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Effects of cadmium exposure and intermittent anoxia on nitric oxide metabolism in eastern oysters, *Crassostrea virginica*

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SUMMARY

Nitric oxide (NO) is an intracellular signaling molecule synthesized by a group of enzymes called nitric oxide synthases (NOS) and involved in regulation of many cellular functions including mitochondrial metabolism and bioenergetics. In invertebrates, the involvement of NO in bioenergetics and metabolic responses to environmental stress is poorly understood. We determined sensitivity of mitochondrial and cellular respiration to NO and the effects of cadmium (Cd) and intermittent anoxia on NO metabolism in eastern oysters, *Crassostrea virginica*. NOS activity was strongly suppressed by exposure to 50 μg l^{-1} Cd for 30 days (4.76 *vs* 1.19 pmol NO min^{-1} mg^{-1} protein in control and Cd-exposed oysters, respectively) and further decreased during anoxic exposure in Cd-exposed oysters but not in their control counterparts. Nitrate/nitrite content (indicative of NO levels) decreased during anoxic exposure to less than 10% of the normoxic values and recovered within 1 h of re-oxygenation in control oysters. In Cd-exposed oysters, the recovery of the normoxic NO levels lagged behind, reflecting their lower NOS activity. Oyster mitochondrial respiration was inhibited by exogenous NO, with sensitivity on a par with that of mammalian mitochondria, and ADP-stimulated mitochondrial respiration was significantly more sensitive to NO than resting respiration. In isolated gill cells, manipulations of endogenous NOS activity either with a specific NOS inhibitor (aminoguanidine) or a NOS substrate (L-arginine) had no effect on respiration, likely due to the fact that mitochondria in the resting state are relatively NO insensitive. Likewise, Cd-induced stimulation of cellular respiration did not correlate with decreased NOS activity in isolated gill cells. High sensitivity of phosphorylating (ADP-stimulated) oyster mitochondria to NO suggests that regulation of bioenergetics is an evolutionarily conserved function of NO and that NO-dependent regulation of metabolism may be most prominent under the conditions of high metabolic flux when the ADP-to-ATP ratio is high.

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Key words: nitric oxide synthase, evolution, expression, anoxia, cadmium, intertidal mollusks.

INTRODUCTION

Nitric oxide (NO) is an intracellular signaling molecule ubiquitously found in animals (Torreilles, 2001). In mammals, where the functions of NO are best characterized, this molecule is involved in a plethora of regulatory processes in the nervous, cardiovascular and immune systems and in cellular bioenergetics (Brown, 1999; Torreilles, 2001). The major pathway of NO synthesis in the cell involves NADPH- and O_2-dependent oxidation of L-arginine (L-Arg) catalyzed by nitric oxide synthases (NOS). Mammals possess three distinct isoforms of NOS: endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) (Alderton et al., 2001). Most NOSs are cytosolic enzymes, although several organelle-specific NOS isoforms have also been described, such as mitochondrial NOS (mtNOS), a splice variant of nNOS found in the inner mitochondrial membrane (Navarro and Boveris, 2008). In recent years, it has become increasingly evident that NOS functions and NO signaling are evolutionarily conserved (Palumbo, 2005). Several NOS isoforms have been described from mollusks, insects and crustaceans (Palumbo, 2005); however, molecular and functional diversity of these enzymes, as well as physiological roles of NO in invertebrates, are not fully understood.

Regulation of bioenergetics and oxygen homeostasis play a prominent role among physiological functions of NO. Recent studies in mammals demonstrated that NO is involved in regulation of aerobic metabolism (Brown, 1999; Brown, 2007), as well as in oxygen sensing and regulation of anaerobic energy production during oxygen-deficient states such as hypoxia and ischemia (Berchner-Pfannschmidt et al., 2007; Benamar et al., 2008). Mitochondria appear to be a central hub of NO-dependent regulation of bioenergetics. In mammalian mitochondria, NO can inhibit oxygen consumption either *via* reversible competitive inhibition of cytochrome *c* oxidase or, irreversibly, *via* NO-dependent modifications (mainly *S*-nitrosylation) of mitochondrial electron transport chain (ETC) proteins (Hüttemann et al., 2008; Navarro and Boveris, 2008). The irreversible inhibition of ETC is especially characteristic of pathophysiological situations when excessive NO is produced whereas the reversible inhibition is considered to be a part of the normal physiological regulation of mitochondrial activity (Valdez et al., 2006; Carreras et al., 2007; Navarro and Boveris, 2008). Recently, it has also been discovered that NO can play a role in metabolic regulation during hypoxia or anoxia and subsequent aerobic recovery (Berchner-Pfannschmidt et al., 2007; Benamar et

al., 2008; Korge et al., 2008 and references therein). Even in severe hypoxia and anoxia, when NOS is not functional due to the lack of oxygen, NO can be formed by either non-enzymatic or xanthine oxidase-catalyzed reduction of nitrite (Zweier et al., 1999; Benamar et al., 2008). Elevated NO levels in hypoxia can prevent stabilization of hypoxia inducible factor-1 (HIF-1),which regulates adaptive transcriptional response to oxygen deficiency (Hagen et al., 2003; Galkin et al., 2007). NO-dependent inhibition of mitochondrial function and associated production of reactive oxygen and nitrogen species (ROS and RNS, respectively) also play a role during post-hypoxic and post-anoxic recovery and can be cytoprotective or damaging, depending on the NO concentrations and tissue type (Schild et al., 2003; Shiva et al., 2007).

While the important role of NO in regulation of metabolism and bioenergetics is well established for mammalian models, involvement of this signaling molecule in metabolic regulation of invertebrates is currently unknown. In mollusks, NOS-like activity has been detected in several species of bivalves, gastropods and cephalopods (reviewed in Palumbo, 2005), and NOS-encoding genes have been isolated from the gastropods *Aplysia californica* and *Lymnaea stagnalis* and the cephalopod *Sepia officinalis* (Korneev et al., 1997; Korneev and O'Shea, 2002; Scheinker et al., 2005). Nitric oxide was shown to be involved in neuromodulation in the central nervous system (Gelperin et al., 2000; Zsombok et al., 2000; Inoue et al., 2001), metamorphosis (Hens et al., 2006) and immune defense of mollusks (Tafalla et al., 2002; Tafalla et al., 2003; Novas et al., 2004; Villamil et al., 2007). By contrast, the possible involvement of NO in regulation of energy metabolism has not been explored in mollusks.

The eastern oysters, *Crassostrea virginica* (Mollusca: Bivalvia), are an ecologically and economically important species in the western Atlantic estuaries (Newell, 2004). As for most estuarine inhabitants, they are exposed to a variety of natural and anthropogenic stressors, including fluctuations in temperature, salinity, oxygen content and pollution. Trace metal cadmium (Cd) is a common pollutant in estuarine and coastal ecosystems worldwide (GESAMP, 1987; Crompton, 1997). Like all marine bivalves, oysters can concentrate Cd in soft tissues to levels that exceed environmental concentrations by orders of magnitude (Roesijadi, 1996), making these mollusks susceptible to the toxic effects of Cd as well as important vectors of Cd transfer to higher levels of the food chain. Cd strongly affects energy metabolism of oysters on both the energy demand and energy supply sides, resulting in elevated costs of the basal maintenance (Cherkasov et al., 2006a; Lannig et al., 2006; Lannig et al., 2008) and suppression of aerobic capacity due to a decrease in activity of mitochondrial enzymes, inhibition of mitochondrial ATP synthesis, partial mitochondrial uncoupling and decline in mitochondrial abundance (Sokolova, 2004; Cherkasov et al., 2006a; Cherkasov et al., 2006b; Cherkasov et al., 2007a; Ivanina et al., 2008). In mammals, NOS activity is very sensitive to the presence of trace metal ions such as Cd^{2+}, Ni^{2+} and Mn^{2+} that inhibit NOS and thus can affect NO signaling in the cell (Weaver et al., 2004). This raises an intriguing possibility that some of the Cd-mediated effects on oyster bioenergetics may be associated with the changes in intracellular NOS activity and/or NO levels. However, the effects of Cd exposure on NOS activity and NO levels have not been comprehensively studied and, for invertebrates including mollusks, these effects are unknown.

The aims of this study were to (1) determine the effects of Cd on NOS activity, NOS expression and NO levels in eastern oysters during normoxia and intermittent anoxia (2) establish sensitivity of oyster mitochondria to NO and (3) test whether experimental manipulation of endogenous NO production affects cellular respiration in oysters. In order to determine NOS mRNA expression levels, a partial transcript of NOS was isolated and characterized from *C. virginica*. To the best of our knowledge, this is the first study to address the links between NO and bioenergetics in mollusks, providing a new insight into the potential roles of this important and versatile signaling molecule in invertebrates.

MATERIALS AND METHODS

Chemicals

Unless otherwise indicated, all chemicals were purchased from Sigma Aldrich (St Louis, MO, USA), Roche (Indianapolis, IN, USA) or Fisher Scientific (Pittsburg, PA, USA) and were of analytical grade or higher.

Animal collection and maintenance

Oysters (*Crassostrea virginica* Gmelin) were purchased from Taylor Shellfish Farms (Shelton, WA, USA), shipped overnight to the University of North Carolina at Charlotte and placed in recirculated aerated tanks with artificial seawater (ASW) (Instant Ocean®, Kent Marine, Acworth, GA, USA) at 20±1°C and 30±1‰ for 10 days. Oyster density was maintained at or below one oyster per 4 liters of ASW. After the preliminary acclimation, half of the tanks were randomly selected, and Cd (as $CdCl_2$) was added to the nominal concentration of 50 $\mu g\,l^{-1}$. The remaining tanks were used as controls. A static-renewal regime with periodical Cd replenishment was used as described in our earlier study (Lannig et al., 2006), allowing us to maintain Cd levels close to the target concentration of 50 $\mu g\,l^{-1}$. This Cd concentration is within the range of Cd levels found in polluted estuaries (15–80 $\mu g\,l^{-1}$) (GESAMP, 1987; Crompton, 1997; Hackney et al., 1998). Throughout the experiments, oysters were fed *ad libitum* on alternate days with a commercial algal blend (2 ml per oyster) containing *Nannochloropsis oculata*, *Phaeodactylum tricornutum* and *Chlorella* with a cell size of 2–20 μm (DT's Live Marine Phytoplankton, Premium Reef Blend, Sycamore, IL, USA) except during anoxic exposure when no feeding was possible because oysters were kept out of water. Mortality during the experimental period (30 days) was <5% and did not significantly differ between control and Cd-exposed oysters.

