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RESEARCH ARTICLE

A role for nitric oxide in the control of breathing in zebrafish (*Danio rerio*)

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ABSTRACT

Nitric oxide (NO) is a gaseous neurotransmitter, which, in adult mammals, modulates the acute hypoxic ventilatory response; its role in the control of breathing in fish during development is unknown. We addressed the interactive effects of developmental age and NO in the control of piscine breathing by measuring the ventilatory response of zebrafish (*Danio rerio*) adults and larvae to NO donors and by inhibiting endogenous production of NO. In adults, sodium nitroprusside (SNP), a NO donor, inhibited ventilation; the extent of the ventilatory inhibition was related to the pre-existing ventilatory drive, with the greatest inhibition exhibited during exposure to hypoxia (P_{O_2}=5.6 kPa). Inhibition of endogenous NO production using L-NAME suppressed the hypoventilatory response to hyperoxia, supporting an inhibitory role of NO in adult zebrafish. Neuroepithelial cells (NECs), the putative oxygen chemoreceptors of fish, contain neuronal nitric oxide synthase (nNOS). In zebrafish larvae at 4 days post-fertilization, SNP increased ventilation in a concentration-dependent manner. Inhibition of NOS activity with L-NAME or knockdown of nNOS inhibited the hypoxic (P_{O_2}=3.5 kPa) ventilatory response. Immunohistochemistry revealed the presence of nNOS in the NECs of larvae. Taken together, these data suggest that NO plays an inhibitory role in the control of ventilation in adult zebrafish, but an excitatory role in larvae.

KEY WORDS: Chemoreceptor, Hypoxia, Hyperoxia, Immunohistochemistry, Neuroepithelial cell, Morpholino

INTRODUCTION

Gasotransmitters are endogenous gaseous signalling molecules that are known to promote several of the physiological responses to hypoxia including O_2 chemoreception by glomus cells of the carotid body. The role of these gasotransmitters, hydrogen sulphide (H_2S), nitric oxide (NO) and carbon monoxide (CO), in carotid body O_2 sensing has been studied extensively in adult (or sexually mature) mammals (Prabhakar and Semenza, 2012; Prabhakar, 2012). However, their role in carotid body chemoreception during early development (e.g. in neonates) is unknown. Although there is an increasing awareness of the physiological significance of gasotransmitters in adult lower vertebrates (Fago et al., 2012; Olson et al., 2012), as in mammals, there are few data concerning their role during early development. To our knowledge, only two studies (Hedrick et al., 2005; Porteus et al., 2014) have systematically compared the interactive effects of gasotransmitters and developmental age and only one of these assessed the O_2 chemosensory response (Porteus et al., 2014). In that study, it was shown that endogenously produced H_2S augmented the hyperventilatory response to hypoxia in both adult and larval (4 days post-fertilization, dpf) zebrafish, *Danio rerio* (Porteus et al., 2014).

Nothing is known of the interplay between developmental age, NO and the hypoxic ventilatory response, and no study has yet assessed the role of NO in O_2 chemoreception in fish. Thus, the intent of the present study was to exploit the rapid generation time of zebrafish, and its utility for gene knockdown, to assess and compare, for the first time, the role of NO in O_2 chemoreception in adults and during early development in a model vertebrate species.

NO is formed from the reaction of L-arginine with O_2, a reaction catalysed by nitric oxide synthase (NOS). Like other gasotransmitters, NO diffuses readily through cellular membranes (Hill et al., 2010). Mammals possess three forms of NOS; neuronal NOS (nNOS or NOSI), inducible NOS (iNOS or NOSII) and endothelial NOS (eNOS or NOSIII). Fish have only two NOS genes; a unique orthologue of eNOS (NOSIII) is not present in any fish genome studied to date (Olson et al., 2012). However, a recent phylogenetic analysis of the evolution of NOS genes in metazoans revealed that teleost fish have a NOSI/III form (referred to as nNOS in the literature) and one NOSII gene (referred to as iNOS) (Andreakis et al., 2011).

