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## How ubiquitous is endothelial NOS?



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### ABSTRACT

The ability to regulate vascular tone is an essential cardiovascular control mechanism, with nitric oxide (NO) assumed to be a ubiquitous smooth muscle relaxant. However, the literature contains reports of vasoconstrictor, vasodilator and no response to nitroergic stimulation in non-mammalian vertebrates. We examined functional (branchial artery myography), structural (immunohistochemistry of skeletal muscle), proteomic (Western analysis) and genomic (RT-PCR, sequence orthologues, syntenic analysis) evidence for endothelial NO synthase (NOS3) in model and non-model fish species. A variety of nitrodilators failed to elicit any changes in vascular tone, although a dilatation to exogenous cyclic GMP was noted. NOS3 antibody staining does not localise to endothelial markers in cryosections, and gives rise to non-specific staining of Western blots. Abundant NOS2 mRNA was found in all species but NOS3 was not found in any fish, while putative orthologues are not flanked by similar genes to NOS3 in humans. We conclude that NOS3 does not exist in fish, and that previous reports of its presence may reflect use of antibodies raised against mammalian epitopes.

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### 1. Introduction

In mammals, nitric oxide (NO) is an important biological signalling molecule synthesised by one of three NO synthase (NOS; EC 1.14.13.39) isoforms: neuronal (nNOS/NOS1), inducible (iNOS/NOS2) or endothelial (eNOS/NOS3). The endothelium of blood vessels express eNOS and on activation NO diffuses to overlying vascular smooth muscle cells to stimulate soluble guanylate cyclase, and produce cyclic GMP (cGMP)-mediated vasodilatation (Moncada and Higgs, 2006). The importance of eNOS in the regulation of vascular tone followed identification of NO as an endothelium-derived relaxing factor (EDRF) (Palmer et al., 1987) and the finding that eNOS gene ablation in mice leads to hypertension and bradycardia (Shesely et al., 1996). The co-expression of eNOS and nNOS in human endothelial cells suggests that nNOS may play a supporting role. Indeed, ablation of either eNOS or nNOS elevated baseline blood pressure and hampered the murine baroreflex response (Carvalho et al., 2006).

The existence of an EDRF similar to that found in mammals has been demonstrated in a number of non-mammalian vertebrates such as birds (Hasegawa and Nishimura, 1991), frogs (Knight and Burnstock, 1996), toads (Broughton and Donald, 2002), and alligators (Skovgaard et al., 2008). However, evidence for the presence of eNOS or that NO is the EDRF in fishes is limited. Such studies have used non-specific NOS blockade using L-arginine analogues, L-NAME (L-nitro-arginine methyl ester) and L-NNA (NG-nitro-L-arginine), to demonstrate NO-dependent

vasodilatation. While the vasoconstriction caused by NOS inhibitors was attributed to blockade of eNOS (Fritsche et al., 2000), neither the eNOS gene nor protein has been identified in zebrafish endothelium. To our knowledge, eNOS mRNA expression has not been studied in any fish species. Rather, immunohistochemical localisation of putative eNOS has used mammalian antibodies to demonstrate apparent eNOS expression (Fritsche et al., 2000; Ebbesson et al., 2005; Amelio et al., 2006; McNeill and Perry, 2006) although extensive literature searches and phylogenetic analyses did not yield a result in fishes (Andreakis et al., 2010; Gonzalez-Domenech and Munoz-Chapuli, 2010). Chemical denudation of the endothelium in trout conduit artery inhibited arginine- and adenosine-mediated vasodilatation and NO<sup>2-</sup>-production, suggesting a role for EDRF in trout (Mustafa and Agnisola, 1998). However, in carp aorta vasodilatation using the calcium ionophore, A23187, is endothelium-dependent but not through the actions of NO (Park et al., 2000). Instead, endothelium-dependent vasodilatation has been shown to be through the actions of cyclooxygenase products in carp (Park et al., 2000) and spiny dogfish Evans and Gunderson (1998). In the eel, NO causes vasodilatation in the ventral aorta Evans and Harrie (2001) but vasoconstriction in the branchial circulation (Pellegrino et al., 2002), suggesting diverse roles for NO in the vasculature. In the western clawed frog, *Xenopus tropicalis*, an eNOS analogue protein has been identified in the kidney, heart and stomach tissue but is not localised to blood vessels (Trajanovska and Donald, 2011). The authors concluded that the protein is more similar to mammalian eNOS than iNOS or nNOS, and contains some, but not all, palmitoylation and myristoylation sites characteristic of mammalian eNOS (Trajanovska and Donald, 2011). Here, we provide evidence for the absence of eNOS mRNA and protein in tissues from model and

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non-model fish species, zebrafish (*Danio rerio*), spotted green pufferfish (*Tetraodon nigroviridis*), common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*). The ideas in this paper were first published in abstract form (Syeda et al., 2010).