To mimic environmental anoxia in the intertidal zone, control and Cd-exposed oysters were exposed to air in plastic trays lined with seawater-soaked paper towels. As in all intertidal bivalves, exposure to air triggers closure of shell valves in oysters, which prevents gas exchange with the environment, causes a rapid onset of tissue anoxia and transition to anaerobic metabolism (Brinkhoff et al., 1983; Kurochkin et al., 2009). In the present study, oyster shells were kept closed using rubber bands to prevent gaping in the air. Under these conditions, oxygen levels in oyster hemolymph drop to 0% within 10–20 min and remain at this low level throughout the duration of air exposure, indicating an onset of deep anoxia (Kurochkin et al., 2009). The maximum duration of air exposure in these experiments was 6 days. This represents an extreme stress but is within the environmentally relevant range for intertidal oysters, which may periodically experience prolonged air exposures (lasting for several days) during neap tides (Brinkhoff et al., 1983; McLusky, 1989). After 6 days of anoxia, oysters were returned to tanks with normoxic ASW and allowed to recover for 12 h. Recovery was conducted at the same Cd levels as during the acclimation period (0 and 50 $\mu g\,l^{-1}$ Cd for control and Cd-exposed oysters, respectively). All exposures were carried out at 20±2°C. Samples for determination of NOS activity

and NO levels were taken in normoxia, after 1, 2, 2.5, 3 and 6 days of air exposure, and after 1, 6 and 12 h of recovery. For cellular and mitochondrial respiration assays, control (non-Cd-exposed) oysters maintained under normoxic conditions were used.

Isolation and characterization of the NOS transcript

Gill tissues were dissected from control *C. virginica* chilled on ice. Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions and quantified with NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). cDNA was obtained from 1–5 μg of total RNA using SuperScriptTM III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Degenerate primers (NOS-RV1moll and NOS-FW1moll; Table 1) were designed against conserved fragments of the molluscan NOS protein using sequences from *Lymnaea stagnalis*, *Sepia officinalis* and *Aplysia californica* (NCBI accession numbers AAC17487, AAS93626 and AAK83069, respectively) and used in PCR under the following conditions: initial denaturation for 5 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 50°C, 40 s at 72°C, followed by the final extension for 7 min at 72°C. Amplified fragments were gel-purified on 1.5% agarose gel using Zymoclean Gel DNA Recovery Kit (Zymo Research, Orange, CA, USA) and subjected to direct sequencing in forward and reverse directions at BioAnalytical Services Laboratory (University of Maryland Biotechnology Institute, Baltimore, MD, USA). These sequences were used to design specific primers for 5′ and 3′ rapid amplification of cDNA ends (RACE)-PCR with a SMART cDNA amplification kit (BD Biosciences, San Jose, CA, USA). For the first PCR of 5′ RACE, 1 μl of SMART cDNA synthesis reaction containing 50 ng of total RNA was amplified with CvNOS5R1 (Table 1) and UPM primers using Advantage Taq DNA polymerase (BD Biosciences). PCR conditions were as follows: initial denaturation for 2.5 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 55°C, 2.5 min at 72°C, followed by the final extension for 7 min at 72°C. This first PCR reaction was 10-fold diluted in nuclease-free water and served as the template for the nested PCR with CvNOS 5R2 (Table 1) and NUP primers. The first 3′-end RACE PCR was carried out using CvNOS3F1 (Table 1) and UPM primers. The PCR product of this first reaction was 10-fold diluted in nuclease-free water and used as the template for the nested PCR with CvNOS3F2 (Table 1) and NUP primers. PCR conditions used for 3′-end RACE-PCR and nested PCRs were identical to those described above for 5′-end RACE-PCR. Products of the two nested PCRs were gel-purified with QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA) and cloned in *E. coli* using pGEMT vector (Promega, Madison, WI, USA). Three positive clones were randomly selected and sequenced with a forward or reverse pUC/M13 primer at BioAnalytical Services Laboratory.

Molecular characterization and evolutionary analysis

Representative sequences of NOS proteins were collected from multiple complete and draft assemblies of vertebrate and invertebrate genomes using GenBank (Benson et al., 2007) and Ensembl (Hubbard et al., 2007) databases (see Table S1 in supplementary material for the list of species and accession numbers). NOS from *Drosophila pseudoobscura* genome was collected from FlyBase (http://flybase.org). If multiple transcripts exist for the same species, only the longest ones were used in the alignment. Multiple sequence alignment was reconstructed using DIALIGN (Morgenstern, 2004).

Phylogenetic trees were reconstructed using the minimum-evolution (ME) method (Rzhetsky and Nei, 1992) as implemented in the MEGA4.0 program (Tamura et al., 2007) and the maximum likelihood (ML) method using PhyML3.0 program (Guindon and Gascuel, 2003). Dayhoff amino acid substitution model was used in the ME method (Dayhoff et al., 1978; Nei and Kumar, 2000), and 1000 bootstrap pseudo-replications were used to evaluate the reliability of internal branches (Felsenstein, 1985). Rate variation among sites was approximated by the gamma distribution, with the alpha parameter 1.2 estimated from the data using PhyML. The Whelan and Goldman substitution model (Whelan and Goldman, 2001) was used in the ML analysis, also taking into account rate variation among sites and proportion of invariable sites estimated from the data (i.e. WAG+gamma+inv). The reliability of internal branches was evaluated by the approximate likelihood-ratio test (aLRT) based on the non-parametric Shimodaira-Hasegawa-like procedure, as implemented in PhyML (Guindon and Gascuel, 2003; Anisimova and Gascuel, 2006). Sites with gaps were excluded from the analyses, resulting in 430 shared amino acid sites. Trees were rooted with an NOS sequence from a coral (Q8MU49).

Protein domains were predicted using the Pfam protein families database (http://pfam.sanger.ac.uk/), with 'global and local (merged)' search strategy against Pfam-A families, with an E value cut-off of 1 (Finn et al., 2008).

Cell isolation

Gill tissues were excised from 2–5 control oysters, minced on ice in 5 ml of digestion buffer (24.72 g l^{-1} NaCl, 0.68 g l^{-1} KCl, 1.36 g l^{-1} $CaCl_2.2H_2O$, 0.18 g l^{-1} $NaHCO_3$ and 30 mmol l^{-1} Hepes, pH 7.5) and washed with an additional 10 ml of digestion buffer. Tissue fragments were digested for 15 min at room temperature with 0.125% trypsin (Fisher Scientific, Suwanee, GA, USA) adjusted to 720 mOsm with sucrose, carefully triturated to release cells and washed twice with 10 ml of digestion buffer. The suspension was filtered through 100 μm sterile nylon mesh and centrifuged for 15 min at 900 ***g*** to pellet the cells. Cells were washed in cell suspension medium (24.72 g l^{-1} NaCl, 0.68 g l^{-1} KCl, 1.36 g l^{-1} $CaCl_2.2H_2O$, 0.18 g l^{-1} $NaHCO_3$, 4.66 g l^{-1} $MgCl_2.6H_2O$, 6.29 g l^{-1}

Table 1. Primer sequences (5′ to 3′) used for isolation and cloning of *NOS* cDNA and for quantitative real-time PCR in *C. virginica*

Gene	Primer name	Primer sequence	T_{ann} (°C)
NOS	NOS-FW1moll	TGG TSI AAG YTI CAR GTI TTY GAY GC	50
	NOS-RV1moll	GTI CCC ATG AAC CAI CCR TTR AAI GG	50
	CvNOS5R1	ACA GTC AAA CAG AAT GCT GGA GAC C	55
	CvNOS5R2	GAT CTT GGG GGA TTT CAA ACA TTT C	55
	CvNOS3F1	GAG AAC TGA TGG GAA ACA CGA CTT C	55
	CvNOS3F2	GCC AAC GTA GAG TTT ACG GAG GTT T	55
β-actin	Act-Cv-F437	CAC AGC CGC TTC CTC ATC CTC C	55
	Act-Cv-R571	CCG GCG GAT TCC ATA CCA AGG	55

T_{ann}, annealing temperature used in PCR. Standard code for degenerate bases is used (R, A/G; Y, C/T; S, C/G); I, inositol.

$MgSO_4.7H_2O$, 30 mmol l^{-1} glucose and 30 mmol l^{-1} Hepes, pH 7.5), centrifuged for 10 min at 900 ***g*** and resuspended in 4 ml of sterile filtered ASW (30‰) with 30 mmol l^{-1} glucose. Cells were counted with a Beckman Coulter Z2 cell counter (100 µm aperture diameter; Beckman Coulter, Inc., Fullerton, CA, USA). The particle size range window was set to 10–30 µm, corresponding to the known size of gill cells from oysters (Cherkasov et al., 2006a). Viability of isolated cells, determined by the Trypan Blue exclusion assay, was >90–95% throughout the experiment. Average cell concentration was $4.5–5.0\times10^6$ cells ml^{-1}.

Isolated cells were incubated for 24 h at 21±1°C in different concentrations of Cd (0–250 µmol l^{-1} as $CdCl_2$) in the presence or absence of a NOS inhibitor, aminoguanidine (AG; 1 mmol l^{-1}), or a NOS substrate, L-Arg (10 mmol l^{-1}). Antibiotics (10,000 U ml^{-1} of penicillin and 10,000 U ml^{-1} streptomycin) were added to the incubation media to prevent bacterial growth. Our earlier studies show that isolated oyster cells maintain viability for over 48 h in culture in Cd concentrations of <1 mmol l^{-1} (Sokolova et al., 2004). After 24 h incubation, NOS enzyme activity, *NOS* mRNA expression and oxygen consumption rate were determined in control and Cd-exposed gill cells incubated in the presence or absence of AG or L-Arg as described below (see 'NOS activity and expression' and 'Cellular oxygen consumption').