NO is involved in the hypoxic ventilatory response of mammals by acting on both peripheral chemoreceptors and the respiratory centres of the central nervous system (CNS). Carotid bodies are innervated by efferent nerves that contain nNOS and acetylcholine (ACh) (Campanucci and Nurse, 2007). In the carotid body, NO is released from efferent nerves and inhibits the release of ACh from glomus cells, thereby inhibiting afferent nerve discharge (Campanucci and Nurse, 2007; Wang et al., 1995a). Additionally, NO causes vasodilation of the vasculature around the carotid body, promoting blood flow to the carotid body, which in turn also inhibits chemoreception (Campanucci and Nurse, 2007; Wang et al., 1995a). However, in the CNS, where respiratory network activity is modulated, NO promotes rhythm generation (Pierrefiche et al., 2002). Therefore, in mammals, NO has an inhibitory role in the carotid body but may be excitatory in the CNS.

In fish, neuroepithelial cells (NECs) (Dunel-Erb et al., 1982) are putative O_2 chemoreceptors (Jonz et al., 2004) which share several properties with mammalian carotid body glomus cells (Jonz et al., 2004). They contain neurotransmitters (Jonz and Nurse, 2003), are innervated, and exhibit membrane depolarization and a presumed increased afferent nerve discharge in response to hypoxia (Burleson et al., 2006; Jonz et al., 2004; Qin et al., 2010). Thus, the NECs are considered to be phylogenetic precursors of glomus cells (Milsom and Burleson, 2007). To study the interplay between developmental

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List of abbreviations

5-HT	serotonin
ACh	acetylcholine
CNS	central nervous system
dpf	days post-fertilization
H_2S	hydrogen sulphide
L-NAME	*NG*-nitro-L-arginine methyl ester
MS-222	tricaine methanesulphonate
NEC	neuroepithelial cell
NO	nitric oxide
nNOS	neuronal nitric oxide synthase
NTS	nucleus tractus solitarius
SNP	sodium nitroprusside

age, NO and NEC chemoreception, we first determined the effects of exogenous NO on ventilation in adult zebrafish experiencing widely different levels of ventilatory drive (i.e. fish were exposed to normoxia, hypoxia or hyperoxia) and in larvae that exhibit an intrinsically low ventilatory drive. We predicted that NO would inhibit the ventilatory response, particularly under conditions of high ventilatory drive (hypoxia). We then tested the effect of inhibiting NO production using either a general NOS inhibitor or specific gene knockdown (larvae only) of nNOS on the ventilatory responses to hypoxia or hyperoxia. In addition, we used immunohistochemistry to determine the location of nNOS in the NECs of adult gill and in zebrafish larvae at 4 dpf.

MATERIALS AND METHODS

Ethical approval

All procedures were conducted in accordance with the guidelines established by the Canadian Council for Animal Care and were approved by the University of British Columbia Animal Care Committee (Protocol A06-1510) and University of Ottawa Animal Care Committee (Protocol BL-226).

Animals

Adult zebrafish, *D. rerio* (Hamilton 1822) were obtained from commercial suppliers (Noah's Pet Ark or Delta Aquatics, Vancouver, BC, Canada, or Big Al's Aquarium Services, Ottawa, ON, Canada) and were maintained on a 12 h:12 h light:dark cycle at 28°C in either dechlorinated city of Vancouver tapwater [the ionic composition of the water was (in mmol l^{-1}): Na^+, 0.08; Cl^-, 0.06; Ca^{2+}, 0.03; K^+, 0.004; pH 7.0; Metro Vancouver Water Quality Report 2012; http://www.metrovancouver.org/services/water/WaterPublications/2012WaterQualityControlAnnualReportVolume1.pdf] or dechloraminated City of Ottawa tapwater [the ionic composition of the water was (in mmol l^{-1}): Na^+, 0.78; Cl^-, 0.4; Ca^{2+}, 0.25; K^+, 0.025; pH 7.6]. Embryos were obtained using standard zebrafish breeding techniques (Westerfield, 2000). Fertilized eggs were placed in Petri dishes under conditions identical to those for the adults except that 0.05% Methylene Blue was added to the holding water.