## 2. Materials and methods

### 2.1. Animals and tissue collection

Rainbow trout (*Oncorhynchus mykiss*, Salmonidae) and common carp (*Cyprinus carpio* Cyprinidae), ~250 g body mass, were purchased from local farms (Leadmill trout farm, Hathersage and Ripples Waterlife, Telford, respectively) and held in recirculating, aerated, charcoal-filtered Birmingham tap water. Spotted green pufferfish (*Tetraodon nigroviridis*, Tetraodontidae) were gifted by F. Mueller and zebrafish (*Danio rerio*, Cyprinidae) by R. Bicknell (both University of Birmingham). Wistar rats (*Rattus norvegicus*) were purchased from Harlan UK Ltd (Bicester, UK). All animals were killed according to the Animals (Scientific Procedures) Act, 1986, using Home Office Schedule 1 methods. Trout, carp and rats were killed by stunning followed by transecting of the brainstem, pufferfish and zebrafish were killed by immersion in 5% (w/v) MS222. Samples were excised immediately and snap-frozen in liquid nitrogen. Human umbilical vein endothelial cell (HUVEC) RNA was gifted by P. Stone (University of Birmingham). HUVEC were isolated from umbilical cords with maternal consent, and cultured in M199, supplemented with 20% heat-inactivated fetal calf serum, 2.5 µg/mL amphotericin B, 1 µg/mL hydrocortisone, 10 ng/mL epidermal growth factor (all from Sigma-Aldrich) and 35 µg/mL gentamycin (Gibco Invitrogen Compounds) until confluent.

### 2.2. Antibodies and chemicals

The following antibodies were used: anti-eNOS, Ab1- BD Transduction Laboratories (cat#610293); Ab2- Chemicon, Temecula, USA (cat#AB16301) and anti-nNOS, Ab3- Santa Cruz Biotechnology, USA (cat#SC-648). All other materials were from Sigma Aldrich, UK, unless otherwise stated.

### 2.3. Extraction of total RNA and reverse transcription

Total RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA, USA); genomic DNA contamination was removed using the RNase-free DNase set (Qiagen). Prior to RNA extraction, all samples were homogenised in lysis buffer using pre-cooled 5 mm stainless steel beads (Qiagen) and a high-speed shaker. Skeletal muscle samples were digested with Proteinase K (Qiagen) as per the manufacturer's instructions, prior to column separation of RNA. The quantity and quality of RNA were assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). RNA was stored at  $-80^{\circ}\text{C}$  until further analysis.

### 2.4. Phylogenetic analysis

A selection of full-length sequences from species randomly selected from each mammalian subclass was assembled from Ensembl ([www.ensembl.org](http://www.ensembl.org)) and the National Centre for Biotechnology Information (NCBI; [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) nucleotide and protein databases. Accession numbers of sequences for eNOS amino acids were as follows: *H. sapiens* [P29474], *M. mulatta* [XP\_002803570], *P. troglodytes* [XR\_02287], *M. musculus* [NP\_032739], *R. norvegicus* [AAT99567], *B. taurus* [NP\_8513380], *C. lupus familiaris* [NP\_001003158], *E. caballus* [XP\_001504700], *O. aries* [NP\_001123373], and *S. scrofa* [NP\_999460]. Accession numbers for eNOS nucleotide sequences were as follows: *H. sapiens* [NM\_00603.4], *M. mulatta* [XR\_012468.1], *P. troglodytes* [XR\_02287], *M. musculus* [NM\_008713], *R. norvegicus* [NM\_021838.2], *B. taurus* [NM\_181037.2], *C. lupus familiaris* [NM\_001003158.1],

*E. caballus* [XM\_001504649], *O. aries* [NM\_001129901], and *S. scrofa* [NM\_2142951]. The accession numbers for the amino acid and nucleotide sequences for *X. tropicalis* were XP\_002943058 and NW\_003164518, respectively. The phylogeny based on multiple sequence alignments was constructed by the widely-used neighbour-joining method, and phylogenetic trees created using ClustalW (Saitou and Nei, 1987; Thompson et al., 1994), to identify sequence similarity, with the branch length of the trees representing the fraction of mismatches at aligned positions. Unlike computationally-intensive maximum likelihood methods, neighbour-joining does not employ a model of sequence evolution. Instead, it uses a pairwise distance matrix to progressively cluster sequences from an initial star phylogeny; details of the clustering heuristic are given in (Saitou and Nei, 1987). This was carried out for all mammalian eNOS sequences. In addition, eNOS amino acid and nucleotide sequences for *X. tropicalis* were compared with human nNOS (NP\_001191147/NM\_001204218.1) and iNOS (NP\_000616.3/NM\_000625.4) and xenopus nNOS (ENSXETP00000048371/ENSXETG00000022354). The statistical significance of each tree branch was evaluated by bootstrapping with 1000 resamples.

