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Dynamic changes in nitric oxide synthase expression are involved in seawater acclimation of rainbow trout, *Oncorhynchus mykiss*

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Running head: Nos/NO system during salinity acclimation
Abstract

Recent research has shown that nitric oxide (NO) produced by nitric oxide synthases (Nos) is an inhibitor of ion transporter activity and a modulator of epithelial ion transport in fish but little is known on changes in the Nos/NO system during osmotic stress. We hypothesized that the Nos/NO system responds to salinity changes as an integrated part of the acclimation process. Expression and localization of nos1/Nos1 and nos2/Nos2 were investigated in gill, kidney and intestine of FW- and SW-transferred trout using qPCR, Western blotting and immunohistochemistry, along with expressional changes of major ion transporters in the gill. The classical branchial ion transporters showed expected expressional changes upon SW-transfer, there among a rapid decrease in Slc26a6 mRNA, coding a branchial Cl/HCO$_3^-$ exchanger. There was a major down-regulation of nos1/nos2/Nos2 expression in the gill during SW-acclimation. A significant decrease in plasma nitrite supported an overall decreased Nos activity and NO production. In the middle intestine, Nos1 was up-regulated during SW-acclimation, whereas no changes in nos/Nos expression were observed in the posterior intestine and the kidney. Nos1 was localized along the longitudinal axis of the gill filament, beneath smooth muscle fibers of the intestine wall and in blood vessel walls of the kidney. Nos2 was localized within the epithelium adjacent to the gill filament axis and in hematopoietic tissues of the kidney. We conclude that down-regulation of branchial Nos is integrated to the SW-acclimation process likely to avoid inhibitory effects of NO on active ion extrusion.

Keywords: Hyper-osmotic stress, Ion transporter, Nitric oxide, Nitric oxide synthase, Osmoregulation.
**Introduction**

The signalling agent nitric oxide (NO) is produced by the enzyme NO synthase (Nos), which is expressed as two isoforms in fish: Nos1 (or neuronal nNOS) and Nos2 (or inducible iNOS) (2). NO is known to influence various physiological functions in fish, including those associated with environmental stress responses. For example, involvement of the Nos/NO system was reported in some fish species exposed to hyper-ammonia, pathological conditions, hypoxia/anoxia, high ambient nitrite, desiccation stress, temperature elevation and acid/base disturbance (3, 7, 8, 9, 10, 11, 20, 26, 31, 34, 37, 53, 64, 67). Surprisingly, dynamics of the Nos/NO system during osmotic stress has not received much attention despite its emerging role as a modulator of ion transport (21, 59). In a recent study, we found that NO was involved in the down-regulation of Cl⁻ secretion elicited by hypotonic exposure in the opercular epithelium of the SW-acclimated killifish (21). Two studies have reported temporary elevation of nos1 mRNA level during hyper-osmotic stress in the preoptic-hypothalamic and caudal neurosecretory system of tilapia (Oreochromis mossambicus) and European flounder (Platichthys flesus), suggesting that NO is a short-term effector in the neuronal response for acclimation to hyper-osmotic stress (12, 48). However, there are no reports of the dynamics of the Nos/NO system in the osmoregulatory gill, kidney and intestine during hyper-osmotic stress.

The euryhaline rainbow trout (Oncorhynhynchus mykiss) is a commonly used model in research on salinity acclimation, and many facets of its acclimatory responses are well described from the molecular to the organismic level. There is an overall shift from an ion-absorptive mode in freshwater (FW) to an ion-secretory mode in seawater (SW), which among other details (see 29, 49) includes an important differential regulation of branchial ion transport proteins, including Na⁺, K⁺-ATPase (Nka) isoforms, Na⁺, K⁺,2Cl⁻ cotransporter (Nkcc), cystic fibrosis transmembrane conductance regulator (Cftr), H⁺-ATPase, and Cl⁻/HCO₃⁻ exchanger (Sle26a6) (4, 27, 45, 63, 66, 70). This regulation is controlled by multiple signalling events, including long-term endocrine transcriptional effectors (e.g. cortisol, growth hormone and prolactin) and rapid but short-term neurotransmitters of the adrenergic system (e.g. catecholamines) (15, 44, 50, 52). NO is likely to be an additional agent, since its signalling functions include modulation of NaCl transport in the opercular epithelium (gill-like epithelium) and intestine of SW fish (16, 21, 71) and modulation of enzymatic activity of ion transporters like Nka and H⁺-ATPase in gill, kidney and intestine of fish (13, 60, 69). We therefore hypothesized that the Nos/NO system responds during salinity changes as an integrated mediator of the acclimatory process. In order to investigate this, we analysed...
expression of nos1/Nos1 and nos2/Nos2 in gill, kidney and intestine of rainbow trout switching from an absorptive (hyper-osmoregulating) to a secretory (hypo-osmoregulating) mode during SW-transfer. Measurement of the NO metabolite nitrite (NO$_2$-) was used as indicator of Nos activity and NO production (43). The kinetics of expressionional changes of classical branchial ion transporters (nka a1a, nka a1b, cftr1, nkcc1 and slc26a6) were used to verify progress of the physiological adjustments. In parallel, Nos1 and Nos2 were localized in the osmoregulatory tissues using immunohistochemistry to further identify the potential role(s) of NO in each tissue.