Series I: adult zebrafish

Ventilatory responses of adult zebrafish to exogenous NO

Ventilation rate in adult zebrafish was measured non-invasively as described previously (Porteus et al., 2014; Vulesevic et al., 2006). Briefly, fish were placed in cylindrical plastic chambers (University of Ottawa, Canada). Each chamber was supplied by gravity with continuous water flow (~1 ml min^{-1}). Two electrodes were submerged in the water inside the chamber and mesh was used to prevent the fish from coming into contact with the electrodes. The analog signals from the electrodes, which represented opercular displacements, were amplified (amplifier was custom built at University of Ottawa) and transferred to a computer as digital data using an A/D interfacing system and data acquisition software (AcqKnowledge, BioPac Systems Inc., Galeta, CA, USA) at a sampling rate of 500 Hz. Although previous studies have used calibration procedures to quantify the magnitude of the linear deflections (in mm) of the opercular movements (Vulesevic and Perry, 2006; Vulesevic et al., 2006), the current study focused exclusively on quantifying breathing frequency because ventilation amplitude in zebrafish is largely unaffected by hypoxia (Vulesevic and Perry, 2006). Thus, in the current study, each fish was assumed to have a resting breathing amplitude of 0.5 mm (maximum combined linear displacement of both operculae during inspiration) and the system was calibrated accordingly. Breathing frequencies were determined by *post hoc* analysis of the AcqKnowledge files.

Adult zebrafish were placed in the breathing recording chambers for 1–3 h prior to the start of experiments. After recording breathing under control conditions, different groups of zebrafish were exposed to 5 min hyperoxia (100% O_2), followed by 5 min of hyperoxia plus 100 µmol l^{-1} of sodium nitroprusside (SNP, a NO donor). Measurements were taken during the last minute of the recording period. The fish were allowed to recover for 5 min and breathing frequency was quantified during the final minute of recovery. This experiment was repeated with different adult zebrafish exposed to either normoxia (P_{O_2}≈21 kPa) or hypoxia (P_{O_2}=5.6 kPa) instead of hyperoxia.

Effect of NO synthesis inhibitors on ventilation in adult zebrafish

The nNOS inhibitor *NG*-nitro-L-arginine methyl ester (L-NAME, Sigma-Aldrich, Oakville, ON, Canada) was used to further test the role of NO in the control of breathing in adult zebrafish. Fish were injected with either a solution of 100 µg L-NAME or a similar volume (3 µl) of saline (sham), 48 h prior to the start of experiments. Ventilation was measured as described above during resting conditions (pre-exposure) and the measurements were continued as fish were exposed to hyperoxia (100% O_2) for 5 min and then allowed to recover under normoxic conditions. Measurements were taken over the final minute of each time period (pre-exposure, hyperoxia and recovery).

A separate group of adult zebrafish were injected with 100 µg L-NAME, 48 h prior to the start of experiments. Ventilation frequency was measured during resting conditions (pre-exposure). Zebrafish were then exposed to 5 min of hyperoxia (100% O_2), followed by 5 min of hyperoxia and 400 µmol l^{-1} SNP, then allowed to recover under pre-exposure conditions (normoxia).

Immunohistochemistry in adult gills

Adult zebrafish were killed by an overdose of MS-222 (tricaine methanesulphonate; 0.5 g l^{-1}) followed by a sharp blow to the head. Whole gill baskets were dissected and placed in 4% paraformaldehyde prepared in PBS containing (in mmol l^{-1}): NaCl, 137; Na_2HPO_4, 15.2; KCl, 2.7; KH_2PO_4, 1.5; buffered to pH 7.8 with 1 mol l^{-1} NaOH overnight at 4°C (Jonz and Nurse, 2003). Afterwards, they were rinsed in PBS and dehydrated by a graded series of ethanol (70%, 80%, 90% and 100%), followed by toluene, and infiltrated and embedded in Paraplast wax. Sections of 5–10 µm were incubated overnight with primary antibodies on slides kept at room temperature in a humidified chamber. The antibodies were anti-5-HT mouse (Dako, 1:50 dilution) and nNOS (nNOS rabbit, Transduction Labs, 1:250 dilution), used in combination and diluted in a permeabilizing solution (PBS with 2% Triton-X). Sections were then treated with fluorescently labelled secondary antibodies diluted in PBS (goat anti-rabbit conjugated with fluorescent isothiocyanate, 1:50 dilution and goat anti-mouse conjugated with Alexa 568, 1:100 dilution). Sections were incubated in the dark at room temperature for 2 h. After washing, the sections were mounted with Vectashield (Vector Laboratories Inc., Burlington, CA, USA) to reduce photobleaching during confocal scanning. Negative control experiments that excluded the primary antibodies showed a scarce non-specific labelling of the structures (data not shown). Images were acquired using a confocal microscope (Zeiss LSM 700, Jena, Germany) equipped with solid state lasers (405, 488, 639 nm) and a spectral variable secondary dichroic beam splitter.