### 2.5. Myography

Blunt dissection revealed the carp efferent branchial arteries (EBA), which were quickly removed and placed in ice-cold teleost Ringers solution (118 mM NaCl, 5 mM KCl, 1 mM MgSO<sub>4</sub>, 1 mM NaH<sub>2</sub>PO<sub>4</sub>, 2.5 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.9 mM NaHCO<sub>3</sub>, 2 mM CaCl<sub>2</sub>, 5.5 mM D-glucose; pH 7.8 at 12 °C) equilibrated with air. A modified wire myograph (Multi Myograph Model 610 M; Danish Myo AB) interfaced with PowerLab (AD Instruments) allowed experiments on sections of vessel (approximately 2 mm) to be performed at the acclimation temperature. Passive tension on the vessels was gradually increased and left until constant basal tone was reached. Thereafter, pre-constriction with 50 mM KCl was used to verify vessel viability. Agonist dose–response curves were constructed using half decades on a log scale (Hill and Egginton, 2010). Attempts were made to obtain a nitro-dilator response using sodium nitroprusside (SNP as sodium nitroferricyanide), 3-morpholinosyndonimine (SIN-1), S-nitroso-N-acetylpenicillamine (SNAP), and bradykinin (BK), and to block basal nitregic tone using L-NNA. A small number of cGMP (as 8-Bromoguanosine-3',5' cyclic monophosphate sodium) dose–response curves were obtained opportunistically. Mass specific change in tension (mN mg<sup>-1</sup>) of the agonist was normalised to the KCl response (%KCl<sub>max</sub>). Dilator responses were also calculated as a proportion of the pre-constriction from which the dilator was added. Dose–response curves were established and the pEC<sub>50</sub> (dose at the midpoint of dilatation) was calculated using R software. StatView 5.0 (SAS Institute Inc.) was used to compare groups by ANOVA, with Fisher's PLSD post-hoc test of significance.

### 2.6. Synteny analysis

The location of the eNOS gene in mammals was identified using the human, mouse and rat genome assemblies in Ensembl (GRCh37, NCBI7 and RGSC3 respectively). Syntenic regions in the zebrafish were identified using zebrafish assembly Zv8.

## 3. PCR

eNOS and nNOS were sought in pufferfish liver, zebrafish liver, carp red muscle and trout red muscle, using degenerate primers (eNOS: forward CCCCAGCACGGNATHGCNCC (amino acid sequence: PGTGIAP), reverse ACAGCACCCGGTGCACYTCNSMNGC (amino acid sequence: ACEVHRVLC); nNOS: forward GCCAAGCACGCTGGMGNAAY, reverse GTACTTGATGTGGTTCADATR), and the validity of the PCR protocol was determined by carrying out the same assay in HUVECs using a TaqMan Gene Expression Assays primer/probe set (Applied Biosystems Inc., USA; Hs\_01574659\_m1). Degenerate primers were designed

by passing the multiple amino acid sequence alignments described above through CODEHOP (Consensus-Degenerate hybrid Oligonucleotide Primer) to generate forward and reverse primers. cDNA synthesis and PCR were performed on the ThermoHybaid PCR Express Thermal Cycler (Middlesex, UK), using the OneStep RT-PCR kit (Qiagen). Amplification for all experiments was replicated three times. Amplification volume was 25  $\mu$ L with 50 ng RNA and 1  $\mu$ g of each primer per reaction. The PCR cycling conditions were: 30 min reverse transcription (50 °C) with SensiScript and Omniscript reverse transcriptases, 15 min Hotstart PCR activation with HotStarTaq®DNA polymerase, 35 cycles of 1 min denaturation (94 °C), followed by 30 s annealing (60 °C) and 1 min extension (72 °C), with a final extension at 72 °C for 10 min. Agarose gel electrophoresis (2%w/v) at 100 V constant voltage was carried out immediately, and gel images were taken using SynGene GeneSnap software (Cambridge, UK), to visualise PCR products.

### 3.1. Western blotting

Frozen samples (rat liver, trout red muscle, carp liver, zebrafish muscle and zebrafish liver) were ground to fine powder and homogenised in ice-cold RIPA buffer (150 mM sodium chloride, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 50 mM Tris–HCl, 1 mM EDTA, 1 mM PMSF, 1 mM general protease inhibitor cocktail (Sigma), pH 8.0), with a Potter-Elvehjem homogeniser. Samples were centrifuged at 16,000 g at 4 °C (EBA12R, Global Medical Instrument, USA), and the protein concentration in the supernatant was measured using the Bradford assay. Samples were diluted to equal concentration in sample buffer (5% glycerol, 0.5% SDS, 3 mM  $\beta$ -mercaptoethanol, 0.02% bromophenol blue and Tris–HCl, pH 6.8). The protein was denatured at 90 °C for 3 min and 60–100  $\mu$ g protein was loaded per gel. Western blots were run on a 4% (w/v) polyacrylamide gel, transferred onto polyvinylidene fluoride membrane with a 0.4  $\mu$ m pore size, and blocked with 5% (w/v) non-fat milk in TBS/Tween-20 buffer (20 mM Tris/HCl (pH 7.6, 137 mM NaCl and 0.1% Tween-20) for 4 h at room temperature. Primary antibodies against eNOS (1:2000 dilution Chemicon and Becton Dickinson) were incubated overnight at 4 °C, followed by 3  $\times$  10 min washes in TBS and 3  $\times$  10 min washes in TBS/0.1% (w/v) Tween-20. The membrane was then incubated with HRP (horseradish peroxidase-conjugated) secondary antibody, 1:2000 for 1 h at room temperature, and washed 3  $\times$  10 min in TBS/0.1% (w/v) Tween-20. Membranes were developed by applying chemiluminescence substrate (Roche Diagnostics) and exposures made on photographic film.