Materials and methods

Animals

Juvenile all-female rainbow trout, O. mykiss (24.5 ± 0.3 g) were obtained from a local fish farm (Lihme, Randbøl, Denmark). Fish were maintained indoor at the University of Southern Denmark (Odense Campus) at 15°C and 12h:12h light:dark cycle in aerated bio-filtered, recirculated freshwater. Fish were fed commercial trout food (Inicio, Biomar, Denmark) until three days before start of experiments. The experimental work followed the guidelines of the Danish Law on Animal Experiments.

Experiment 1

A preliminary experiment was conducted to track the detailed kinetics of changes in mRNA expression of nos and various ion transporter genes in the gill during SW-acclimation and to report changes in branchial Nos protein abundance 168 h after SW-transfer. Groups of eight fish were directly transferred from FW to 80% artificial SW (25 ppt, Red Sea Salts, Verneuil s/Avre, France) and sampled at 4 h, 24 h, 72 h and 168 h after the transfer. A time 0 FW group served as control. Upon sampling, individual fish were netted and anesthetized with 2-phenoxy-ethanol (Sigma-Aldrich, Steinheim, Germany) in either FW or SW and then euthanized by cutting the spinal cord. The 2nd left gill arches were dissected out and stored at -80°C until analysis by qPCR.

Experiment 2

Based on results from experiment 1, a full experiment was designed with parallel sampling of both FW-FW and FW-SW transferred fish. In this experiment, changes in nos gene and Nos protein expression were evaluated in gill, kidney and intestine, along with the transcriptional change in branchial ion transporters. Groups of eight fish were placed in separate tanks before the experiment
and sampled 24 h and 168 h after FW-FW transfer (control groups) or FW-80% artificial SW-transfer. A FW control group was sampled at 0 hr. Upon sampling, individual fish were netted and anesthetized with 2-phenoxy-ethanol (Sigma) in either FW or SW. Blood was drawn from the caudal vessels before the fish was killed by cutting the spinal cord. The 2nd and 3rd left gill arches, middle intestine (section between the pyloric caeca and the ileorectal valve), posterior intestine (section after the ileorectal valve) and the posterior trunk of kidney were dissected out. Samples were taken for multiple analyses by qPCR, immunohistochemistry and immunoblotting. Blood samples were immediately centrifuged (2 min at 12,000 g) and the plasma was frozen in liquid nitrogen and stored at -80 °C for later measurements of NO metabolite nitrite and osmolality.

**Extraction of RNA, cDNA synthesis and real-time qPCR**

Total RNA was extracted with TRIzulre™ reagent (Bioline, London, UK) and DNase treated (Promega Biotech AB, Stockholm, Sweden) prior single-stranded cDNA synthesis using a High Capacity cDNA reverse transcriptase kit (Applied Biosystems, Foster, CA, USA) with 1 μg total RNA in a total volume of 20 μl. All steps were conducted following the manufacturer's instructions. Real-time PCR analysis was carried out on a Mx3000P instrument (Stratagene, La Jolla, CA, USA) using standard software setting. Reactions were carried out with 1 μl cDNA, 3 μl primer mix (200 nmol l⁻¹ final concentration) and 7.5 μl SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma) in a total volume of 15 μl nuclease-free water. Cycling conditions: 95°C for 3 min followed by: 95°C for 10 s, 60°C for 60 s (ef1a, nka a1a, nka a1b, cftr1, nkcc1 and slc26a6) or 64°C for 60 s (nos1 and nos2) for 40 cycles. Melting curve analysis was carried out routinely with 30 s for each 1°C temperature interval from 55°C to 95°C.

Primer sequences used in this study are listed in Table 1. nos2 and nos1 primers were designed using O. mykiss nos2 and nos1 nucleotide sequences. slc26a6 primers were developed from an O. mykiss cloned sequence (4). nka a1a, nka a1b, nkcc1, cftr1 and ef1a primers were identical to those previously described (42). Amplification efficiency of each primer set (established by a six point cDNA four-fold dilution series in duplicate and analysed by qPCR) was used for calculation of relative copy numbers of the individual target genes (61); using the formula:

\[(1+E_{tar})^{\Delta C_{tar}}/(1+E_{ef1a})^{\Delta C_{ef1a}}\]

where E is the amplification efficiency for the primer pairs, Ct is the threshold cycle value of the PCR products, and tar is the target gene. ef1a was used as normalization/housekeeping gene after confirmation of its stability among groups.
Primary antibodies

A homologous Nos2 affinity purified rabbit polyclonal antibody was designed and generated by GenScript (Piscataway, New Jersey - USA) for this study. The peptide sequence SHQTKPNKWETRAN near the N-terminus of Nos2 of rainbow trout origin was chosen as epitope for antibody generation (Genbank acc. No. CAC82808.1). We validated the specificity of the antibody by Western blot and immunohistochemistry by pre-absorption with up to 100-fold molar excess of the antigenic peptide. Cross-reactivity between Nos2 and Nos1 antibody was assessed and shown to be non-existent. The molecular masses of Nos1 and Nos2 were determined by denaturing polyacrylamide gel electrophoresis (SDS-PAGE) using SeeBlue® Pre-stained Protein Standard (Invitrogen, Carlsbad, CA, USA).

