a2*, *AVTRV2* or *IRT* in response to hormonal stimulation.

To cover the range of circulating plasma values in the sea bream (Kleszczynska et al., 2006; Mancera et al., 2008) for both hormones, swelling assays were performed in the absence (basal group) or presence of AVT (AVTR V1a2-type, AVTR V2-type) or IT (ITR) receptors at concentrations of 10^{-12}, 10^{-10}, 10^{-8} or 10^{-6} mol l^{-1}. For hormonal/pharmacological manipulations, oocytes were pre-incubated for 15 min in the presence of the agent, which was also present during the swelling assay in diluted medium ($NDE_{swelling}$).

Statistics

Results are presented as means±s.e.m. All statistics were performed after assessing homogeneity of variance and normality. Expression analysis of aquaporins was assessed with a two-way analysis of variance (ANOVA), considering intestinal region and external salinity as main factors. As the interaction between both factors was highly significant (P<0.001), subsequent statistical analysis of the data was carried out using, as appropriate, Student's t-test (intestinal region) and one-way ANOVA followed by the *post hoc* Bonferroni test (salinity). Analysis of covariance (ANCOVA) was used to compare regression lines in swelling assays by studying the interaction of the categorical variable (presence of hormone) with the continuous independent variable (time). In those cases where a regulatory action of hormones on aquaporin function was significant, P_f values were used for calculation of individual EC_{50} values (mol l^{-1}) and adjusted to sigmoid curves. All statistical analyses were performed with Prism 5.0 (GraphPad Prism 5.0 for Macintosh, GraphPad Software, San Diego, CA, USA) and groups were considered significantly different at P<0.05.

Competing interests

The authors declare no competing or financial interests.

Author contributions

J.F. conceived the study. J.A.M.-S., M.A.C. and J.F. performed the experiments. J.A.M.-S. and J.F. drafted the manuscript. J.A.M.-S., M.A.C., J.M.M., G.M.-R. and J.F. wrote the paper.

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