NOS activity and expression

Specific NOS activity was determined in gill tissues from oysters exposed to intermittent anoxia and/or long-term (30 days) incubation with 50 µg l^{-1} Cd and in isolated gill cells of control oysters exposed for 24 h to different Cd levels (0–250 µmol l^{-1}) in the presence or absence of AG or L-Arg. Gill tissues or isolated gill cells were flash-frozen in liquid nitrogen and homogenized in five volumes of ice-cold buffer containing 75 mmol l^{-1} Tris-HCl pH 7.6, 10 µg ml^{-1} aprotinine and 100 µmol l^{-1} phenylmethylsulphonyl fluoride (PMSF). Homogenate was centrifuged at 20,000 ***g*** and 4°C for 20 min to remove cell debris, and supernatant was used to measure NOS activity using Ultrasensitive Colorimetric Assay for Nitric Oxide Synthase (Oxford Biomedical, Oxford, MI, USA). NOS activity determination was carried out according to the manufacturer's protocol with a slight modification in that 6 mmol l^{-1} of $CaCl_2$ and 6 µmol l^{-1} of tetrahydrobiopterin were added to fully activate oyster NOS. Protein concentrations were measured in tissue and cell homogenates using a modified Biuret assay (Bergmeyer, 1985). Specific NOS activity was expressed as pmol NO min^{-1} mg^{-1} protein.

For determination of NOS mRNA levels (*NOS* gene expression), total RNA was isolated from isolated gill cells incubated for 24 h in different Cd concentrations using mini RNA Isolation II Kit™ (Zymo Research) according to the manufacturer's protocol. RNA concentration and quality were verified by UV spectroscopy. The first-stranded cDNA was obtained from 5 µg of total RNA using 200 U µl $^{-1}$ SuperScript III Reverse Transcriptase (Invitrogen) and 50 µmol l^{-1} of oligo$(dT)_{18}$ primers. The level of *NOS* expression was determined using quantitative real-time PCR (qRT-PCR) with a LightCycler® 2.0 Real Time PCR System (Roche Applied Science, Indianapolis, IN, USA) and QuantiTect SYBR Green PCR kit (Qiagen). The qRT-PCR reaction mixture (10 µl) consisted of 5 µl of 2× QuantiTect SYBR Green master mix, 0.3 µmol l^{-1} of each forward and reverse gene-specific primers (Act-Cv-F437 and Act-Cv-R571 for *β-actin* and CvNOS3F2 and CvNOS5R2 for *NOS*; Table 1) designed against *C. virginica* sequences (NCBI accession numbers: GQ844865 and X75894 for *NOS* and *β-actin*, respectively), 1 µl of 10× diluted cDNA template in water. The reaction mixture was loaded into LightCycler capillaries (Roche Applied Science) and subjected to the following cycling: 15 min at 95°C to denature DNA and activate Taq polymerase; 50 cycles of 15 s at 94°C, 20 s at 55°C and 15 s at 72°C. SYBR Green fluorescence (acquisition wavelength 530 nm) was measured at the end of each cycle for 2 s at the read temperature of 78°C (to melt all primer dimers but not the amplified gene product). Serial dilutions of a cDNA standard were amplified in each run to determine amplification efficiency, and an internal standard was included to test for run-to-run amplification variability. Amplification efficiencies (E) were 1.68±0.10 and 2.15±0.07 (N=3) for *NOS* and *β-actin*, respectively. Expression of *NOS* gene was normalized relative to the expression of *β-actin* and against the internal standard as described elsewhere (Sanni et al., 2008).

Nitric oxide content

Gill or muscle tissues were ground to fine powder under liquid nitrogen, resuspended at a 1:5 (w/v) ratio in ice-cold buffer containing 75 mmol l^{-1} Tris-HCl pH 7.8 and centrifuged at 20,000 ***g*** and 4°C for 10 min to remove cell debris. The supernatant was filtered through Microcon Ultracel YM-30 ultrafilters (Millipore Corporation, Pittsburgh, PA, USA) at 14,000 ***g*** and 4°C for 20 min. Concentrations of the stable end-products of NO oxidation (nitrate and nitrite) were measured in the ultrafiltrate as a proxy for NO levels using the Nitrate/Nitrite Fluorometric Assay Kit (Cayman Chemical, Ann Arbor, MI, USA). Values were expressed as µmol NO g^{-1} wet tissue mass.

Mitochondrial oxygen consumption (mitochondrial $\dot{M}_{O_2}$)

Mitochondria were isolated using a method modified from Sokolova (Sokolova, 2004). Briefly, 6–8 g of oysters gills (pooled from 2–3 oysters) was homogenized in the ice-cold homogenization buffer (100 mmol l^{-1} sucrose, 200 mmol l^{-1} KCl, 100 mmol l^{-1} NaCl, 8 mmol l^{-1} EGTA and 30 mmol l^{-1} Hepes, pH 7.5). Mitochondria were obtained by differential centrifugation. First, tissue homogenate was centrifuged at 2000 ***g*** and 4°C for 8 min to remove tissue and cellular debris. The supernatant was collected and centrifuged at 8500 ***g*** and 4°C for 8 min to collect mitochondria. The mitochondrial pellet was surface-washed in homogenization buffer without EGTA and resuspended in 2 ml of ice-cold assay medium [150 mmol l^{-1} sucrose, 250 mmol l^{-1} KCl, 10 mmol l^{-1} glucose, 10 mmol l^{-1} KH_2PO_4, 10 mg ml^{-1} BSA (fatty acid free) and 30 mmol l^{-1} Hepes, pH 7.2]. Mitochondrial respiration was assessed using a Clark-type oxygen electrode (Qubit Systems, Kingston, ON, Canada) in a water-jacketed respirometry chamber at 20°C. Pyruvate (3.2 mmol l^{-1}) was used as a substrate, and 0.5 mmol l^{-1} of malate was used to spark respiration. State 3 respiration was achieved by the addition of 60–150 µmol l^{-1} ADP to mitochondrial chambers. State 4 respiration (determined after ADP is exhausted) is considered a good upper-level estimate for proton leak of resting mitochondria when most energy of substrate oxidation is used to counteract the futile proton and cation cycles (collectively known as proton leak) across the mitochondrial membrane (Brand et al., 1994). Calibration of oxygen electrodes, data acquisition and $\dot{M}_{O_2}$ calculations were performed as described elsewhere (Sokolova, 2004). To test the effects of NO donors on mitochondrial respiration, state 3 and state 4 $\dot{M}_{O_2}$ was assessed in the presence of different concentrations of the NO donors diethylenetriamine/*NO* (DETA/NO) and *S*-nitroso-*N*-acetylpenicillamine (SNAP). The reported half-lives of DETA/NO and SNAP in aqueous media at physiological pH (7.0–7.5) are 5 h and 20 h, respectively (Ramirez et al., 1996; Wang et al., 2005). Due to the faster rates of NO release by SNAP than DETA/NO, we

used 500–2000 μmol l^{-1} of DETA/NO and 50–400 μmol l^{-1} of SNAP in our experiments. In a separate experiment, the effects of L-Arg (10 mmol l^{-1}) or a specific NOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME) (10 mmol l^{-1}), on mitochondrial state 3 and 4 respiration were also tested. Protein concentrations were measured in mitochondrial suspensions using a modified Biuret method with added 1% Triton X-100 to solubilize mitochondria (Bergmeyer, 1985). Mitochondrial $\dot{M}_{O_2}$ was expressed in nmol l^{-1} O mg^{-1} protein.

Cellular oxygen consumption (cellular $\dot{M}_{O_2}$)

To test for the effects of Cd levels and NOS activity on cellular $\dot{M}_{O_2}$, isolated gill cells were exposed for 24 h to different Cd levels (0–250 μmol l^{-1}) with or without a NOS inhibitor (AG; 1 mmol l^{-1}) or a NOS substrate (L-Arg; 10 mmol l^{-1}). Cell suspensions ($0.9–1.0\times10^6$ cells) were placed in antibiotics- and glucose-supplemented ASW (30 mmol l^{-1} glucose, 10,000 U ml^{-1} penicillin and 10,000 U ml^{-1} streptomycin) in water-jacketed chambers at 20°C, and $\dot{M}_{O_2}$ was determined using Clarke-type oxygen electrodes (Qubit Systems). AG and Cd were added as appropriate to maintain the same concentration as in the incubation media, and the rate of oxygen consumption was recorded. Total $\dot{M}_{O_2}$ and $\dot{M}_{O_2}$ in the presence of 3 μg ml^{-1} oligomycin (to inhibit mitochondrial F_0,F_1-ATPase; $\dot{M}_{O_2,oligo}$) and 100 μmol l^{-1} KCN (to inhibit mitochondrial respiration, $\dot{M}_{O_2,KCN}$) were determined. Cell respiration rates were corrected for electrode drift and/or non-mitochondrial oxygen consumption and expressed as μmol O_2 min^{-1} 10^{-6} cells. Total respiration ($\dot{M}_{O_2,total}$), mitochondrial respiration ($\dot{M}_{O_2,total}$–$\dot{M}_{O_2,KCN}$) and mitochondrial proton leak ($\dot{M}_{O_2,oligo}$–$\dot{M}_{O_2,KCN}$) were compared in gill cells exposed to different concentrations of Cd and AG.

