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Sexual maturation and changes in water and salt transport components in the kidney and intestine of three-spined stickleback (*Gasterosteus aculeatus* L.)

Steffen S. Madsen [a,*], Claus Weber [a], Andreas M. Nielsen [a], Mohammad Mohiseni [c], Maryline C. Bosssus [b], Christian K. Tipsmark [b], Bertil Borg [d]

[a] Department of Biology, University of Southern Denmark, Campusvej 55, DK-5230 Odense Denmark
[b] Department of Biological Sciences, University of Arkansas, SCEN 601, Fayetteville, AR 72701, USA
[c] Behbahan Khatam Alanbia University of Technology, Iran
[d] Department of Zoology, Stockholm University, S-106 91 Stockholm, Sweden

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ABSTRACT

Mature three-spined stickleback males use spiggin threads secreted from their kidney to glue together nest material. This requires strongly hypertrophied renal proximal tubular cells, which compromises renal osmoregulatory function during the breeding period. Experimental evidence suggests that the intestine takes over hypotonic fluid secretion at that stage but the mechanism is unexplored. To unravel the molecular mechanism we analyzed and compared transcript levels of several membrane proteins involved in water and salt transport in intestinal and renal tissues, in non-mature males (NM), mature males (MM), and mature females (MF). Aquaporin paralogs *aqp1a*, *-3a*, *-8aa*, *-8ab*, *-10a*, *and -10b*, two Na^+,K^+-ATPase alpha-1 subunit isoforms (*nka547*, *nka976*), Na^+,K^+,$2Cl^-$-, and Na^+,Cl^--cotransporters (*nkcc1a*, *nkcc2*, *ncc*), the cystic fibrosis transmembrane conductance regulator (*cftr*) and two *claudin isoforms* (*cldn2*, *cldn15a*) were expressed in the intestine and kidney in all groups. There were no differences in *aqp* and *cldn* expression between intestines of NM and MM; *nkcc2* was lower and *nka* levels tended to be higher in intestines of MM than in NM. In the kidney, *aqp1* and *aqp8ab* levels were lower in MM than in NM, whereas *aqp3a*, *nkcc1a*, *cldn15a*, and *spiggin* were markedly elevated. This was accompanied by marked hypertrophy of kidney tubules in MM. The data support an altered kidney function in terms of water handling in mature males, whereas there was no support for modified trans-epithelial water permeability or salt-secretory activity in the intestine of mature males. Salt-absorptive activity in the intestine may, however, be down-regulated during male maturation.

1. Introduction

Among invertebrates, there are several examples of extraorganismal proteins being secreted in bulk amounts with the purpose of attachment, building hunting webs, cocoons, etc. (Tsuda et al., 1979; Devore et al., 1984; Gosline et al., 1999; Mondal et al., 2007). Vertebrates, on the other hand, only rarely use such engineering skills. One example which has long fascinated zoologists occurs among males of the teleostean stickleback family (Gasterosteidae), where nest building occurs aided by a glue-like proteinaceous substance secreted from the kidney tubules and stored in the urinary bladder. A pioneering record (Möbius, 1885) pointed out that the secreted thin threads originated from the kidney in the male fifteen-spined stickleback, *Spinachia spinachia*. Later, Hess (1918) described the fascinating anatomical transformation of the kidney in the five-spined stickleback *Culaea (Eucalia) inconstans*, during its annual reproductive cycle. Primarily, the cells of the secondary proximal tubular epithelium transform into a secretory epithelium, producing bulk amounts of a sticky protein, which years later was characterized, sequenced, and given the name "spiggin," named after "spigg" meaning stickleback in Swedish (Jakobsson et al., 1999; Jones et al., 2001).