Series II: larval zebrafish

Ventilatory responses

Ventilatory responses of zebrafish larvae were obtained as described previously (Coccimiglio and Jonz, 2012; Jonz and Nurse, 2005). Briefly, 4 dpf larvae were anaesthetized with 0.05 mg ml^{-1} Tris-buffered MS-222 (Westerfield, 2000) to minimize gross movements. The larvae were placed

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in a groove in a plastic chamber and were confined to a small area of the well using fine mesh. The dish was perfused with solutions using gravity. The experimental chamber was placed on the water jacket stage of a dissecting microscope (Model SZX10, Olympus, Richmond Hills, ON, Canada), which was maintained at 28°C using a water bath (Model RC 6, Lauda-Birkmann, Delran, NJ, USA). Larvae were left undisturbed in the chamber for 20 min before the experiments were started. Hypoxia was achieved by bubbling a mixture of air and nitrogen into the perfusion chamber using a gas mixer (Model GF-3/MP, Cameron Instruments, Port Aransas, TX, USA). After a pre-exposure period of 3 min, the larvae were exposed for 5 min to either hypoxia (P_{O_2}=3.5 kPa, measured in the reservoir) or SNP (100, 200 or 400 µmol l^{-1} – made fresh several times per day). The larvae were then allowed to recover for 5 min; breathing rate was determined during the last minute of each of these time periods. Depending on the position and orientation of the larvae, breathing frequency was determined by counting either buccal or opercular movements. Control experiments were performed to make sure the response observed to SNP was not being caused by one of the breakdown products of SNP (i.e. cyanide). To test this, 400 µmol l^{-1} SNP was aerated near a source of bright light and bubbled with air overnight to deplete the NO. The solution was used exactly as described above to test the ventilatory response of 4 dpf zebrafish larvae. The response to freshly made SNP was also tested.

Inhibition of NO production

Larvae were exposed to 1 mmol l^{-1} L-NAME from 2 to 4 dpf. At 4 dpf, the ventilatory response to hypoxia (P_{O_2}=3.5 kPa) was measured before exposure, after 5 min of hypoxia exposure, and after 5 min of recovery from hypoxia.

Knockdown of nNOS (NOSI)

To provide additional evidence that NO is involved in the control of breathing, the NO biosynthetic enzyme nNOS was knocked down by injecting fertilized single cell embryos with antisense oligonucleotide morpholinos (designed by Gene Tools, Philomath, OR, USA; http://www.gene-tools.com/) targeting nNOS (5′-ACG CTG GGC TCT GAT TCC TGC ATT G-3′). This is a translational block morpholino, with the target in the translation start site between 11 and 35 bp on NM_131660.1.

The morpholinos were prepared to a final concentration of 4 ng nl^{-1} in 1× Danieau buffer [58 mmol l^{-1} NaCl, 0.7 mmol l^{-1} KCl, 0.4 mmol l^{-1} $MgSO_4$, 0.6 mmol l^{-1} $Ca(NO_3)_2$ and 5.0 mmol l^{-1} Hepes (pH 7.6)] and 0.05% Phenol Red. Injections were performed using a microinjector system (model IM 300, Narishige, Long Island, NY, USA). Control groups were injected with a standard control morpholino (5-CCTCTTACCTCA-GTTACAATTTATA-3; GeneTools) prepared in the same way as the nNOS morpholinos. Morpholinos were injected at a dose of 4 ng/embryo. No significant mortality or deformities were observed up to 4 dpf. Morpholinos were conjugated with fluorescein isothiocyanate (FITC) and 1 day after injection, embryos were screened using a microscope (Model SMZ1500, Nikon Instruments, Melville, NY, USA) for the presence of widely distributed carboxyfluorescein. Embryos that were FITC-positive were raised to 4 dpf in dechloraminated University of Ottawa tapwater (see above) supplemented with 0.05% Methylene Blue. For the larvae experiencing gene knockdown, the protocol was modified slightly so that the hypoxia and recovery periods were extended to 10 min. In these experiments, breathing rate was measured after 2, 4, 6 and 9 min of hypoxia and after 4 and 9 min of recovery from hypoxia. Confirmation of nNOS knock down was performed using immunohistochemistry as described below.