Statistics

Statistical analysis was performed using repeated-measures ANOVA (for cellular and mitochondrial $\dot{M}_{O_2}$) or generalized linear model ANOVA (for all other endpoints) followed by *post hoc* procedures [Fisher's Least Significant Difference (LSD) test for unequal *N*]. For cellular $\dot{M}_{O_2}$, individual cell isolates were used as a repeated-measures variable, and Cd concentration and presence/absence of AG or L-Arg were used as fixed factors in ANOVA. For mitochondrial $\dot{M}_{O_2}$, individual mitochondrial isolates were used as a repeated-measures variable, and concentrations of NO donors, L-Arg or L-NAME were used as fixed factors. For all other analyses, no repeated measures were employed, and anoxia/reoxygenation and Cd exposure were used as fixed factors. Pearson product-moment correlation coefficients between *NOS* mRNA levels and NOS enzyme activity, as well as between NOS activity and cellular $\dot{M}_{O_2}$, were determined using data on individual cell isolates. Factor effects and differences between the means were considered significant if the probability of Type I error (*P*) was less than 0.05, and marginally significant if $0.05\leq P<0.10$. NO levels and specific activity of NOS during intermittent anoxia were measured in tissue samples from individual oysters; therefore, the number of samples (*N*) corresponds to the number of individual oysters used in the analyses. For the end points measured in isolated mitochondria or gill cells, *N* represents the number of individual mitochondrial or cell isolates, each obtained from pooled tissues of 2–5 individual oysters. Data are presented as means ± standard error of means (s.e.m.).

RESULTS

Molecular characterization of oyster NOS and phylogenetic relationships among animal NOSs

Amino acid alignment of the putative translation of the *NOS* gene of *C. virginica* (*CvNOS*) with other molluscan NOS proteins and comparison with the Pfam database reveals the presence of a full-length NO-synthase domain (i.e. nitric oxide synthase, oxygenase domain), shared among all four proteins, and a flavodoxin domain that harbors an FMN binding site (Fig. 1). On the ME tree of representative animal NOS amino acid sequences (Fig. 2), the CvNOS protein sequence is clustered with the *Sepia* NOSa sequence (100% bootstrap support). Together they form a cluster with NOS from other molluscs (*Lymnaea* and *Aplysia*), which in turn is most closely related to NOS sequences from arthropods (including insects *Drosophila* and *Anopheles*, and a crab *Gecarcinus*). Notably, CvNOS is closer related to NOS sequences from other invertebrates than to a particular NOS isoform from vertebrate genomes, and this

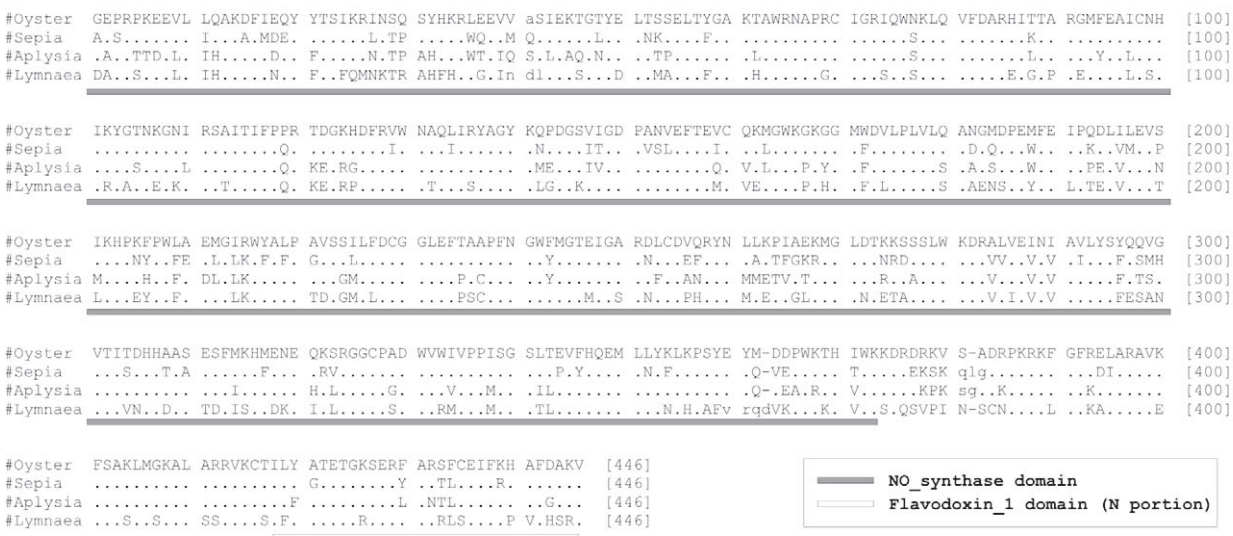

Fig. 1. Amino acid alignment of partial NOS protein from *C. virginica* with other molluscan NOS sequences. Residue identity is marked with dots. Nitric oxide synthase and flavodoxin (partial) domains are underlined. First and last positions of this alignment correspond to residues 31 and 475 of *Sepia NOSa* sequence (AAS93626). Lower-case letters denote residues that are not considered to be aligned by DIALIGN (Morgenstern, 2004).

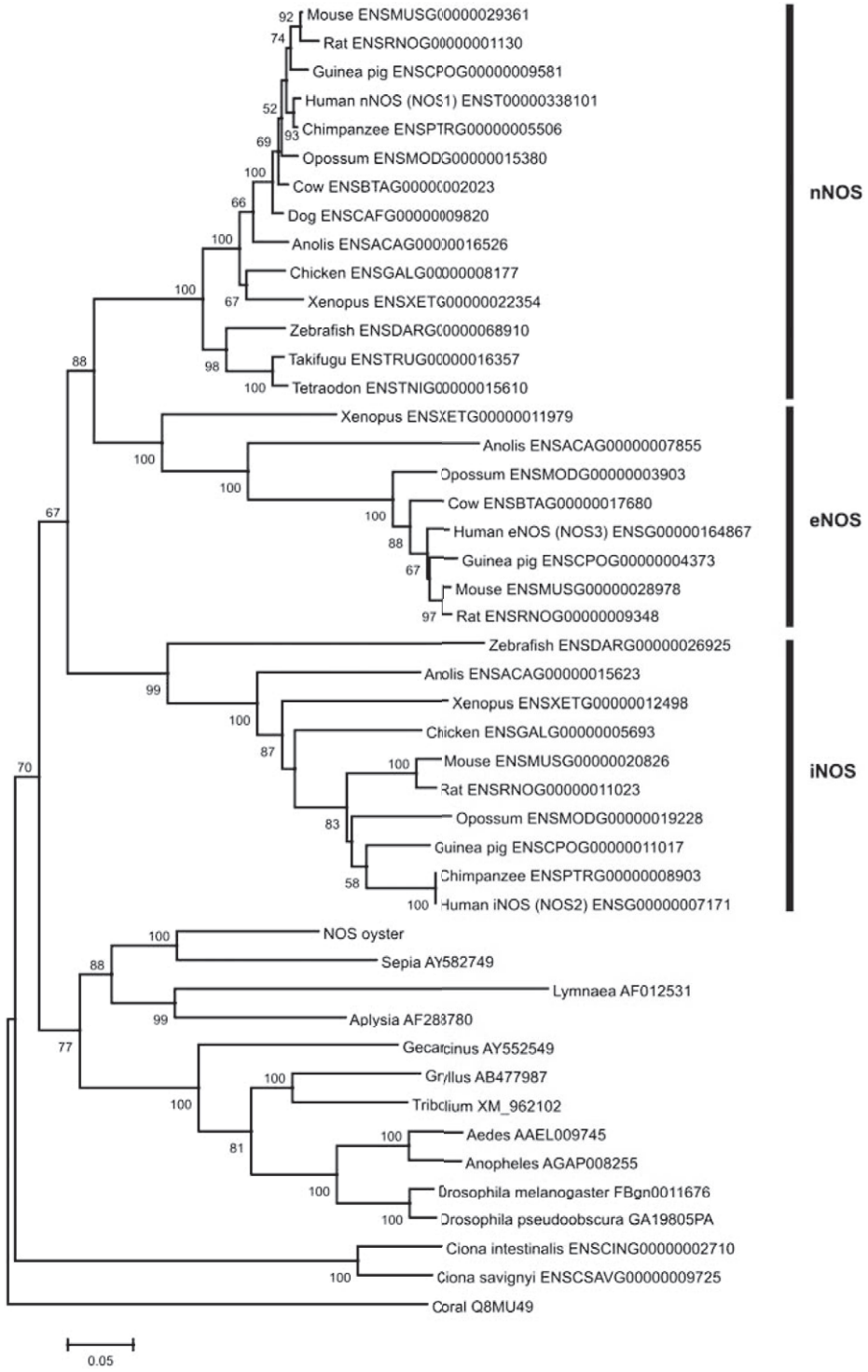

Fig. 2. Minimum evolution tree of representative NOS sequences based on the Dayhoff distance at 430 shared amino acid sites. Support of internal branches was evaluated using the bootstrap method, and only values higher than 50% are shown. NOS sequence from a coral was used as an outgroup. Each sequence is identified by its Ensembl or GenBank accession number and name of the species. nNOS, eNOS, iNOS correspond to the vertebrate branches of neuronal, endothelial and inducible NOS, respectively. Detailed list of sequences used is given in Table S1 in supplementary material.

clustering is supported by the bootstrap value of 77%. Similar results were observed with the ML tree (supplementary material Fig. S1), where molluscan and arthropod sequences also clustered separately from the three strongly supported clusters formed by nNOS, eNOS and iNOS vertebrate isoforms (99–100% bootstrap support or 1.0 approximate likelihood ratio test value for ME or ML trees, respectively). Unlike the ME tree, where the placement of tunicates was not well resolved, in the ML tree the tunicate sequences were clustered with the vertebrate sequences, with the internal branch supported by an approximate likelihood ratio test value of 0.98 (supplementary material Fig. S1). Essentially the same ML tree topology (supplementary material Fig. S2) was obtained using the improved general amino acid replacement matrix (LG) model proposed by Le and Gascuel, which has been shown to outperform both WAG and JTT substitution models (Le and Gascuel, 2008).