### 3.2. Immunohistochemistry

Trout white muscle was embedded in an inert mounting medium (Optimum Cutting Temperature compound, BDH, UK) and frozen onto cork discs at –160 °C. Samples were stored at –80 °C and warmed to –20 °C for cutting into sections. Samples were cut at 10  $\mu$ m and mounted onto polysine™-coated glass slides (VWR, UK), in a Bright Clinicut Cryostat, and air-dried at room temperature. Sections were fixed with ice-cold acetone (5 min), washed 3  $\times$  5 min in PBS and blocked for 30 min with BSA and normal goat serum in PBS. Tissues were stained with anti-eNOS (1:200 dilution; Chemicon) and anti-nNOS (1:200, Santa Cruz) antibodies overnight at 4 °C and finally washed for 3  $\times$  5 min in PBS. Appropriate controls (secondaries without primary antibody treatment) were included, which resulted in blank images (results not shown). All preparations were mounted with a mounting medium (Vector Laboratories), containing 4',6'-diamidino-2-phenylindole (DAPI), for identifying nuclei, and visualised using a fluorescence microscope (Zeiss Axioscop) and CCD camera (Zeiss, MRC). Images were captured using Axiovision imaging software.

## 4. Results

### 4.1. Phylogenetic analysis

A systematic search for the NOS3 gene across all species within Ensembl showed that no known NOS3 orthologues were present in any fish species, and that most available NOS3 sequences are from mammals. *X. tropicalis* and *A. carolinensis* were the closest non-mammalian vertebrates that were allocated a NOS3 orthologue (Fig. 1). Phylogenetic trees comparing the amino acid and nucleotide sequences of xenopus with some mammals grouped the putative xenopus NOS3 separately from all other species (Suppl. Fig. 1), but grouped the xenopus NOS3 closer to human NOS3 than NOS1 or NOS2 (Suppl. Fig. 2).

### 4.2. Myography

The various nitrodilators used (SNP, SIN-1, SNAP, BK) elicited no reaction in any vessel from carp (n = 5), tested variously after pre-constriction or from baseline tension. Similarly, L-NNA had no effect on baseline tension. However, and surprisingly given the absence of any response to the above nitrodilator agonists, a dilatation was found in response to cGMP in carp, resulting in a change in tension of  $0.83 \pm 0.01$  mN  $\text{mg}^{-1}$ , from a baseline of  $2.18 \pm 0.17$  mN  $\text{mg}^{-1}$  ( $P < 0.05$ ), equivalent to  $89 \pm 38\%$   $\text{KCl}_{\text{max}}$ . In contrast, the same preparations gave a potent NO-dependent dilatation in trout vessels (Young & Egginton, unpublished).

### 4.3. Synteny analysis

The human and mouse NOS3 genes are found on chromosomes 7 and 5, respectively (Fig. 2) and are flanked by KCNH2 (potassium voltage-gated channel, subfamily H (eag-related) gene, member 2) and ATG9 (autophagy-related protein 9 gene). A NOS3 gene could not be found in the zebrafish genome after a thorough search using Ensembl and PubMed, and the KCNH2 gene is located on a different chromosome to the ATG9B in the zebrafish.

### 4.4. PCR profiling

mRNA expression profiling of NOS3 and NOS2 in human, pufferfish, zebrafish, carp and trout tissues is shown (Fig. 3). This revealed abundant eNOS mRNA in isolated human endothelial cells but none in any of the fish species. There was abundant NOS2 mRNA in all species, confirming the method of amplification was suitable for the tissues used.

### 4.5. Western blot analysis

Western blot analysis in trout and carp muscle, and zebrafish and rat liver, comparing the polyclonal antibody that had been used to visualise NOS3 in fishes in earlier studies (Ab2) with a monoclonal antibody (Ab1) is shown (Fig. 4). Ab1 revealed a band in rat liver tissue, but the band was absent in fishes. Conversely, Ab2 demonstrated a band in trout at the expected molecular weight (MW), as seen in previous studies, but there was no band in rat liver tissue. The lack of a band for the rat liver suggests that the band seen in fishes in previous publications was a result of non-specific binding. This seems especially likely because in fish, particularly in the carp, there is a high level of non-specific binding giving rise to 5 bands, none of which correspond with the expected MW of NOS3 (~132 kDa).

### 4.6. Immunohistochemistry

Immunohistochemical staining with antibodies used to visualise NOS3 in vessels is shown (Fig. 5). High levels of staining were seen with NOS3 antibody, but this was not due to auto-fluorescence caused by the secondary antibody as NOS2 staining using the same secondary