Immunohistochemistry of nNOS in larvae

A sequential staining procedure was used to label structures with antibodies raised in the same host (Inoue and Wittbrodt, 2011). Zebrafish larvae were killed by an overdose of MS-222 and placed in 4% paraformaldehyde prepared in PBS. Whole larvae were then rinsed in PBST (PBS with 0.1% Tween). The larvae were dehydrated gradually using 50% MeOH followed by 100% MeOH and left at −20°C for 1 h, after which larvae were rehydrated using 50% MeOH then PBST. The PBST was replaced with 1 ml Tris HCl (150 mmol l^{-1}, pH 9) and fish were incubated at room temperature for 10 min, then at 65°C for 15 min. The larvae were washed 5× with PBST and placed in a blocking solution (2% BSA, 2% goat serum in PBS with 0.8% Triton-X) for 1 h at room temperature. The blocking solution was

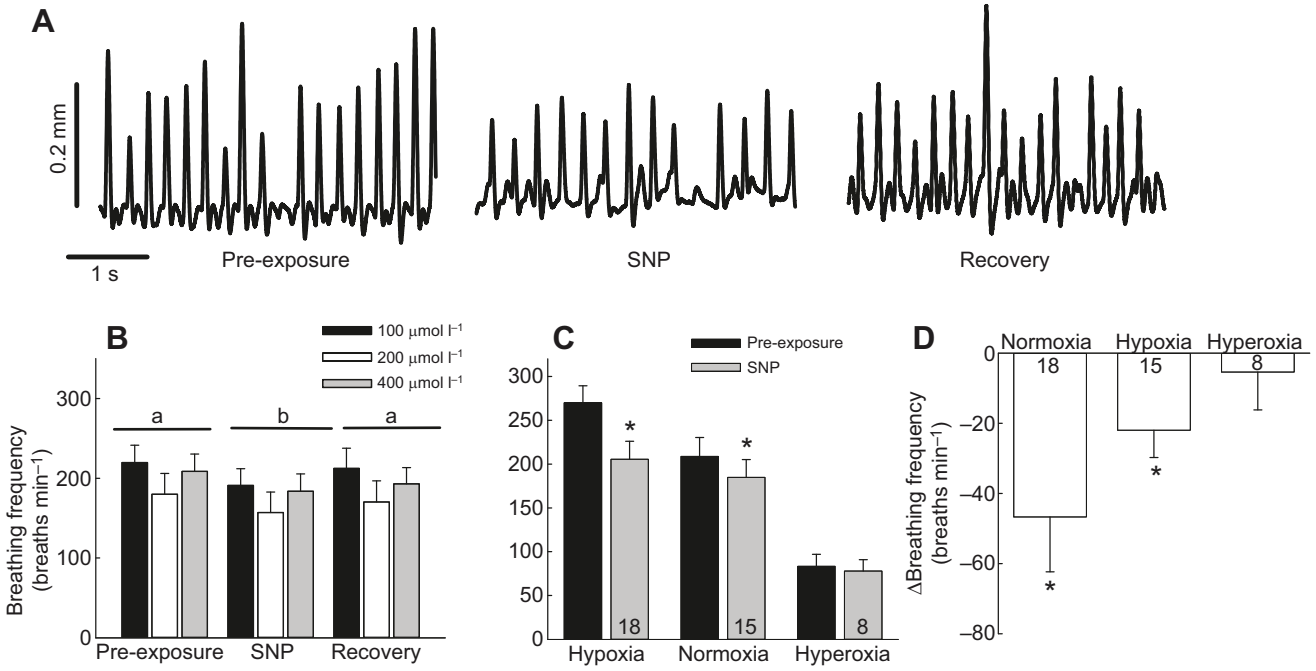

Fig. 1. Effects of exogenous nitric oxide (NO) on gill breathing ventilation in adult zebrafish (*Danio rerio*). (A) Ventilation traces obtained from a representative zebrafish before (pre-exposure), during and after (recovery) exposure to 100 µmol l^{-1} sodium nitroprusside (SNP), a NO donor. (B) Average breathing frequency of adult zebrafish exposed to various concentrations of SNP. (C) Average breathing frequency of adult zebrafish exposed to hypoxia (5.6 kPa), normoxia (~21 kPa) or hyperoxia (100% O_2) followed by the addition of 100 µmol l^{-1} SNP. (D) Changes in breathing frequency caused by the addition of 100 µmol l^{-1} SNP during exposure to normoxia, hypoxia or hyperoxia. *Significant (P<0.05) difference from pre-exposure values. Bars not sharing the same letters are significantly different from one another. Values are means+s.e.m.; N is indicated within the bars.