For detection of Nos1, a commercial affinity purified rabbit polyclonal antibody (SC-648; 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used. This antibody was raised against a peptide near the C-terminus of Nos1 of rat origin, and it was previously validated for Western blotting by pre-absorbing the primary antibody with Nos1 blocking peptide on rainbow trout head kidney tissue (53).

Western Blotting

Relative abundance of Nos2 and Nos1 protein in gill, middle intestine, posterior intestine and trunk kidney were determined by SDS-PAGE and Western blotting. Tissues from five 168 h FW-transferred and five 168h SW-transferred fish were homogenized in ice-cold SEI buffer (in mmol l⁻¹: 300 sucrose, 20 EDTA, 50 imidazole - pH 7.3) with 1% protease inhibitor cocktail (P8340, Sigma). The homogenates were centrifuged at 2,000 g for 10 min at 4°C to remove cell debris. The resulting supernatants were then centrifuged at 45,000 g for 30 min to pellet an enriched plasma membrane fraction. The supernatant and the re-suspended (in SEI buffer) pellet were used for further analyses. Optimal protein concentration and fractions (supernatant or pellet) to be loaded were determined in preliminary work and found to be 50 µg of gill supernatant, 50 µg of kidney pellet and 20 µg of intestine pellet. Protein determination was performed using absorbance at 280 nm with a NanoDrop ND-1000 spectrophotometer (NanoDrop technologies, Wilmington, DE). Samples were size-fractionated using 12% separating and 4% stacking polyacrylamide gels for 35 min at 200 V using NuPAGE® MES SDS running buffer (Invitrogen). Proteins were then blotted onto a nitrocellulose membrane (Amersham™ Protran™ 0.45 µm NC, GE healthcare, Germany) using a Tris-Glycine transfer buffer (7.5 mmol l⁻¹ Tris, 60 mmol l⁻¹ Glycine, 20% v/v methanol) for
2 hours at 25 V. Membranes were blocked overnight in 2% bovine serum albumin (BSA) in Tris-buffered saline containing Tween 20 (TBS-T; 20 mmol l⁻¹ Tris, 140 mmol l⁻¹ NaCl, 1% Tween 20). For immunodetection, the blots were double labelled for 1 hour at room temperature in a cocktail solution containing either the heterologous polyclonal rabbit anti-Nos1 (0.8 µg ml⁻¹) or the homologous polyclonal rabbit anti-Nos2 (1 µg ml⁻¹) antibody and a heterologous monoclonal mouse anti-β-actin (1:5000; [mAbcam 8224], Abcam®, Cambridge, UK) antibody diluted in blocking buffer. Membranes were washed 3 x 5 min in TBS-T before incubation with goat-anti-rabbit IgG Cy5® (1:3000, Invitrogen) and goat-anti mouse IgG Cy3® (1:3000, Invitrogen) secondary antibodies in blocking solution for 30 minutes at room temperature. Membranes were then washed 4 x 5 min in TBS-T. After final washing, membranes were air-dried and scanned using a Typhoon™ FLA 9500 laser scanner (GE healthcare, Little Chalfont, UK) set to either Cy5® or Cy3® excitation and emission wave-lengths (Cy5®: ~650 and ~670 nm, Cy3®: ~550 and ~570 nm respectively). Densitometric analysis of Nos bands was performed using ImageJ gel analyser (ver. 1.50i; National Institutes of Health; 65). β-actin was used as a loading control and for normalization of relative Nos abundance.

**Immunohistochemistry**

Preparation of gill, trunk kidney, middle and posterior intestine tissues from FW- and SW-acclimated trout was performed using the procedure described previously (14). Prior to immunostaining, the sections were blocked for 2 h in PBS (in mmol l⁻¹: 137 NaCl, 2.7 KCl, 1.5 KH₂PO₄, 4.3 Na₂HPO₄, pH 7.3) containing 3% BSA at room temperature. The slides were then double-labelled with either the rabbit anti-Nos1 (1 µg ml⁻¹) or the rabbit anti-Nos2 (4 µg ml⁻¹) and a mouse monoclonal anti-chicken Nka α5 antibody (0.5 µg ml⁻¹; developed by Douglas M. Fambrough and obtained from the Developmental Studies Hybridoma Bank created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA). Primary antibodies were diluted in PBS containing 3% BSA and incubated overnight at 4°C. In control slides, primary antibodies were omitted. After three washes in PBS, sections were incubated for 2 h at 37°C with goat anti-mouse IgG Oregon Green® 488 (1:3000; Invitrogen) and goat anti-rabbit IgG Alexa Fluor® 568 (1:3000; Invitrogen) secondary antibodies diluted in PBS containing 3% BSA. Sections were washed 3 x 5 min in PBS followed by a wash in distilled water. Coverslips were mounted on the slides with ProLong® Gold antifade reagent (Invitrogen). Representative
pictures of the stain tissues were obtained with a Zeiss LSM510 META confocal microscope (Carl Zeiss, Oberkochen, Germany).