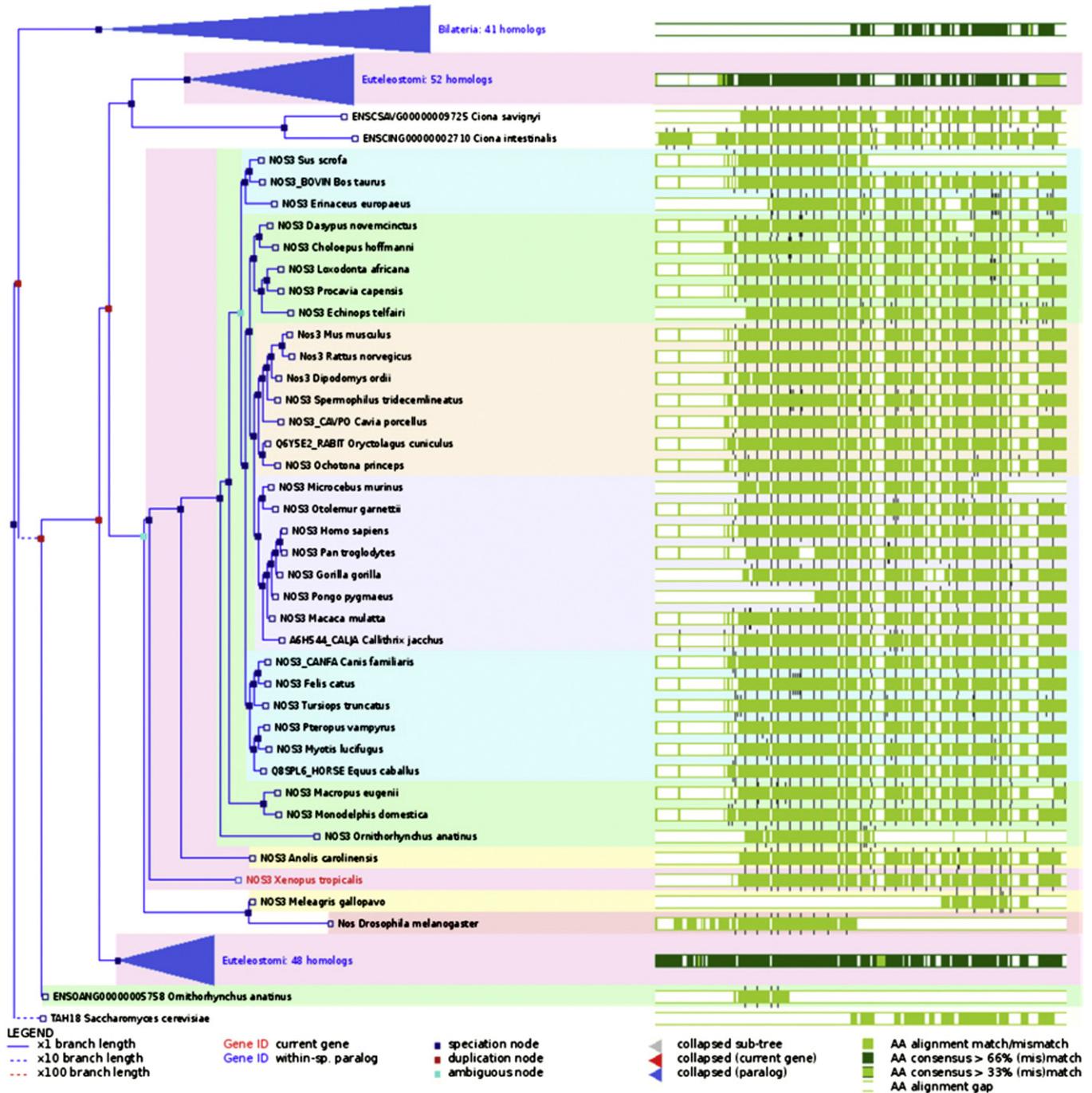


Fig. 1. Maximum likelihood gene tree displaying distances between annotated eNOS (NOS3) gene orthologues in Ensembl. Note the lack of a NOS3 orthologue in fish species. There are NOS3 orthologues in the African clawed frog (*X. tropicalis*), and a lizard, the Carolina Anole (*A. carolinensis*), but the distance evident here suggests that these may have low identity to mammalian NOS3, therefore may be similar but not homologous to NOS3.

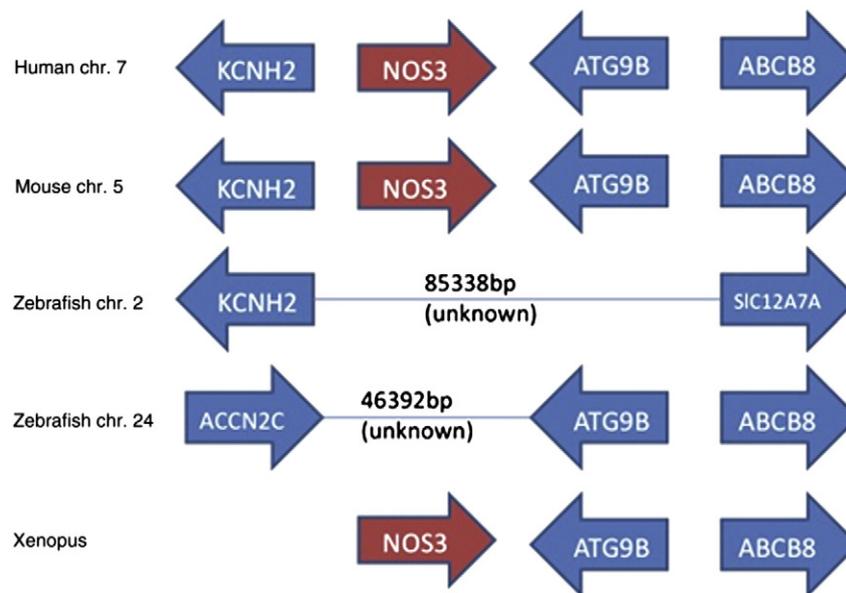
antibody showed distinct staining. Alkaline phosphatase (AP; endothelial cell marker) staining on a serial section shows that the NOS3 antibody binds to regions where there are no vessels, and conversely, cannot be seen where AP staining reveals that there are vessels present (Fig. 5D). These findings point to non-specificity of the antibodies used in other studies that appeared to demonstrate NOS3 staining in fishes (Fritsche et al., 2000; Ebbesson et al., 2005; Amelio et al., 2006; McNeill and Perry, 2006).