The three-spined stickleback (*Gasterosteus aculeatus*) occurs widespread in the Holarctic region and may live and reproduce in freshwater (FW), brackish water as well as coastal marine environments. In anadromous and thus euryhaline populations, sexual maturation occurs in the late spring, where the fish seek residence in FW or low-salinity brackish water. In the male, 11-ketoandrogens are efficient inducers of a pronounced structural reorganization and hypertrophy of the secondary proximal and collecting tubule epithelia cells (De Ruiter and Mein, 1982; Borg et al., 1993), which become functionally compromised by the switch to synthesize and secrete spiggin. The agglutinate is stored in the bladder until used as a structural and highly elastic adhesive thread to assemble a nest in which the female lays her eggs (Hess,

* Corresponding author. Tel.: +45 6550 2450.
E-mail address: steffen@biology.sdu.dk (S.S. Madsen).

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1918). Very little is known about the consequences of this functional specialization for the osmoregulatory function of the kidney. Fish living in a hypotonic environment generally depend on their filtrating kidney to secrete surplus water taken up by osmosis across the gills and it appears that the production and storage of bulk amounts of a slimy, proteinaceous fluid is incompatible with a high urine output. Indeed this is what De Ruiter (1980b) reported in his thesis and subsequent publications on the hydromineral regulation of three-spined stickleback during sexual development. The pronounced kidney hypertrophy is accompanied by reduced glomerular filtration rate (GFR) and loss of ion reabsorptive capacity and leaves the kidney with a reduced osmoregulatory function. In order to maintain water balance, FW sticklebacks must therefore be expected to use one or more extra-renal compensatory mechanisms for water elimination. De Ruiter and Wendelaar Bonga (1985) elegantly demonstrated by use of micro-catheterization that sexually maturing males initiate secretion of a hypotonic fluid originating from their intestine, which suggests a reorganization of organ function specific to sexually mature males, since mature females do not induce this function. This was supported by development of a more extensive basolateral membrane system of the enterocytes (basal labyrinth; De Ruiter et al., 1985), which led the authors to hypothesize the development of a "backward channel standing gradient osmotic flow function" of the intestine (Diamond and Bossert, 1967, 1968). According to this model, fluid secretion may occur across an epithelium if salt secretion is accompanied by the presence of appropriate transepithelial water permeability. Water may in principle penetrate an epithelium via para- or transcellular routes: De Ruiter proposed a transcellular pathway based on an expectation of water tight junctions between the enterocytes. No studies have, however, investigated the nature and molecular composition of intestinal tight junctions in sticklebacks or of the presence and distribution of aquaporins to facilitate water transport in this epithelium.

To the best of our knowledge, intestinal fluid secretion as a natural contribution to hyperosmoregulation in fishes has never been reported before or after this discovery. In mammals, the pathophysiological condition of diarrhea is induced by bacterial endotoxins and is due to stimulated salt secretion in association with reduced water reabsorptive capacity due to mis-localization of aquaporin 2 and 3 (Field, 2003). A similar phenomenon was, however, induced in seawater-acclimated killifish (*Fundulus heteroclitus*) intestines when intracellular levels of Ca^{2+} and cAMP were stimulated pharmacologically *in vitro* (Marshall et al., 2002). Whether this condition reflects a physiological scenario is unknown. Recently, fluid secretion from the distal part of the intestine was induced by guanylin peptides in the Gulf toadfish, *Opsanus beta*, which may facilitate deposition of $CaCO_3$ precipitates (Ruhr et al., 2014). Fluid secretion from the intestine is counterintuitive to hypo-osmoregulation in marine fishes, but it may theoretically be advantageous in a FW fish. Whereas evidence for intestinal fluid secretion in fishes is scarce, it is well accepted that intestinal fluid absorption is a common compensatory mechanism for dehydration in marine fishes (Edwards and Marshall, 2013). There is evidence that osmotic water flow may occur transcellularly through aquaporins as well as paracellularly through tight junctions (Grossell, 2011; Madsen et al., 2011). There are 13 members of the aquaporin family in mammals and even more in fishes (Tingaud-Sequeira et al., 2010). All aquaporins are water permeable; a subfamily—the aquaglyceroporins—have additional permeabilities to small nonpolar solutes such as urea and glycerol, so their function may be linked to metabolic events as well. In order to establish transcellular water movement though aquaporins, they must be present in both apical and basolateral membrane domains and a favorable osmotic gradient must be established. The permeability of tight junctions is critically controlled by proteins of the claudin family, i.e. junctions guarded by specific claudin isoforms may serve as ionic barriers or display selective permeabilities to anions, cations, and water (see Angelow et al., 2008; Hou et al., 2013). Two particular claudin isoforms, claudin-2 and -15, have been shown to create water permeable junctions in other systems (zebrafish (*Danio rerio*) intestine: Bagnat et al., 2007; transfected MDCK C7 cells: Rosenthal et al., 2010).