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removed and the larvae were incubated on a shaker overnight at 4°C with an anti-5-HT primary antibody raised in rabbit to label the NECs (1:250, Sigma Aldrich) and sometimes with primary antibody raised in mouse to label innervation (zn-12, 1:200 dilution; Developmental Studies Hybridoma Bank, University of Iowa). The anti-5-HT antibody was washed 3× with PBST and the larvae were then incubated in the secondary antibody (anti-rabbit Alexa 488, 1:500; Sigma-Aldrich) for 1 h. Five washes with PBST were performed to remove any remaining antibodies. The larvae were then incubated with a rabbit anti-nNOS primary antibody (1:250; Abcam, product no. PA3-032A) diluted in PBS (0.8% Triton-X) overnight at 4°C. The larvae were washed 3× with PBST and a secondary antibody fluorescing red (anti-rabbit Alexa 564, 1:500; Sigma-Aldrich) was applied for 1 h. All antibodies were diluted in PBST. Final washes (5× with PBST) were performed before mounting the larvae in PBST on concave slides for imaging. The larvae were observed and images were captured using a confocal microscope (A1 MP, Nikon, NY, USA) equipped with solid state lasers emitting at 405, 473 and 559 nm. *Z*-stacks of 20–50 optical sections taken 1.0 µm apart were captured using the 25× objective of this microscope.

Statistics

Data are routinely presented as means±1 s.e.m.; all statistical analyses were performed using SigmaPlot (v 10.0, Systat Software). A significance level of 0.05 was used throughout. Paired *t*-tests were used to determine the effect of exogenous NO (SNP) on ventilation in adult fish exposed to normoxia, hypoxia or hyperoxia. To evaluate the effect of nNOS inhibition in adult zebrafish (using L-NAME), a Kruskal–Wallis one-way ANOVA on ranks was used to test for differences between treatments (sham or L-NAME) because data were not normally distributed. This analysis was followed by Dunn's *post hoc* multiple comparison test. A paired *t*-test was used to test for differences between exposure to hyperoxia and addition of SNP. To assess the effect of exogenous NO (SNP) on larval ventilatory frequency, a two-way RM ANOVA was used to test for differences within and among different concentrations of SNP, followed by a Holm–Sidak *post hoc* comparison. To test for the effect of inhibiting NO biosynthesis in larvae (either by knockdown or by inhibition with L-NAME), a two-way RM ANOVA was used, followed by a Holm–Sidak *post hoc* comparison.

RESULTS

Series I: adult zebrafish

Ventilatory responses of adult zebrafish to SNP

Exposure to exogenous NO via a NO donor, SNP, inhibited ventilation (Fig. 1A,B); however, the response was not concentration dependent (Fig. 1B). Moreover, the extent of the inhibition depended on the level of ventilatory drive. Zebrafish exposed to hypoxia had an average ventilation frequency of about 270±19 breaths min^{-1} and addition of 400 µmol l^{-1} SNP decreased breathing frequency to 223±18 breaths min^{-1} (Fig. 1C,D; $P<0.01$, $t=3.0$). Zebrafish exposed to hyperoxia had an average ventilation frequency of 83±13 breaths min^{-1} and addition of 400 µmol l^{-1} SNP did not significantly decrease ventilation rate ($P=0.6$, $t=0.5$).