## 5. Discussion

This study provides genomic, immunological and functional evidence that NOS3 mRNA and protein do not exist in fishes. *Xenopus*

*tropicalis* is the closest non-mammalian vertebrate thought to express NOS3. The putative xenopus NOS3 sequence was grouped with the mammalian NOS3 because of its relative sequence identity to NOS3 compared with NOS1 or NOS 2, its conserved synteny with mammalian NOS3, and its possession of palmitoylation and myristoylation sites that are not characteristic of NOS1 or NOS2. However, mRNA expression of the putative xenopus NOS3 was not seen in the vascular endothelium (Trajanovska and Donald, 2011).

The lack of a direct effect of nitrodilators on carp vascular tone may seem a surprise given that NOS activity has been determined immunohistochemically using specific antibodies, and by NADPH-diaphorase histochemistry, in the peripheral nervous system as well as throughout the digestive tract, swim bladder and heart of the goldfish *Carassius*



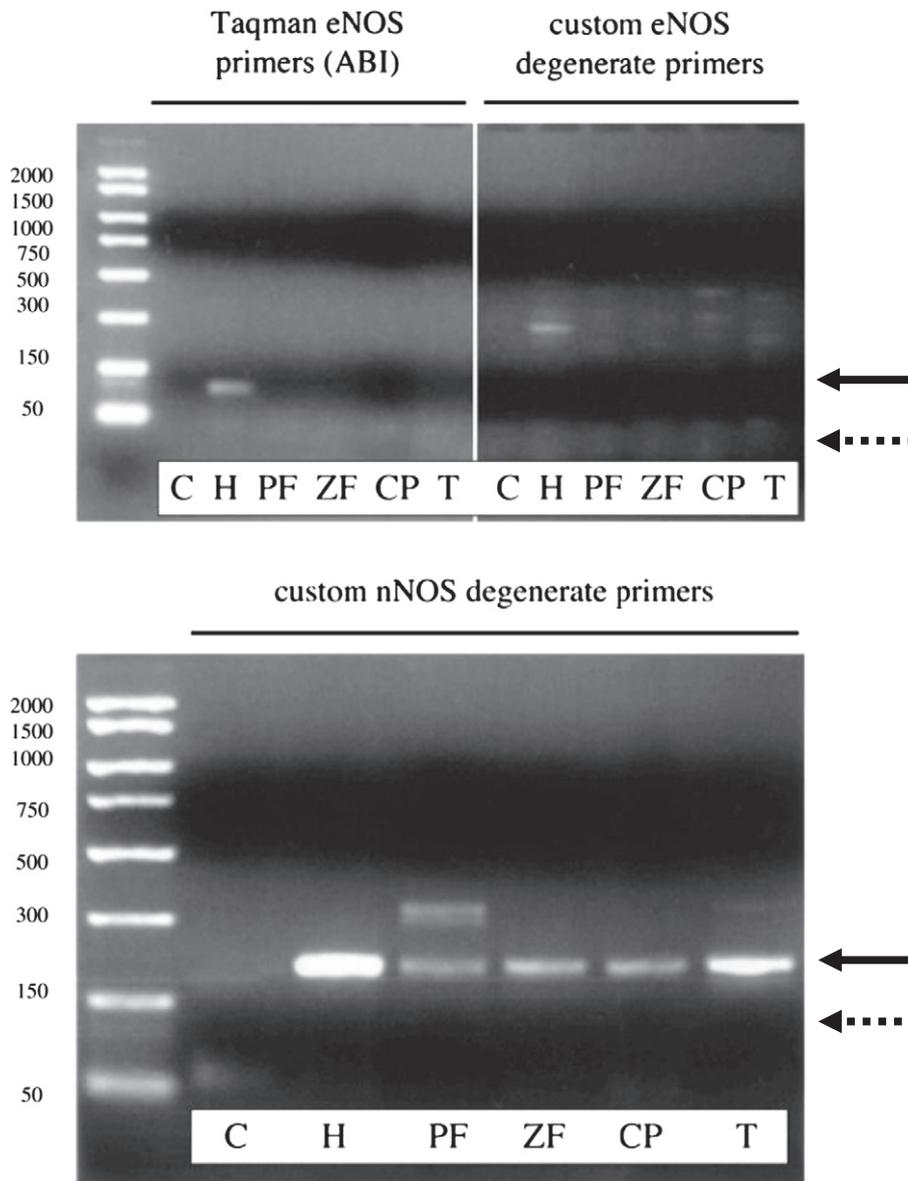
**Fig. 2.** Comparison of NOS3 genes and neighbouring gene loci in human, mouse and zebrafish genomes. Xenopus genes shown for comparison, but chromosome location is unknown. Arrows indicate the direction of transcription. Information collated from Ensembl genome browser.