The energetic drive for any fluid transport across an epithelium is the simultaneous (active) transport of osmolytes (typically NaCl) in order to establish favorable osmotic conditions. Thus to absorb fluid, NaCl must be absorbed in parallel and fluid secretion requires salt secretion followed by osmosis. This mechanism will usually transport more or less isotonic fluid. In order to secrete a hypotonic fluid, subsequent reabsorption of salt must therefore take place. This process is thus more complex and requires spatial separation of fluid secretion and salt reabsorption. Among the various ion-transport proteins involved in epithelial NaCl transport are basolateral Na^+,K^+-ATPase in combination with apical Na^+,K^+,$2Cl^-$ (NKCC2)- or Na^+,Cl^- (NCC)-cotransport for absorption or in combination with apical CFTR and basolateral NKCC1-cotransport (for secretion). Paracellular transport of NaCl mediated by leaky pores created by claudins may also be involved in certain epithelia. In this study, we tested the hypothetical model of these processes shown in Fig. 1.

In particular, we wanted to test if there is evidence of a modified transport activity in the intestine of sexually maturing stickleback males which could facilitate increased hypotonic fluid secretion. This was accomplished by analyzing and comparing transcript levels of selected aquaporins, ion-transport, and claudin proteins in maturing and non-maturing males. First, we analyzed the tissue distribution of the selected targets to assure their relevance and then focused the investigation on transcript levels in the intestine and kidney of sexually mature and non-mature males. Mature females were used for comparison. In line with the hypothesis of De Ruiter (1980b), our expectation was that there would be strong signs of developmental changes in both the intestine and kidney during male sexual maturation, assuming a shift in the respective roles of these two organs in eliminating hypotonic fluid.

2. Materials and methods

2.1. Fish and sampling

2.1.1. Experiment 1: Tissue distribution of aquaporins

For this part of the study, 10 non-mature adult three-spined sticklebacks (*Gasterosteus aculeatus*, mixed sexes) were caught in brackish water (10 ppt) in the Kerteminde Fjord (Denmark) in September 2012. The fish were transported to the lab in aerated containers, and sampling took place the same day. Each fish was anaesthetized in 0.5 ppm 2-phenoxyethanol and subsequently killed by cervical dislocation. The following tissues were excised and immediately frozen in liquid nitrogen: liver, esophagus, brain, kidney, anterior intestine, posterior intestine, gill, and gonads. The experimental procedures were approved by the Danish Animal Experiments Inspectorate in accordance with the European convention for the protection of vertebrate animals used for experiments and other scientific purposes (#86/609/EØF).

2.1.2. Experiment 2: Effects of sexual maturation

Three-spined sticklebacks were originally obtained from the sea (10 ppt) near Skanör (Sweden) in the spring of 2011 and brought to the University of Stockholm. Prior to experimentation, two subgroups had been held at different photo- and temperature regimes in 5 ppt artificial saltwater.

Some of the fish were transferred to LD 16:8 at 20 °C in 5 ppt artificial seawater for maturation soon after capture. At the time of the experiment, these fish (males) were in a post-breeding state and denoted as non-mature males (NM). The other fish were kept under winter conditions (LD 8:16 at 8 °C) in similar water until September 8 when they brought into LD 16:8 and 18 °C to mature. On September 16, all fish were transferred to FW and held at LD 16:8 and 18 °C until sampling. The FW was Stockholm tap water in a thoroughly cleansed