**Plasma and water measurements**

The NO metabolite nitrite (NO$_2^-$) was measured in plasma by highly sensitive reductive chemiluminescence, using a Sievers (Boulder, CO, USA) Nitric Oxide Analyzer (NOA, model 280i) and previously described procedures (25, 33). Tank water [NO$_2^-$] was also measured by the chemiluminescent method, and water [Cl$^-$] was determined by coulometric titration (Sherwood Scientific Chloride Analyzer 926S, Cambridge, UK). Osmolality of plasma and water were assessed with a cryoscopic osmometer (OSMOMAT® 030, Gonotec GmbH, Berlin, Germany).

**Statistical analysis**

All data are presented as mean ± s.e.m and mRNA expression data were normalized to the FW control group at 0 h. mRNA data from experiment 1 (inserts in Fig. 1 and 2) were analysed with one-way ANOVA followed by Dunnett’s multiple comparisons tests (GraphPad Prism v5.02 software, La Jolla, CA, USA). mRNA expression and [NO$_2^-$] data from experiment 2 were analysed by generalized linear models (GLM) with gamma distribution error, and the relative abundance of Nos protein was analyzed with unpaired two-tailed Student t-tests with Welch correction for non-homogeneity of variance (RStudio, Version 0.99.903). Statistical significance was accepted at P = 0.05.

**Results**

**Plasma osmolality and transcriptional regulation of ion transporters**

Figure 1A shows that fish transferred into 80% SW experienced hyper-osmotic stress with a significant increase in plasma osmolality at 24 h. After 168 h plasma osmolality was regulated back to a level only slightly higher than in FW fish (Fig. 1A). This was accompanied by major changes in mRNA levels of key ion transporter genes. Similar transcriptional patterns were observed in gills from experiments 1 and 2. Results from experiment 1, giving more detailed insight into the kinetics, are presented in small inserts together with the corresponding results from experiment 2 (Fig. 1; Fig. 2). nka α1a was significantly down-regulated (Fig. 1B), whereas nka α1b (Fig. 1C), cftrI (Fig. 1D) and nkcc1 (Fig. 1E) were significantly up-regulated within 24 h (Fig. 1B, C, D, E and inserts) following SW-transfer. Our evaluation of slc26a6 expression kinetics during SW-acclimation
documented a drastic and rapid down-regulation of *slc26a6* mRNA levels within 4 h in SW (Fig. 1F insert), and the gene was barely expressed after 168 h (Fig. 1F).

**Change in nos expression during SW-acclimation**

Branchial mRNA levels of *nos2* and *nos1* were significantly down-regulated during SW-acclimation compared to the FW-transferred groups (Fig. 2 A, B). *nos2* expression in the FW-transferred group was also transiently decreased at 24 h but after 168 h *nos2* levels were similar to the FW control group at 0 h (Fig. 2A).

Renal (Fig. 2 C, D) and posterior intestine (Fig. 2 E, F) mRNA expression of both *nos2* and *nos1* did not differ between FW and SW-transferred groups. The levels of *nos* mRNA in the middle intestine are not reported, as no stable normalization genes were found in this tissue.

**Detection of Nos proteins and their expression during SW-acclimation**

Expression of Nos2 and Nos1 proteins in tissues of rainbow trout was investigated by Western blotting. The anti-Nos2 antibody, designed for this study, recognized a protein at 134 ± 0.8 kDa in the gill (supernatant fraction) and kidney (pellet fraction) homogenate (Fig. 3A) corresponding to the predicted molecular mass of Nos2 (~131 kDa). The specificity of the anti-Nos2 antibody was demonstrated with both the pellet fraction of kidney (by Western blot; Fig. 3B) and gill tissue sections (by immunohistochemistry; see below) and showed that the band at 134 kDa (i.e. the Nos2 specific staining) was eliminated after pre-incubation of the primary antibody with the specific blocking peptide. The specific Nos2 band at 134 kDa was not detected in the homogenate from middle and posterior intestine.

The anti-Nos1 antibody recognized a protein at 157 ± 0.2 kDa (Fig. 3C) in both pellet and supernatant fractions of intestine corresponding to the predicted molecular mass of Nos1 (~160 kDa). However, a band with a lower molecular mass was detected by the anti-Nos1 antibody at 121 ± 0.1 kDa in the supernatant fraction that was not found in the pellet fraction. Using the current methodology, the Nos1 band at 157 kDa could not be detected in gill and kidney homogenates.

In the gill, Nos2 protein level was significantly lower in SW-transferred fish than in FW fish (Fig. 4A and insert), whereas SW-transfer did not affect Nos2 protein level in the kidney (Fig. 4B). Nos1 protein level increased in middle intestine after SW-transfer (Fig. 4C) but no change in Nos1 protein level was observed in the posterior intestine (Fig. 4D).
**Plasma and water measurement**

Plasma [NO$_2^-$] was 1.8 ± 0.3 µmol l$^{-1}$ in FW-trout and decreased gradually after SW-transfer to 0.7 ± 0.3 µmol l$^{-1}$ at 24 h and 0.4 ± 0.1 µmol l$^{-1}$ at 168 h (Fig. 5). Fresh water [NO$_2^-$] was < 4.4 µmol l$^{-1}$ and a water [Cl$^-]$ of 9 mmol l$^{-1}$ precluded any active branchial nitrite uptake.