*auratus* (Bruning et al., 1996). NOS has also been found in the branchial vasculature of Atlantic cod (Gibbins et al., 1995) and pufferfish (Funakoshi et al., 1999). In the Australian short-finned eel *Anguilla australis* NOS could be found in perivascular nerves, but not in the endothelium, leading to the suggestion that nNOS, but not eNOS, is present in this species (Jennings et al., 2004). However eNOS has been reported in dorsal aorta and vein of developing zebrafish *Danio rerio* (Fritsche et al., 2000). In the gills of Atlantic salmon all three forms of NOS have been demonstrated immunohistochemically (Ebbesson et al., 2005), and different NOS isoforms have been found in zebrafish brain (Holmqvist et al., 2000). In this study, we used the same antibody sources used in previous studies to identify the locality of eNOS in zebrafish, pufferfish, rainbow trout and carp, and have demonstrated the non-specificity of the antibodies used, and therefore cannot confirm the identity of the protein that the antibodies bound in this study. Further, we have demonstrated that eNOS mRNA is probably absent, using both commercially optimised eNOS primers and custom-made degenerate primers.

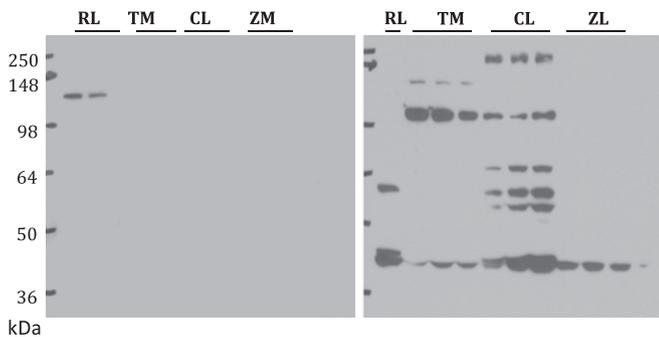
Functionally, SNP (an NO donor) has been found to exert a dilatatory effect on coronary vessels of rainbow (Small and Farrell, 1990) and steelhead trout (Small et al., 1990). Whilst SNP also dilated steelhead trout branchial arteries and ventral aorta, BK failed to elicit a response, suggesting NO donors act directly on vascular smooth muscle, but that there is no endothelially-derived NO response (Olson and Villa, 1991). BK also failed to dilate coronary arteries from steelhead trout (Farrell and Johansen, 1995). However, there is some evidence that an NO-dependent vasodilatation operating *via* guanylate-cyclase is present in coronary arteries of rainbow trout (Farrell and Johansen, 1995) and dorsal aorta of Australian short-finned eel *Anguilla australis* (Jennings et al., 2004). In contrast, SNP has been found to constrict dogfish ventral aorta (Evans and Gunderson, 1998). It has been postulated that this may be due to formation of peroxynitrite ( $\text{ONOO}^-$ ), a by-product of reactive oxygen species (ROS) and NO. In branchial basket preparations from the eel *Anguilla anguilla* SNP, as well as the  $\text{ONOO}^-$  donor SIN-1 caused vasoconstriction, which could be potentiated by incubation with the ROS scavenger superoxide dismutase that likely reduces  $\text{ONOO}^-$  formation, resulting in an increased concentration of NO available to mediate constriction. Based on a dilatation in trout aorta to SNP but not to direct application of NO, it was concluded that SNP was activating particulate guanylate cyclase and causing membrane hyperpolarisation, rather than acting

*via* soluble guanylate cyclase, as is the case with NO (Miller and Vanhoutte, 2000). However, in carp aorta, nitrodilators such as SNP and SIN-1 had no effect on the vascular tone, leading to the conclusion that a functional NO system is not present in this species (Park et al., 2000). This agrees with the findings of the present study, suggesting an apparent lack of an NO-mediated dilatatory system in this species, and therefore that this phenomenon may not be as ubiquitous as previously thought. Indeed, heterogeneity of EDRF in the coronary circulation, with effect diminishing as vessel size increases, may be responsible for negative reports concerning the existence of this system in arteries (Mustafa and Agnisola, 1998). However, cGMP did produce a small dilatation in carp EBA, indicating that part of the nitroergic pathway is present in carp.

NO can be broken down by superoxide anions ( $\text{O}_2^-$ ), leading to a short half life of less than 50 seconds *in vitro* (Gryglewski et al., 1986; Rubanyi and Vanhoutte, 1986). However, some consider that the method of preparation of NO, choice of donor and, in *in vitro* experiments, the media used, are crucial in ensuring that NO is in fact being donated (Feelisch, 1991). For instance, SIN-1 acting as a nitrodilator produces  $\text{ONOO}^-$  rather than NO (Beckman and Koppenol, 1996) and the quantity of NO released by the commonly used donor SNP may not be sufficient to account for its cGMP-releasing ability and associated dilatatory action (Feelisch, 1991). Cyanide has been shown to inhibit the relaxant effects of SNP and the associated increase in cGMP levels in rat aorta (Ignarro et al., 1981; Rapoport and Murad, 1984), although with a lesser (Rapoport and Murad, 1984) or no (Ignarro et al., 1986) inhibition on other substances causing relaxation by cGMP-dependent mechanisms. It has been suggested that the inhibition of SNP by cyanide is due to chemical inactivation (Ignarro et al., 1986). As SNP releases cyanide in addition to NO (Feelisch, 1991), it could be postulated that antagonistic effects of these two compounds may account for the constrictive effect of SNP sometimes observed, although in the present study this may be discounted due to the number of nitrodonors used. Some studies have also suggested that it is not NO itself that is EDRF, but a related compound such as a nitrosothiol (Ignarro et al., 1981; Myers et al., 1990), and based upon observed differences in its half-life a phylogenetically attractive hypothesis has been proposed that the identity of EDRF is species-dependent (Forstermann et al., 1984). Therefore, it is possible that claims of a 'NO-mediated' response may be experimental artefacts, instead reflecting a response to e.g. nitrosothiols or peroxynitrite.