Effects of anoxia and Cd exposure on NOS activity and NO content

Long-term exposure to Cd ($50\,\mu g\,l^{-1}$ for 30 days) resulted in a dramatic decrease in NOS activity in gills, by more than 80%

compared with control oysters (P<0.0001) (Fig. 3A,B). In control oysters, long-term anoxia and subsequent reoxygenation had no effect on NOS activity in the gills (P>0.05; Fig. 3A). By contrast, in Cd-exposed oysters, NOS activity in the gills was suppressed in anoxia and quickly returned to control levels after reoxygenation (Fig. 3B). During acute short-term (24 h) Cd exposure of isolated gill cells, there was also a trend to a decreased NOS activity with increasing Cd concentrations, down to 67 and 25% of the control levels at 50 and 250 μmol l^{-1}, respectively. However, this decrease was only marginally significant at the highest tested Cd concentration, 250 μmol l^{-1}, due to the high variability between cell isolates (Table 2). *NOS* mRNA expression and enzyme activity were not significantly correlated in isolated gill cells (R=0.88, P>0.05) (Table 2). Similarly, there was no significant difference in *NOS* mRNA levels between muscle and gill tissues of control oysters (0.46±0.14 and 0.59±0.20 *NOS/β-actin* ratio, respectively; N=4, P>0.05), despite large differences in measurable NOS activity (4.76 pmol NO min^{-1} mg^{-1} protein *vs* non-detectable levels in the gills and adductor muscle, respectively), suggesting that NOS activity is regulated by post-transcriptional mechanisms in oysters.

NO content (determined by the tissue concentrations of nitrate and nitrite) was significantly lower in the adductor muscle but not in the gills of Cd-exposed oysters compared with their control counterparts (Fig. 4A,B). Prolonged anoxia resulted in a gradual decrease in tissue NO levels in control and Cd-exposed oysters. While there was no change in tissue NO concentration during the first day of anoxic exposure, the NO levels decreased to 3–10% of the normoxic values after 2.5–3 days of anoxia and remained at these low levels throughout the remainder of the anoxic period (6 days) (Fig. 4A,B).

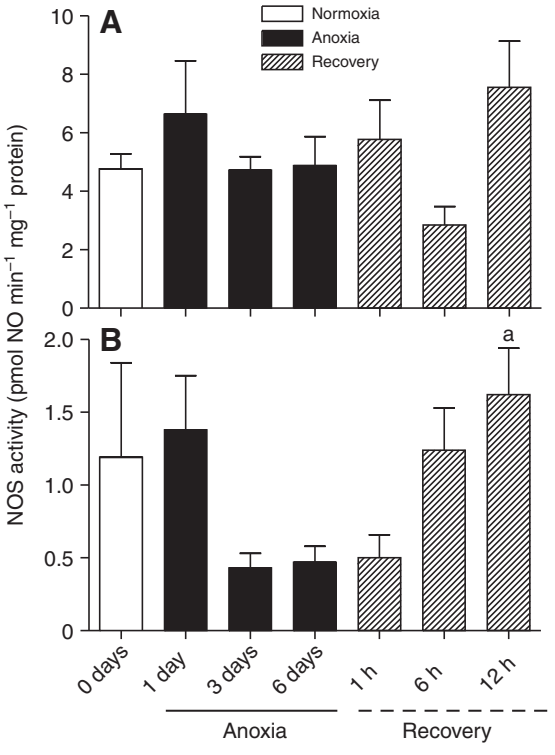

Fig. 3. Specific NOS activity in gills of control (A) and Cd-exposed (B) *C. virginica*. Note that different scales were used for control and Cd-exposed oysters for the sake of visual clarity. Differences in NOS activity between control and Cd-exposed oysters were significant at all time points during normoxia and anoxia/reoxygenation (P<0.01). The effect of anoxia/reoxygenation on NOS activity was not significant in control oysters (P=0.16). In Cd-exposed oysters, NOS activity was significantly different after 12 h of reoxygenation compared with after 3 and 6 days of anoxia and 1 h of reoxygenation in Cd-exposed oysters (indicated by letter a, P<0.05) but not significantly different from the normoxic control (P>0.05). All other groups were not significantly different (P>0.05, N=5–6).

Reoxygenation resulted in a rapid recovery of tissue NO levels in gill and muscle of control oysters, reaching 50–60% of the normoxic levels within the first hour of recovery. In Cd-exposed oysters, restoration of the NO levels was delayed; thus, one hour of reoxygenation restored only about 15–20% of the normoxic NO concentrations in gills and muscle of Cd-exposed oysters (Fig. 4). Later during recovery (6–12 h), tissue NO levels returned to normoxic values in gills of control and Cd-exposed oysters and in the muscle of Cd-exposed oysters (Fig. 4A,B). By contrast, the NO levels in the adductor muscle of control oysters did not fully recover even after 12 h of reoxygenation.

Cellular respiration

In order to test whether low NOS activity in Cd-exposed oysters may partially explain their higher oxygen consumption rates (Lannig et al., 2006; Lannig et al., 2008), we measured $\dot{M}_{O_2}$ of isolated gill cells in the presence of different concentrations of Cd (0–250 μmol l^{-1}), a NOS inhibitor (1 mmol l^{-1} AG) or a NOS substrate (10 mmol l^{-1} L-Arg). On average, incubation with AG resulted in a 60% decrease of NOS activity except in the cells incubated with 250 μmol l^{-1} Cd, where NOS activity was very low (Table 2) and NOS decreased by 20% in response to AG addition. Incubation with AG had no effect on the total cellular respiration, mitochondrial respiration or proton leak in isolated gill cells (P=0.47, 0.73 and 0.92, respectively). Similarly, L-Arg had no effect on NOS activity, total respiration, mitochondrial respiration or proton leak in isolated gill cells (P=0.80–0.96) (data not shown).

Total cellular respiration and mitochondrial $\dot{M}_{O_2}$ increased by ~40% during incubation with low and intermediate Cd concentrations (10–50 μmol l^{-1}) and declined at high Cd levels (250 μmol l^{-1}) (P=0.01 for Cd effects; Table 2). By contrast, mitochondrial proton leak was not affected by Cd exposure (P=0.34). There was no significant correlation between NOS activity and total $\dot{M}_{O_2}$, mitochondrial $\dot{M}_{O_2}$ or proton leak in isolated gill cells (Pearson product-moment correlation, P=0.46–0.91).

Mitochondrial respiration rate

NO donors (SNAP and DETA/NO) significantly affected oxygen consumption ($\dot{M}_{O_2}$) of mitochondria respiring on pyruvate (Fig. 5). The effects were stronger on ADP-stimulated (state 3) respiration than on the resting (state 4) respiration. As a result, mitochondrial respiratory control ratio (RCR) decreased in the presence of NO donors (Fig. 5). Overall, state 3 $\dot{M}_{O_2}$ was significantly inhibited by 100 μmol l^{-1} SNAP or 1 mmol l^{-1} DETA/NO (consistent with the faster rates of NO release by SNAP), while state 4 respiration significantly declined only in the presence of 400 μmol l^{-1} SNAP or 2 mmol l^{-1} DETA/NO (Fig. 5). Incubation of oyster mitochondria with 10 mmol l^{-1} L-Arg or 10 mmol l^{-1} L-NAME had no effect on state 3 or 4 mitochondrial respiration with pyruvate (data not shown).

DISCUSSION

Evolutionary relationships of *NOS* gene from *C. virginica* (CvNOS)

Evolutionary relationships between invertebrate NOS and the three mammalian NOS isoforms (eNOS, iNOS and nNOS) have remained poorly resolved due to insufficient taxon sampling, lack of proper

Table 2. NOS activity, *NOS* mRNA expression and respiration rate ($\dot{M}_{O_2}$) of isolated oyster cells incubated for 24 h with 0–250 μmol l^{-1} Cd

	Cd^{2+} in incubation medium (μmol l^{-1})			
	0	10	50	250
NOS mRNA/*β-actin* ratio	0.81±0.30	1.28±0.49	0.91±0.33	0.70±0.21
NOS activity (pmol NO min^{-1} mg^{-1} protein)	1.44±0.33	2.16±0.82	0.97±0.60	0.37±0.27†
Total $\dot{M}_{O_2}$ (μmol O_2 min^{-1} 10^{-6} cells)	1.29±0.15	1.76±0.20*	1.75±0.18*	1.11±0.07
Mitochondrial $\dot{M}_{O_2}$ (μmol O_2 min^{-1} 10^{-6} cells)	1.20±0.11	1.38±0.15	1.69±0.24*	0.98±0.06
Proton leak (μmol O_2 min^{-1} 10^{-6} cells)	0.61±0.06	0.62±0.07	0.72±0.13	0.51±0.04

Asterisks represent values significantly different from the control (0 μmol l^{-1} Cd) (P<0.05); daggers represent a value marginally different from the control (P=0.07). N=4–7 for mRNA expression and 10 for all other end points.

outgroups and low support of internal branches (e.g. Cox et al., 2001; Wang et al., 2001). Recent availability of numerous completely sequenced or draft assemblies of vertebrate and invertebrate genomes allowed us to overcome these limitations. In the present study, using representative NOS sequences from genomes of 13 vertebrates (representing major vertebrate groups), two tunicates and 12 invertebrates (including mollusks, insects, crustaceans and corals), we show that diversification into three NOS isoforms has occurred inside the vertebrate lineage after the vertebrate–tunicate divergence. Within vertebrates, iNOS sequences form the most basal clade in both ME and ML trees, indicating that it may be the ancestral NOS isoform. However, this clustering pattern received relatively weak bootstrap support (less than 90%), although strongly supported in the ML tree by the approximate likelihood ratio test value of about 0.98 (Fig. 2 and supplementary material Fig. S1).