**Localization of Nos immunoreactivity**

In the gill, Nos2-immunoreactivity (IR) was found within the epithelium of the gill filament, close to the base of the lamellae, and was not associated with Nka-IR, used to localize ionocytes (Fig. 6A and 6B). Nos2-IR was found scattered in the haematopoietic tissues of the trunk kidney (Fig. 6C, D) but not in tubular regions positive to Nka. No IR for Nos2 was detected in the middle and posterior intestine (not shown). When the primary antibody was blocked with the antigenic peptide (Fig. 6E) or omitted (Fig. 6F) no IR was observed. Nos1-IR was specifically observed along the midline of the gill filament, below Nos2-IR but not co-localized with it (Fig. 7A, B). Yet, Nos1-IR was absent in sagittal gill sections on both the afferent and efferent sides of the midline. The distribution of Nos1-IR relative to Nos2-IR in the gill filaments is sketched in Figure 8. In the kidney, Nos1-IR was occasionally found associated with the wall of small (<20 µm diameter) blood vessels (Fig. 7C, D). In the middle and posterior intestine Nos1-IR was observed running along circular muscles layer in the intestinal wall (Fig. 7E, F).

The immunolocalization of Nos1 and Nos2 is only shown in FW fish (Fig. 6 and Fig. 7) and was similar in SW-acclimated fish (not shown).

**Discussion**

The long-term rearrangement of ion transporter protein expression occurring during salinity acclimation and its control by various endocrine effectors has been widely studied (24, 44). On the other hand, the short-term (rapid) modulation of ion transport, which may be crucial during the initial phase of the acclimation process, is poorly understood. The short-lived gasotransmitter NO displays physiological functions that make it a good candidate for rapid control/modulation of ion transport in fish (21, 59). The present study is the first to report tissue-specific changes in the Nos/NO system in a euryhaline fish during SW-acclimation. The euryhaline rainbow trout was chosen as model for our studies, because the osmoregulatory mechanisms and the endocrinology of long-term adjustment are well characterized and, additionally, some background information on Nos expression and NO physiology was available. The major findings include a decrease in
branchial Nos expression and NO production and an increase in intestinal Nos expression. We suggest that the Nos/NO system is involved in the readjustment of branchial and intestinal osmoregulatory function during salinity transfer.

Transcriptional changes in branchial ion transporter genes

After SW-transfer, trout responded as expected with a temporary and significant (25%) increase in plasma osmolality at 24 h, which was homeostatically corrected at 168 h. We evaluated the transcriptional regulation of several branchial ion transporters during SW-acclimation to verify a normal osmoregulatory response in the fish and to obtain additional information. nka a1a (coding the Nka α isoform involved in FW ion uptake) was down-regulated (Fig. 1B), whereas nka a1b (coding the Nka α isoform involved in SW ion secretion) was up-regulated during SW-acclimation (Fig. 1C) as previously reported (63). nkcc1, coding for the basal secretory isoform of the Na+, K+, 2Cl⁻ co-transporter involved in NaCl secretion, and the apical chloride channel (cftr1), involved in Cl⁻ secretion, were both up-regulated during SW-acclimation (Fig. 1D, E), as expected (66, 70). slc26a6 that codes an apical HCO₃⁻/Cl⁻ exchanger (involved in active Cl⁻ uptake in FW) was down-regulated during SW-acclimation and barely expressed in SW fish after 168 h, which agrees with two recent studies comparing slc26a6 expression in gill (4) and isolated ionocytes (45) of FW and SW-acclimated trout. Our study is the first to report on the kinetics, and we show a rapid and extensive down-regulation of slc26a6 expression already within 4 h after transfer to SW (Fig. 1F), which is the fastest transcriptional regulation among all examined transporter genes (Fig. 1). The rapid down-regulation of slc26a6 complies with the idea that the branchial Cl⁻/HCO₃⁻ exchanger is central for active ion uptake in FW but redundant in the secretory SW gill, except for a small amount of protein for acid-base regulatory purpose (17).