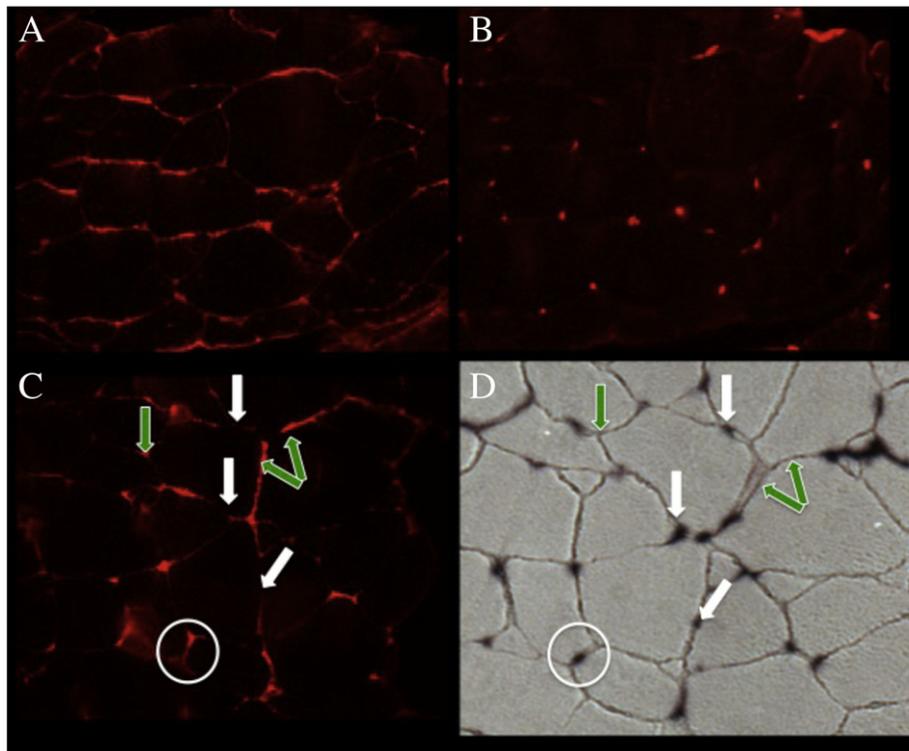


**Fig. 3.** PCR amplicons from 35 cycles using Taqman eNOS primers, custom-made eNOS degenerate primers, and custom-made nNOS degenerate primers. C = control (water), H = human umbilical vein endothelial cells, PF = pufferfish liver, ZF = zebrafish liver, CP = carp red muscle, T = trout red muscle. Top panel: Dotted arrow points to the expected size of the PCR amplicon using the Taqman eNOS primer. Solid arrow points to the expected size of the amplicons using the degenerate eNOS primer. Bottom panel: Dotted arrow represents the expected size for the amplicon using the nNOS degenerate primer. Solid arrow points to an unidentified amplicon.



**Fig. 4.** Western blots demonstrating monoclonal (Ab1; left) and polyclonal (Ab2; right) antibody binding in rat liver (RL), trout red muscle (TM), carp liver (CL), zebrafish red and white muscle (ZM) and zebrafish liver (ZL). Arrow denotes expected molecular weight of NOS protein.

Immunohistochemical and western blot analysis of tissue from non-mammalian species is currently difficult. Unlike the readily available antibodies against mouse, rat and human proteins, such antibodies do not exist for fish species. Consequently, the existing literature on NOS activity relies on analyses carried out using antibodies generated against mammalian epitopes. In this study, we have demonstrated the possible pitfalls of such an approach and the necessity of generating antibodies specific to the protein in the species concerned, in this case NOS3, which we do not believe exists in fishes. This possibility has been raised previously (Olson and Villa, 1991), and interesting questions for future investigation are when the NOS3 gene appeared during vertebrate evolution, why (or if) NOS3 activity is advantageous, which alternatives (e.g. prostanoids) permit sensitive regulation of microvascular function in non-mammalian vertebrates, and whether this knowledge can be exploited in mammals.



**Fig. 5.** Immunohistochemistry in parallel trout glycolytic muscle sections comparing polyclonal eNOS staining with Ab2 (A) to polyclonal nNOS staining with Ab3 (B). Ab2 staining was also compared with alkaline phosphatase (AP) staining for capillaries (C vs. D). Ab2 staining produced high background whereas Ab3 staining was distinct with little background. Green arrows: Ab2 staining where there was no AP staining; white arrows: no Ab1 staining where there was AP staining; white circle: Ab1 and AP stains were adjacent but not overlapping.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.cbpa.2013.05.027>.

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