Interestingly, our genome-wide search did not identify any eNOS homologs from zebrafish, tetraodon or takifugu genomes, suggesting that eNOS was either lost in the fish lineage or appeared due to a gene duplication in the tetrapod lineage after it had split from their common ancestors with fish. This agrees with earlier studies that found only nNOS and iNOS but not eNOS homologues in fish (Cox et al., 2001; Wang et al., 2001; Hyndman et al., 2006; Reddick et al., 2006). By contrast, genomes of all invertebrates (including

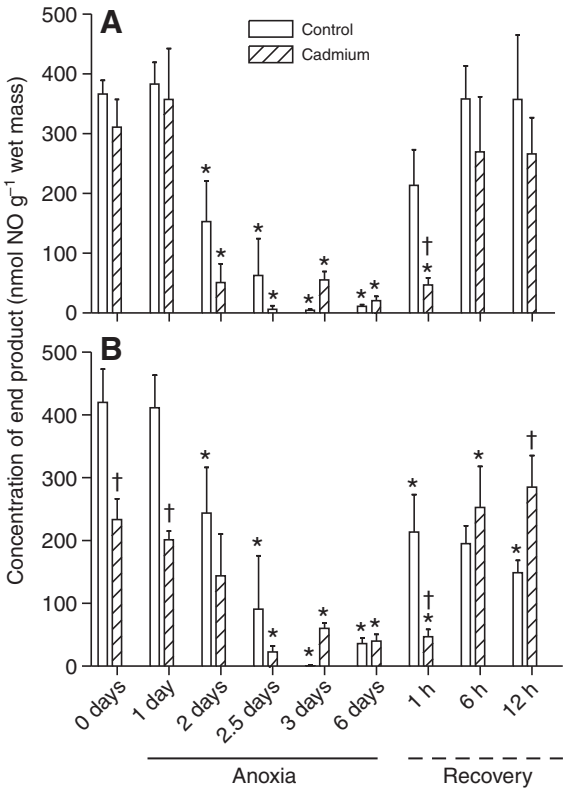

Fig. 4. Levels of stable end products of NO oxidation in gills (A) and adductor muscle (B) in control and Cd-exposed oysters during prolonged anoxia and normoxic recovery. Concentration of stable end products of NO oxidation (nitrite and nitrite) was measured as an index of NO levels and expressed in μmol l^{-1} NO g^{-1} wet tissue mass. Asterisks indicate values significantly different from the respective normoxic values (P<0.05). Daggers show the values that differ between control and Cd-exposed oysters (P<0.05, N=6).

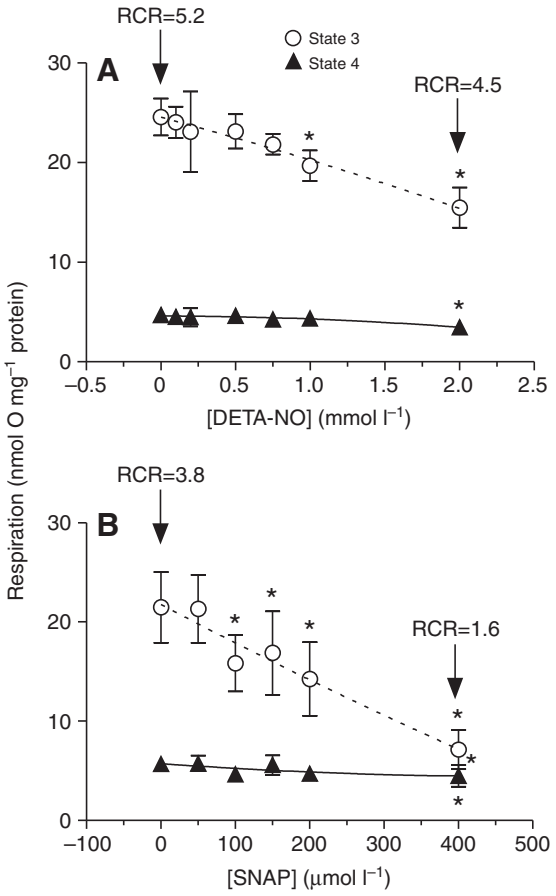

Fig. 5. Effect of NO donors SNAP and DETA/NO on respiration of isolated oyster mitochondria. State 3 (ADP-stimulated) and state 4 (resting) respiration of isolated gill mitochondria was determined in the presence of different concentrations of two nitric oxide donors with pyruvate as a substrate. Asterisks indicate values significantly different from the respective controls (in the absence of the NO donors) (P<0.05, N=6). RCR, respiratory control ratio.

mollusks, arthropods and a lower chordate) contain a single NOS isoform, likely ancestral to the eNOS–iNOS–nNOS vertebrate branch. Although there is no evidence of molecular differentiation of NOS orthologs in invertebrates, there may be functional differentiation *via* alternative transcription start sites and alternative splicing as was shown in *Drosophila* (Stasiv et al., 2001). In the present study, we did not detect alternative splicing isoforms of NOS in oysters. However, dramatic differences in activity of NOS between gill and muscle tissues in oysters suggest some functional differentiation of this enzyme at the level of the maximum enzyme velocity V_{max}.

NOS activity and NO metabolism during environmental stress

Similar to insect NOS (Davies, 2000) and mammalian nNOS and eNOS (Alderton et al., 2001), but unlike mammalian iNOS, CvNOS required Ca^{2+} for full activation, which may suggest that Ca^{2+} dependence is an ancestral trait for the NOS family of enzymes. NOS activity considerably differed between the two studied tissue types in oysters. In the muscle tissue, NOS activity was undetectable under the assay conditions of this study, whereas in gills it averaged 4.76 and 1.19 pmol NO $min^{-1} mg^{-1}$ protein for control and Cd-exposed oysters, respectively. These values are within the broad range of specific activities reported for constitutive NOS enzymes from mammalian tissues, which vary from 1–2 pmol NO $min^{-1} mg^{-1}$ protein in adipose tissue, lungs and liver to 20 pmol NO $min^{-1} mg^{-1}$ protein in the brain (Ribiere et al., 1996; Tagliente et al., 1997; Newaz et al., 2005).

Cd exposure *in vivo* and *in vitro* resulted in a strong inhibition of NOS activity in oyster gills. Thus, NOS activity in gills of oysters exposed to 50 $\mu g l^{-1}$ (0.44 $\mu mol l^{-1}$) Cd for 30 days was 25% of the respective values for control oysters, and short-term exposure of isolated cells to 250 $\mu mol l^{-1}$ Cd resulted in a similar decrease of NOS activity, to approximately 25% of the respective controls. Earlier studies in mammals showed that Cd inhibits activity of Ca^{2+}-dependent constitutive NOS, likely due to the competitive displacement of Ca^{2+} from calmodulin (Mittal et al., 1995; Weaver et al., 2002; Weaver et al., 2004). Such direct inhibition by Cd may explain the observed decline in NOS activity in oyster cells incubated for a short time (24 h) at high concentrations of Cd (250 $\mu mol l^{-1}$). However, it is remarkable that even after 30 days of *in vivo* incubation with 50 $\mu g l^{-1}$ (0.44 $\mu mol l^{-1}$) Cd, which allows sufficient time for a compensatory response, NOS activity remains significantly suppressed in Cd-exposed oysters. It is unlikely that this suppression reflects a direct inhibition of NOS by high Cd levels accumulated in the cytosol of oysters. Indeed, in the present study, NOS activity was determined under the conditions of excess Ca^{2+} (6 $mmol l^{-1}$) in the assay medium, which would be sufficient to overcome the potential carryover of cytosolic Cd; based on our earlier studies of Cd accumulation under identical exposure regimes, such carryover would not exceed 1 $\mu mol l^{-1}$ in homogenates from Cd-exposed mollusks (Lannig et al., 2006; Cherkasov et al., 2007b). This raises an intriguing possibility that the reduced NOS activity is part of an adaptive response to long-term Cd exposure in oysters. Indeed, it has been shown that nitric oxide releases metals from metallothioneins (MTs) and affects MT gene expression (Kroncke et al., 1994; Aravindakumar et al., 1999; Katakai et al., 2001; Zangger et al., 2001). Under non-stressed conditions, elevated NO levels induce MT expression, most likely due to the NO-stimulated release of Zn^{2+} (Katakai et al., 2001; Datta and Lianos, 2006). In line with these findings, short-term pre-treatment with NO donors enhances MT induction and can protect against subsequent Cd-induced cytotoxicity (Liu et al., 2004; Poliandri et al., 2004; Qu et al., 2005; Poliandri et al., 2006). By contrast, long-term sustained elevation of NO levels may blunt MT expression, lead to the release of MT-bound metals, including Cd, and reduce cell viability (Misra et al., 1996; Molinero et al., 1998; Pearce et al., 2000; Zangger et al., 2001; Ostad et al., 2008). Thus, suppression of NOS activity during long-term exposure to Cd may be a cytoprotective response in oysters. Further studies will be needed to test this hypothesis and to determine whether physiologically relevant changes in NO levels modulate Cd toxicity in oysters.

Steady-state levels of nitrite/nitrate (indicative of the NO levels) tended to be lower in Cd-exposed oysters compared with their control counterparts; these differences were significant in the muscle but not the gill tissues. As expected, prolonged anoxia resulted in a decrease of nitrite/nitrate levels in oyster tissues, likely reflecting a decrease in NO production due to the absence of O_2, a required substrate for NOS. Interestingly, although hemolymph O_2 levels in oysters decrease to zero within 10–20 min of shell closure (Kurochkin et al., 2009), nitrate/nitrite levels in oyster tissues did not significantly decline until the second day of anoxic exposure and dropped below 10% of the normoxic levels only after 3–6 days in anoxia. Given strict O_2 dependency of NOS, the persistence of tissue nitrate/nitrite for the first 24–48 h of anoxic exposure is unlikely to be due to the continuing NO production by NOS and probably reflects delayed metabolism and excretion of nitrate/nitrite (Wagner et al., 1984). Although unrelated to NOS activity, this persistence of nitrate/nitrite in tissues during anoxia may have important physiological and methodological implications. From the physiological viewpoint, tissue nitrite may serve as a source of non-enzymatic NO production under the acidic and highly reduced conditions of anaerobic tissues (Zweier et al., 1999; Benamar et al., 2008), thus possibly maintaining some of the regulatory and signaling functions of NO in anoxia. This persistence also implies that tissue nitrate/nitrite levels should be interpreted as an indicator of NO content with extreme caution, especially when NOS activity and/or NO levels are undergoing a rapid change in response to altered physiological or pathological conditions, in which case changes in the tissue nitrate/nitrite levels are expected to significantly lag behind.