Branchial Nos regulation during SW-acclimation and localization

The Nos/NO system underwent major changes in the gill in response to the change in salinity. Both nos1 and nos2 mRNA expression were rapidly and significantly decreased following SW-transfer (Fig. 2A, B). A temporary and moderate decrease of branchial nos2 mRNA expression was also observed following FW sham-transfer (Fig. 2A), which may be related to handling stress that produce a transient peak of cortisol secretion. In a recent study (22), we demonstrated that cortisol treatment induces branchial nos2 down-regulation in FW and SW rainbow trout. The suspected transient stress-induced change in cortisol was, however, not sufficient to induce changes in ion transporter expression. The decrease in nos2 mRNA expression following SW-transfer was long-
lasting and it further translated to the protein level (Fig. 4A), as confirmed by Western blotting, suggesting a decreased Nos2 activity and NO production. Direct measurement of NO production is complicated due to its short half-life, but the more stable NO metabolite nitrite (NO$_2^-$) is routinely used as an indicator of Nos-derived NO production (43). The plasma level of NO$_2^-$ measured in FW- and SW-transferred trout was within the natural physiological range observed in teleost (18). Yet, the level of NO$_2^-$ in the plasma decreased significantly in fish during SW-exposure (Fig. 5), suggesting a decreased Nos activity and NO production. This does not necessarily reflect a decreased NO production in a specific tissue (6), but since hypoxia-induced up-regulation of nos2 in the gill of trout is paralleled by an increased plasma [NO$_2^-$] (34), there appears to be some correlation between branchial Nos activity and plasma [NO$_2^-$]. Indeed, the gill is the only organ whose capillary bed is perfused with the whole cardiac output, and the branchial Nos activity/NO production is therefore likely to influence the systemic NO$_2^-$ level. While down-regulation of branchial Nos2 was observed at both mRNA and protein levels, the down-regulation of branchial nos1 mRNA levels could not be confirmed at the protein level. We simply could not detect branchial Nos1 protein with the methodological approach which worked for the intestine. This is likely due to a low Nos1 protein expression in the tissue, which was corroborated by immunohistochemistry, where Nos1-IR was exclusively observed in a few sagittal sections (Fig. 7A, B). The distribution of Nos1-positive cells along the longitudinal axis of the gill filament resembles that observed in the zebrafish gill using 5-HT (serotonin) and SV2 (synaptic vesicle glycoprotein 2A) antibodies (36, 74) that suggests Nos1-positive neuroepithelial cells. In addition, the present localization of Nos1-IR in gill of rainbow trout resembles previous Nos1 staining of Indian catfish (Heteropneustes fossilis; 51) and of killifish (Fundulus heteroclitus; 30), where Nos1 was suggested present in nerve fibers. Although the innervation of the gill was not investigated in the present study, the distribution of nerve fibers along the gill filament of zebrafish (74) and goldfish (68), reported in previous studies using the zebrafish-derived neuronal marker zn-12, suggest that the Nos1-IR observed in the present study is likely closely apposed to nerve fibers. In other studies, Nos1-IR was found in the lamellae (zebrafish, Danio rerio; 62; Indian catfish; 51, 73) and co-localized with Nka (Atlantic salmon Salmo salar; 13). That was not observed in the present study.

Nos2 localization in fish tissues remains largely unexamined. Few Nos2-positive cells were observed deep in the gill filament of Atlantic salmon (13) using a heterologous Nos2 antibody. This study is the first to localize Nos2 in the gill of fish using a homologous antibody. Our Nos2
antibody recognized a single band at ~134 kDa in the gill and kidney of rainbow trout that corresponds well to the theoretical molecular mass of Nos2 around 131 kDa (Fig. 3A). Nos2-positive cells were detected within the epithelium of the gill filament (Fig. 6A, B). The signal was found in close proximity of Nka-IR but not co-localized or associated with ionocytes. The Nos2-IR disappeared by pre-incubation with the antigenic peptide, supporting its specificity (Fig. 3B and 6E). The localization of the two Nos isoforms in gill tissues is strengthening the contribution of the NO system as a paracrine-signalling molecule involved in the regulation of branchial functions. The present down-regulation of branchial Nos and NO production during SW-acclimation complies very well with the documented inhibition of branchial Nka activity in both FW and SW gill tissue (13, 69) and with our recent demonstration of NO as an inhibitor of Cl⁻ secretion in the opercular epithelium of SW killifish and during hypotonic exposure (21).

*Intestinal Nos regulation during SW-acclimation and localization*

Two intestinal regions with distinctive involvement in ion and water transport (72) were investigated. Nos1 seems to be the major isoform present and active in the intestine. Nos2 protein was not detected in the intestine by Western blot or immunohistochemistry. The absence of Nos2 is in agreement with results on sea bass (*Dicentrarchus labrax*), where Nos2 was only found in epithelial cells of the larval gut, whereas it was absent in the gut of adults (58). However, the mRNA transcript of *nos2* was found in the intestine of *O. mykiss* (Fig. 2E, F) and might be translated to protein under certain physiological conditions, e.g. as part of the immune response (3, 7). Nos1 protein was detected in both regions of the intestine by Western blot and immunohistochemistry, but Nos1 protein abundance was only affected by SW-acclimation in the anterior region of intestine, where it increased (Fig. 4C). The localization of Nos1 in tissues helped identifying the potential role played by Nos1 elevation. In both regions, Nos1-IR was localized in the intestinal wall along and beneath the circular muscle layer (Fig. 7E, F), which is consistent with previous studies (28, 40, 58). In addition, the intestinal circular muscle layer of trout is known to be innervated by reactive nerve fibres where some Nos1-containing cell bodies can be found (46), which suggests that the present Nos1-IR is associated with nerve fibers. Several other studies support the localization of Nos1 in the enteric nervous system of the intestinal wall (5, 32, 57). Therefore, Nos1 is likely involved in the well-known functional regulation of gut motility by NO (56) that includes descending inhibition of the peristaltic reflex (39, 54) and regulation of smooth muscle vascular tone (23, 32, 55). Hence, an increase of Nos1 expression and activity during SW-
acclimation might contribute to reduce gut motility to further retention of water in the gastrointestinal tract and facilitate selective absorption of water and electrolytes. Only a few studies have addressed NO effect on intestinal ion transport. A previous study showed that NO may modulate the permeability of tight junctions and inhibit HCO₃⁻ transport in the middle intestine of SW eel (Anguilla Anguilla; 71). More recently, the inhibition of Nka and H⁺-ATPase activity by a NO donor was reported in the intestine of FW climbing perch (Anabas testudineus; 60). Thus, based on the scattered available knowledge it is difficult to settle on the role of the apparent up-regulation of Nos1 protein in the intestine after SW-entry. It may not link directly to an osmoregulatory function.

Renal Nos regulation during SW-acclimation and localization

The contribution of the kidney in mono-valent ionic balance during SW-acclimation is minor compared to the gill and intestine, and no change in whole-renal expression and activity of Nka was reported in salmonids following SW-transfer (38, 47). Concomitantly, the renal mRNA expression of nos1 and nos2 was similar in FW- and SW-transferred fish (Fig. 2C, D). No change was observed in Nos2 protein abundance and Nos1 transferred fish could not be detected with the methodological approach. The present Nos1 antibody was previously used to detect Nos1 protein in the posterior cardinal vein in the vicinity of the head kidney of rainbow trout (53). Thus, Nos1 may be distinctively and scarcely expressed in different regions of the kidney, and our results suggest low overall expression in the trunk kidney. Furthermore, the two regions have different functions; with the head kidney involved in hematopoietic and immune defense, and the trunk kidney involved in ion transport and filtration (35). The low abundance of Nos1 in the trunk kidney was confirmed by immunohistochemistry, where Nos1-IR was found associated with the wall of a few minor blood vessels (Fig. 7C, D). The finding of Nos1-IR in vessel walls is interesting, as it suggests that other Nos isoforms than Nos3 (which is absent in teleost) may serve a vasodilatory role like Nos3 does in tetrapods. Nos2-IR was found scattered in hematopoietic tissues (Fig. 6C, D), which is in agreement with a previous study where Nos2-IR was also found in haematopoietic tissues in the kidney of African lungfish (Protopterus dolloi; 1). No Nos-IR was found associated with Nka-IR, normally used to identify renal tubules in fish (41). The two Nos isoforms have also been previously localized in other regions of the kidney of rainbow trout; in nerve fibers and in ganglion cells of the head kidney (Nos1; 19), associated with chromaffin cell in PCV tissues (Nos1; 53), in nitrergic fibers close to arteries in the middle trunk of kidney (Nos1; 35) and at the peri-arterial level of the
The present localization of Nos in the kidney suggests a potential role for NO in regulating vascular and immune functions. However, the lack of change in overall expression of Nos isoforms suggests that the role is not related to adjustments of the kidney's osmoregulatory function during salinity acclimation.

**Perspectives and Significance**

This first report on the dynamics of the Nos/NO system in osmoregulatory tissues of an euryhaline fish during SW-acclimation provides insights into the regulation and potential role of NO during osmotic stress. The major response was seen in the gill, where NO previously has been shown to inhibit branchial Nka activity (13) and opercular membrane Cl⁻ secretion (21) in SW fish. The observed down-regulation of branchial nos/Nos upon SW-exposure is likely an integrated element of the acclimation process to hyper-osmotic stress to promote the initiation of Cl⁻ secretion. The present finding of a down-regulation of the Nos/NO system during exposure to hyper-saline conditions supports well our previous demonstration of increased Nos activity during exposure to hypo-osmotic stress (21). Future studies of the response of the branchial Nos/NO system during FW-acclimation (reverse transfer experiment) should provide additional evidences.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

Author contributions: S.S.M. and F.B.J. conception of the study; F.B.J., S.S.M. and L.G. design of the research; L.G. and S.S.M. performed the experiments; L.G. analysed the data and drafted the
manuscript; S.S.M. and F.B.J. edited the manuscript; S.S.M, F.B.J. and L.G. approved final version of manuscript.

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Figure 1: Plasma osmolality and branchial mRNA expression of ion transport proteins in rainbow trout. Plasma osmolality (A, in Osmol kg\(^{-1}\)) and branchial mRNA expression of nka a1a (B), nka a1b (C), cftrl (D), nkcc1 (E) and scl26a6 (F) in rainbow trout in FW (0 h) and [4 h], 24 h, [48 h] and 168 h after transfer to 80% sea water. Times in [ ] were only investigated in experiment 1 and are shown as insert in the respective graphs (the axis scales are as in the respective panels). The overall statistical effect of each factor (salinity and time) and their interaction (if any) are indicated in the figures by an asterisk (* P < 0.05). N=8 in each group.