Interestingly, anoxia and subsequent normoxic recovery affected NOS activity in Cd-exposed oysters but not in their control counterparts. Prolonged anoxic exposure (3–6 days) resulted in a notable decrease of NOS activity in Cd-exposed oysters, which was not reverted until 6 h of post-anoxic recovery. By contrast, NOS enzyme activity did not change during anoxia/reoxygenation in control oysters. Recovery of the normoxic steady-state levels of tissue nitrite/nitrate was also slower in Cd-exposed oysters, hand-in-hand with their lower NOS activity. In Cd-exposed oysters, it took 6 h of aerobic recovery for the nitrate/nitrite levels to reach normoxic values, whereas in their control counterparts, tissue nitrate/nitrite content was restored within one hour of reoxygenation.

NO in mitochondrial and cellular bioenergetics of oysters

In mammals, NO can modulate aerobic metabolism and is, in fact, the only known direct regulator of mitochondrial respiration (Brown, 1999; Brown, 2007). At low, physiological levels, NO inhibits mitochondrial respiration *via* the competitive inhibition of Complex IV (cytochrome *c* oxidase) activity (Brown, 1999; Brown, 2007). At higher levels, NO may also inhibit upstream ETC complexes and other mitochondrial proteins (such as F_0,F_1-ATPase and aconitase) due to nitrosylation by highly reactive peroxynitrite, which forms from NO and superoxide in mitochondria (Brown, 1999; Brown and Borutaite, 2006; Brown, 2007). Given the high

degree of evolutionary conservatism of mitochondrial structure and function among animals, it is important to explore whether this molecule may also be involved in regulation of aerobic respiration of mollusks. Moreover, elevated rates of cellular and whole-organism oxygen consumption of Cd-exposed oysters described previously (Cherkasov et al., 2006a; Lannig et al., 2006; Lannig et al., 2008), along with their lower NOS activity discovered in this study, raise a possibility that the lower rate of NO synthesis may be partially responsible for the higher respiration rates of Cd-exposed oysters due to the release of NO-dependent inhibition of mitochondrial ETC.

Exposure of oyster mitochondria to NO donors SNAP and DETA/NO resulted in a dose-dependent decrease of mitochondrial oxygen consumption. State 3 (ADP-stimulated) respiration was significantly more sensitive to inhibition by NO than state 4 (resting) respiration, indicating that regulation by NO may be especially important under conditions of high mitochondrial flux. Based on the published half-lives of the NO donors (5 and 20 h for SNAP and DETA/NO, respectively) (Ramirez et al., 1996; Wang et al., 2005), concentrations of donors that inhibit 20–30% of state 3 respiration in oyster mitochondria correspond to the rate of NO release of 7–9 nmol l^{-1} s^{-1} (1 mmol l^{-1} DETA/NO and 200 μmol l^{-1} SNAP). State 4 respiration was not significantly changed by these levels of NO donors. At the concentrations of NO donors releasing 15–19 nmol l^{-1} NO s^{-1} (2 mmol l^{-1} DETA/NO and 400 μmol l^{-1} SNAP), state 3 and state 4 respiration was inhibited by 40–65% and 20–25%, respectively. Given a short half-life of NO in air-saturated aqueous solutions (<15 s) (Hakim et al., 1996), we expect that NO levels have remained in nanomolar range throughout the duration of our mitochondrial assays. It is worth noting that we determined NO sensitivity of oyster mitochondria in air-saturated assay media (at approximately 200 μmol l^{-1} O_2), and it is likely to be higher under the low oxygen conditions of oyster tissues *in vivo*. Thus, in isolated mitochondria from mammalian brown adipose tissue, half-inhibition of respiration occurred at 364 nmol l^{-1} NO at 180 μmol l^{-1} O_2 but only required 11 nmol l^{-1} NO at 32 μmol l^{-1} O_2, close to the typical tissue O_2 levels (Koivisto et al., 1997). To the best of our knowledge, this is the first report of the direct effects of NO on invertebrate mitochondria, which suggests that NO-dependent regulation of bioenergetics may be an ancient, evolutionarily conserved function of this important signaling molecule.

Sensitivity of oyster mitochondrial respiration to NO donors is within the range of values reported earlier for mammalian tissues, isolated cells and mitochondria. Thus, 100 μmol l^{-1} SNAP inhibited approximately 35–60% of oxygen consumption in rat and human cardiac muscle and in rat aortic rings (Xie et al., 1998; Mital et al., 2004; Nunez et al., 2005) whereas 1 mmol l^{-1} SNAP reduced respiration of mouse macrophages by approximately 30% (Szabo and Salzman, 1995). Similarly, 1 mmol l^{-1} DETA/NO led to an approximately 10% decrease in respiration of isolated mitochondria from rat hearts (Jekabsone et al., 2003), while rat aortic rings were more sensitive, with more than 60% inhibition found at 100 μmol l^{-1} DETA/NO (Nunez et al., 2005). In contrast to the strong effects of exogenous NO, supplementation of oyster mitochondria with L-Arg to stimulate endogenous NO production did not affect mitochondrial respiration. This may suggest that either endogenously produced NO levels in isolated oyster mitochondria are low (due to the absence and/or low activity of mitochondrial NOS) or that mitochondrial NOS activity is saturated with respect to L-Arg and no further increase is possible in response to L-Arg supplementation. With respect to the latter hypothesis, it is worth noting that, in mammals, mitochondrial NO production is negligible in the absence of exogenous L-Arg and is strongly stimulated by L-Arg supplementation (French et al., 2001). However, this situation may be different in oysters that contain high intracellular levels of free amino acids including L-Arg (0.6–0.9 μg g^{-1} wet mass) (Kurochkin et al., 2009). Similarly, L-Arg supplementation of isolated oyster cells did not affect cellular $\dot{M}_{O_2}$, suggesting that 'L-arginine paradox' (Bode-Böger et al., 2007) may not be present in oysters.

Incubation of isolated cells with low Cd concentrations (10–50 μmol l^{-1}) resulted in elevated total and mitochondrial $\dot{M}_{O_2}$, consistent with our earlier findings of elevated energy demand during sublethal Cd exposures (Cherkasov et al., 2006a; Lannig et al., 2006; Lannig et al., 2008). The highest tested Cd concentration (250 μmol l^{-1}) inhibited respiration of isolated cells, likely due to the acute toxic effects of the metal (Sokolova and Lannig, 2008). The opposing effects on respiration of high *vs* low concentrations of Cd agree with the earlier studies showing that, at low levels, Cd and other trace metals stimulate respiration due to the additional energy demand for detoxification and damage repair whereas at high metal levels respiration is typically inhibited as many cellular systems (including mitochondrial ETC) shut down due to the excessive metal-induced damage (reviewed in Sokolova and Lannig, 2008). Notably, mitochondrial state 4 was not affected by Cd exposure, consistent with our earlier findings of low sensitivity of mitochondrial proton leak to Cd (Sokolova, 2004; Cherkasov et al., 2006b). There was no correlation between NOS activity and cellular or mitochondrial $\dot{M}_{O_2}$ in control or Cd-exposed gill cells, suggesting that the observed elevation of cellular respiration due to Cd exposure is not related to lower NOS activity and slower rates of endogenous NO production in oysters. An earlier study in mammalian endothelial cells also found no correlation between Cd-induced changes in endogenous NO production and cellular oxygen consumption by showing that Cd exposure inhibits cellular respiration despite a considerable suppression of endogenous NO release (Majumder et al., 2008).

Experimental modulation of NOS activity in isolated gill cells of oysters also had no effect on the overall cellular respiration, mitochondrial respiration or proton leak. This may reflect the fact that in resting, non-stimulated cells most mitochondria are in state 4, which, as shown in the present study, is relatively insensitive to NO inhibition. In mammalian models, the effects of endogenous NO on cellular respiration are controversial; in some cell types, inhibition of background NOS activity stimulates oxygen consumption whereas in other cell types the effect of NOS modulation on $\dot{M}_{O_2}$ is negligible (reviewed in Brown, 1999; Brown, 2007). Overall, the effects of endogenous NO production on respiration appear to be dependent on the cell type, the rate of background NO generation and/or physiological state of the cell (e.g. respiration of resting cells is typically less sensitive to NO inhibitors than that of stimulated cells or cells in pathophysiological states when NO production is abnormally high) (Brown, 1999; Brown, 2007). Given our finding that phosphorylating mitochondria are considerably more sensitive to NO inhibition than resting ones, one may also expect that sensitivity of cellular respiration to NO will be most prominent when metabolic flux is high so that most mitochondria function near state 3. To the best of our knowledge, such a relationship between metabolic flux and NO-dependent regulation of cellular metabolism has yet to be explored and would represent an exciting new avenue for future studies. Invertebrates, including mollusks, may serve as excellent model organisms for such studies given their NO-sensitive mitochondria and their ability to rapidly and significantly modify metabolic rates in response to

the environmental conditions, such as temperature stress, entrance into the dormant or metabolically depressed states, post-anoxic and post-hypoxic recovery, and others.