Figure 2: nos mRNA expression in rainbow trout tissues. nos2 and nos1 mRNA expression in gill (A, B), kidney (C, D) and posterior intestine (E, F) in rainbow trout in FW (0 h) and [4 h], 24 h, [48 h] and 168 h after transfer to 80% sea water. N=8 in each group. Other details as in Fig. 1.

Figure 3: Western blot analysis using Nos2 and Nos1 antibodies. (A) Immunoblot of gill (lane 1) and kidney (lane 2) tissues using a homologous anti-Nos2 antibody, the antibody binds specifically to a protein at 134 ± 0.8 kDa; (B) the band at 134 kDa (lane 1) was eliminated after pre-absorption of the primary antibody with the blocking peptide (lane 2). (C) Immunoblot of anterior intestine tissues using a mammalian anti-Nos1 antibody. The antibody binds to a protein at 157 ± 0.2 kDa and at 121 ± 0.2 kDa in the supernatant fraction (lane 1); the lower molecular band was not found in the re-suspended pellet fraction following centrifugation of the supernatant at 45,000 g for 30 min (lane 2).

Figure 4: Immunoblot analysis of Nos protein level in rainbow trout tissues. Relative abundance of Nos2 protein in gill (A and insert) and kidney (B) and of Nos1 in middle (C) and posterior intestine (D) from FW and 80% SW fish after 168 h. Densitometric analysis of Nos bands was conducted with imageJ and β-actin was used as loading control and for normalization. The upper left inserts show the representative Nos and β-actin blots. N=5, each lane represents one individual fish. The upper right insert (graph 4A) shows branchial Nos2 protein abundance in gill of FW trout and 168 h after transfer to 80% SW from experiment 1.

Figure 5: NO metabolite nitrite in plasma of FW- and SW-transferred rainbow trout. Level (in µmol l\(^{-1}\)) of NO\(_2^-\) in plasma in rainbow trout in FW (0 h) and 24 h and 168 h after transfer to 80%
The overall statistical effect of each factor (salinity and time) and their interaction (if any) are indicated in the figures by an asterisk (* P < 0.05). N=8 in each group.

Figure 6: Nos2 localization in FW-acclimated rainbow trout. Double labelling with an anti-Nos2 (red) and an anti-Nka antibody (green). Nos2-IR was observed within the gill filament, adjacent to Nka-IR found in ionocytes of the gill (A, B) and scattered in hematopoietic tissues of the trunk kidney of FW rainbow trout (C, D) but not in the tubular regions where Nka-IR was observed. Specificity of Nos2-IR was confirmed by blocking the gill section with the antigenic peptide (E) and omission of Nka- and Nos2-primary antibodies (F; negative control). ct: collecting tubule, dt: distal tubule, ew: environmental water, hpt: hematopoietic tissue, ic: ionocyte, lam: lamella, n: nucleus, pt: proximal tubule. Scale bar 20µm.

Figure 7: Nos1 localization in FW-acclimated rainbow trout. Double labelling with an anti-Nos1 (red) and an anti-Nka antibody (green). Nos1-IR was observed along the midline of the gill filament (A, B) adjacent to Nos2-IR, and occasionally found in the wall of minor blood vessels (C, D) of the trunk kidney and beneath the smooth muscle layer of the intestinal wall of FW rainbow trout (E, F) but was not found in the tubular regions positive to Nka. bv: blood vessel, dt: distal tubule, e: epithelium, ew: environmental water, gc: goblet cells, ic: ionocyte, lam: lamella, n: nucleus, pt: proximal tubule, sm: smooth muscle. Scale bar 20µm.

Figure 8: Diagram depicting the distribution of Nos1-immunoreactive cells relative to Nos2-immunoreactive cells in the gill filaments of rainbow trout. Nos1-IR (blue) is limited to the longitudinal axis of the gill filament (marked by a dashed line), whereas Nos2-IR (yellow) is observed within the epithelium of the gill filaments. Note that Nos1-IR is close to Nos2-IR, but the two isoforms do not co-localize.
Table 1: Sequences and target genes of primers used to investigate mRNA levels during SW acclimation in osmoregulatory tissues of rainbow trout.
Figure 1

A) Osmolarity (Osmol.kg⁻¹) vs. time (h)

B) nka α1a mRNA expression (% vs. salinity)

C) nka α1b mRNA expression (% vs. salinity)

D) cftr1 mRNA expression (% vs. salinity)

E) nkcc1 mRNA expression (% vs. salinity)

F) slc26a6 mRNA expression (% vs. salinity)
Figure 2

Branchial mRNA expression (%)

A. nos2

B. nos1

Salinity

Time

 FW control

SW transferred

Pre-experiment

Renal mRNA expression (%)

C. nos2

D. nos1

Posterior intestine mRNA expression (%)

E. nos2

F. nos1

FW control

SW transferred

Pre-experiment

time (h)

0 24 48 72 96 120 144 168
Figure 4

Relative abundance of Nos

Gill

A Nos2

B Nos2

C Nos1

D Nos1

Middle intestine

Posterior intestine

FW', SW'

*
Figure 5

Plasma [nitrite]

Salinity *

$[\text{NO}_2^-]$ in $\mu$mol l$^{-1}$

FW control
SW transferred

time (h)

0 24 48 72 96 120 144 168