LIST OF ABBREVIATIONS

AG	aminoguanidine
aLRT	approximate likelihood-ratio test
ASW	artificial seawater
cDNA	complementary DNA
DETA/NO	diethylenetriamine/*NO*
ETC	electron transport chain
eNOS	endothelial NOS
E	amplification efficiencies
HIF-1	hypoxia inducible factor-1
iNOS	inducible NOS
L-Arg	L-arginine
L-NAME	N^G-nitro-L-arginine methyl ester
ME	minimum-evolution method
ML	maximum likelihood method
$\dot{M}_{O_2}$	oxygen consumption
NO	nitric oxide
NOS	nitric oxide synthase
nNOS	neuronal NOS
qRT-PCR	quantitative real-time PCR
RACE-PCR	rapid amplification of cDNA ends PCR
RCR	respiratory control ratio
RNS	reactive nitrogen species
ROS	reactive oxygen species
SNAP	*S*-nitroso-*N*-acetylpenicillamine

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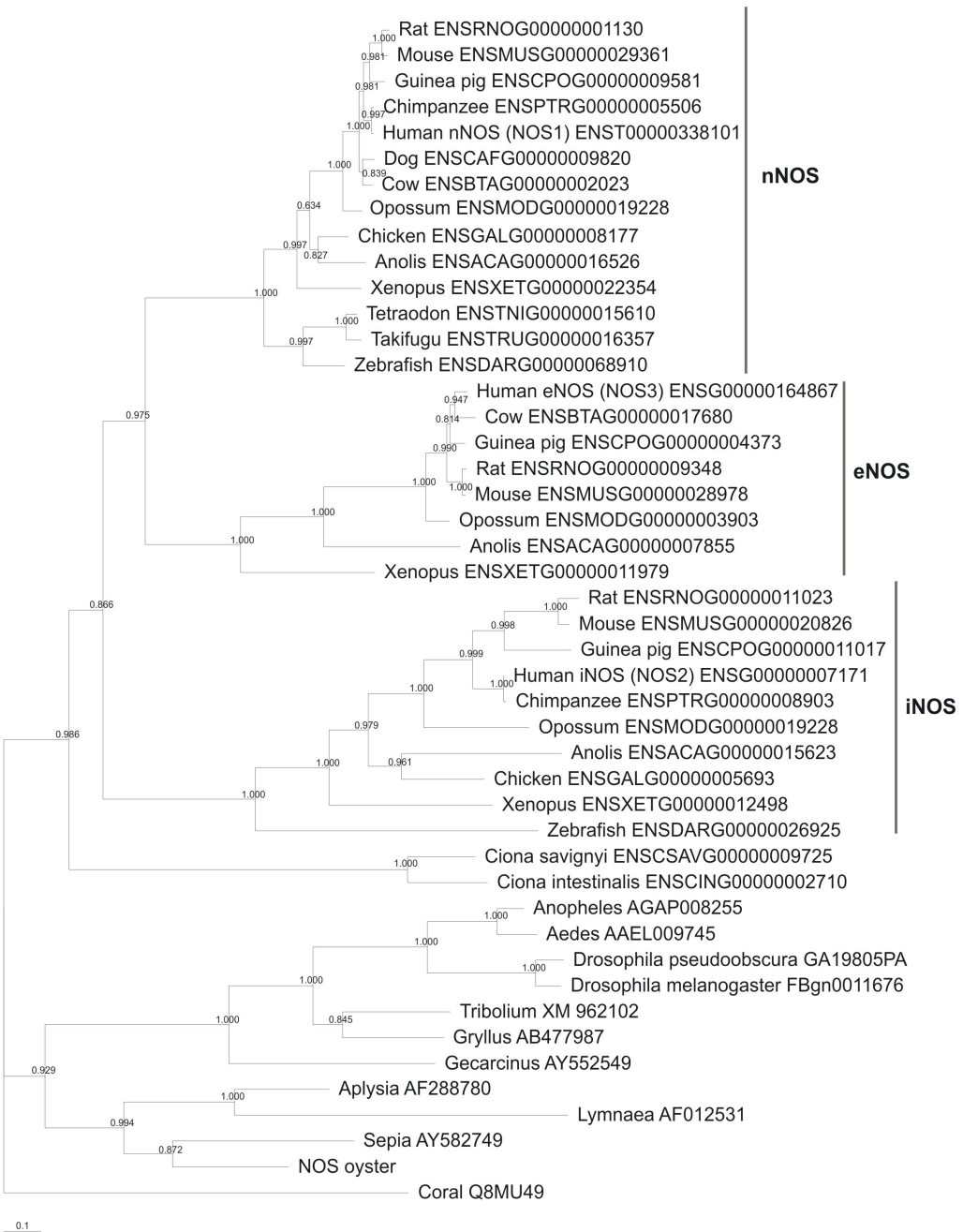
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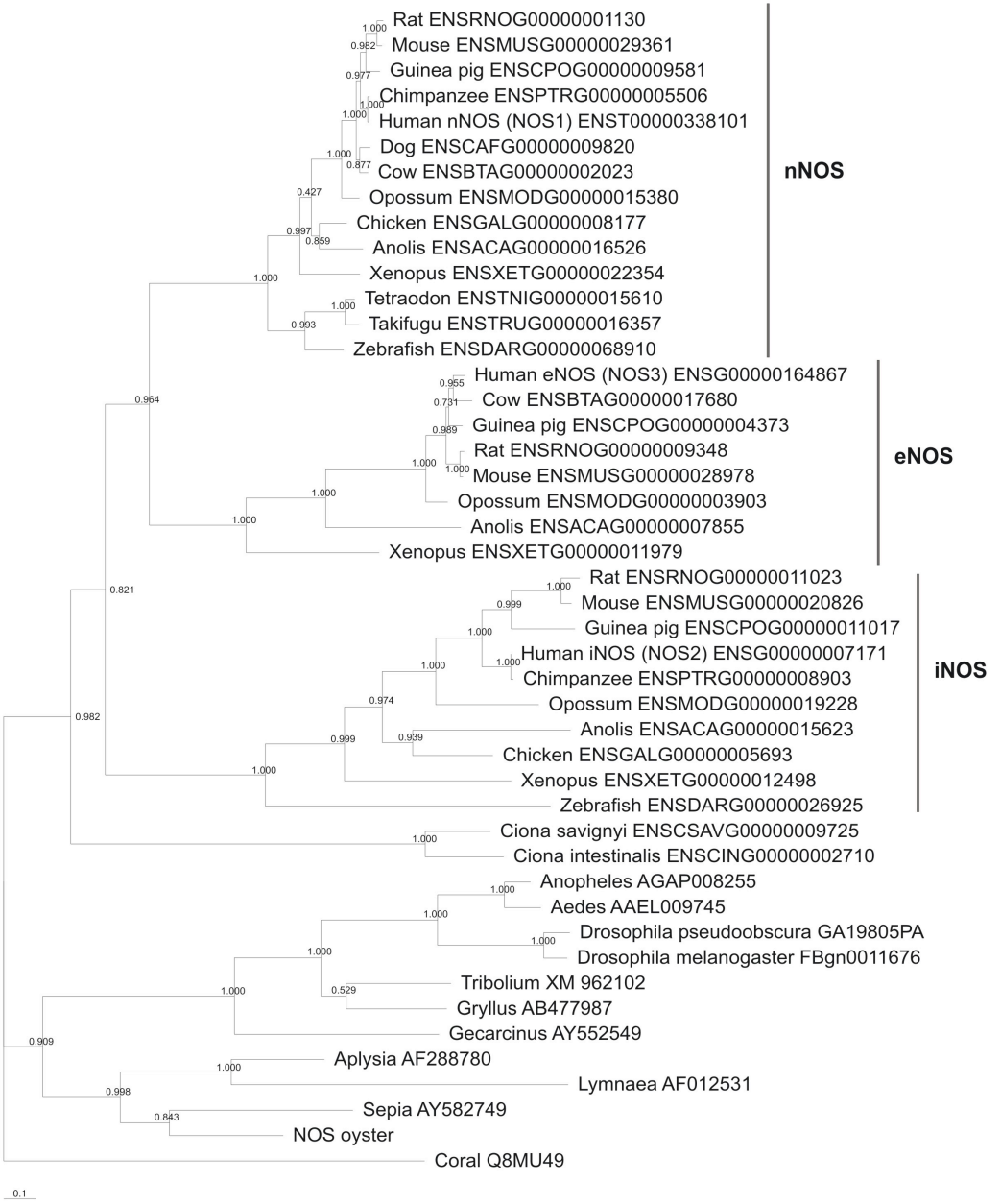
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Drosophila melanogaster FBgn0011676
Tribolium XM 962102
Gryllus AB477987
Gecarcinus AY552549
Aplysia AF288780
Lymnaea AF012531
Sepia AY582749
NOS oyster
Coral Q8MU49
0.1

Table S1. List of NOS sequences used for the construction of phylogenetic trees (Fig. 2, Fig. S1, Fig. S2) and their respective GenBank or Ensembl (ENSXXX) accession/identification numbers

Species name	GenBank or Ensembl ID
Aedes aegypti	AAEL009745
Anolis carolinensis	ENSACAG00000016526
	ENSACAG00000015623
	ENSACAG00000007855
Anopheles gambiae	AGAP008255
Aplysia californica	AF288780
Chicken	ENSGALG00000008177
	ENSGALG00000005693
Chimpanzee	ENSPTRG00000008903
	ENSPTRG00000019868
	ENSPTRG00000005506
Ciona intestinalis	ENSCING00000002710
Ciona savignyi	ENSCSAVG00000009725
Coral	Q8MU49
Cow	ENSBTAG00000002023
	ENSBTAG00000017680
	ENSBTAG00000006894
Drosophila melanogaster	FBgn0011676
Drosophila pseudoobscura	GA19805PA
Gecarcinus lateralis	AY552549
Gryllus bimaculatus	AB477987
Guinea pig	ENSCPOG00000009581
	ENSCPOG00000011017
	ENSCPOG00000004373
Human nNOS	ENST00000338101
Human eNOS	ENSG00000164867
Human iNOS	ENSG00000007171
Lymnaea stagnalis	AF012531
Mouse	ENSMUSG00000029361
	ENSMUSG00000020826
	ENSMUSG00000028978
Opossum	ENSMODG00000015380
	ENSMODG00000019228
	ENSMODG00000003903
Rat	ENSRNOG00000001130
	ENSRNOG00000011023
	ENSRNOG00000009348
Sepia officinalis	AY582749
Takifugu rubripes	ENSTRUG00000016357
Tetraodon nigroviridis	ENSTNIG00000015610
Tribolium castaneum	XM_962102
Xenopus laevis	ENSXETG00000022354
	ENSXETG00000012498
	ENSXETG00000011979
Zebrafish	ENSDARG00000068910
	ENSDARG00000026925