Effect of inhibiting endogenous NO production on ventilation

Zebrafish injected with saline (sham) exhibited a normal hyperoxic ventilatory response, decreasing ventilation from 204±15 to 58±8 breaths min^{-1} (Fig. 2A). Zebrafish injected with L-NAME decreased ventilation frequency to a lesser extent, from 220±16 to 92±4 breaths min^{-1} during hyperoxia (Fig. 2A; $P<0.001$, $H=45.8$). In a separate group of fish injected with L-NAME, ventilation decreased significantly from 243±23 to 122±10 breaths min^{-1} during exposure to hyperoxia, and addition of SNP significantly decreased ventilation to 81±17 breaths min^{-1} ($P<0.05$, $t=3.3$; Fig. 2B). Pre-treatment with L-NAME did not affect the hyperventilatory response to acute hypoxia; breathing frequency increased from 180±14 to 313±26 breaths min^{-1} and from 175±17 to 301±14 breaths min^{-1} in sham- and L-NAME-treated fish, respectively (data not shown).

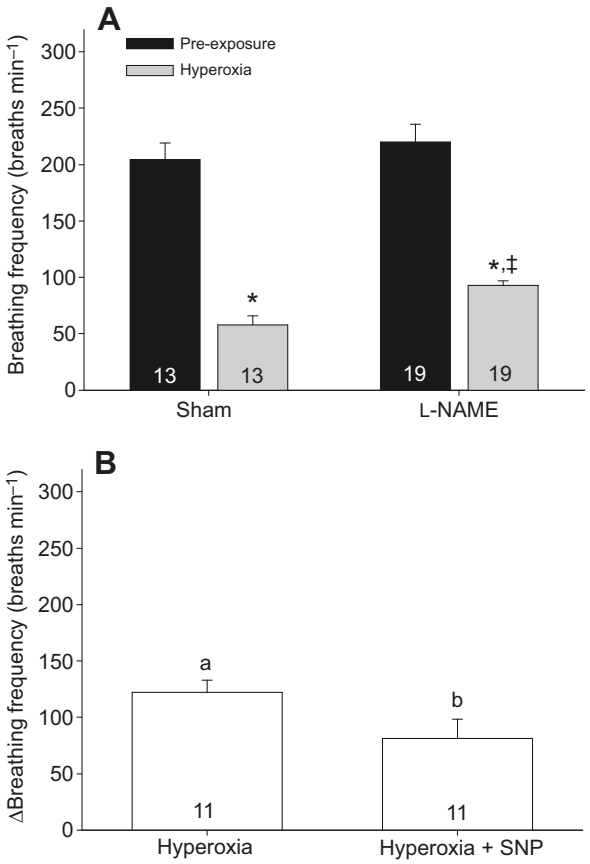

Fig. 2. Effects of inhibiting neuronal nitric oxide synthase (nNOS) on the hyperoxic ventilatory responses of adult zebrafish. (A) Average ventilatory response of adult zebrafish exposed to hyperoxia after injection with 100 µg of L-NAME or saline (sham). (B) Fish injected with L-NAME exhibited a hypoventilatory response during exposure to hyperoxia; following addition of SNP (a NO donor) to the water, ventilation significantly decreased ($P<0.05$). *Significant ($P<0.05$) difference from pre-exposure values; ‡significant difference from control. Bars not sharing the same letters are significantly different from one another. Values are means+s.e.m.; *N* is indicated within the bars.

Immunohistochemistry of NECs

NECs found on the gill lamellae and filaments displayed co-labelling of serotonin (5-HT) and nNOS, showing that the NECs of adult zebrafish contain nNOS (Fig. 3). Omission of the primary antibodies did not produce any specific immunolabelling of gill structures (data not shown).

Series II: larval zebrafish

Ventilatory responses

Zebrafish larvae had a resting ventilatory frequency of 10–40 breaths min^{-1}, which increased to 52, 56 and 135 breaths min^{-1} with the addition of 100, 200 and 400 µmol l^{-1} SNP, respectively (Fig. 4A; $P<0.001$, $F_{2,56}=24.6$). To ensure that this response was specifically caused by the NO released by SNP and not another product of SNP degradation, the response of larvae to SNP aerated and exposed to light overnight was also measured. These larvae were raised in Ottawa tapwater and had a lower resting breathing rate of 20 breaths min^{-1} (compared with 40 breaths min^{-1} in the previous experiment), which increased to 55 breaths min^{-1} (compared with 135 breaths min^{-1} in the previous experiment) when exposed to 400 µmol l^{-1} SNP. Despite the difference in the magnitude of the response, larvae did not increase ventilation when

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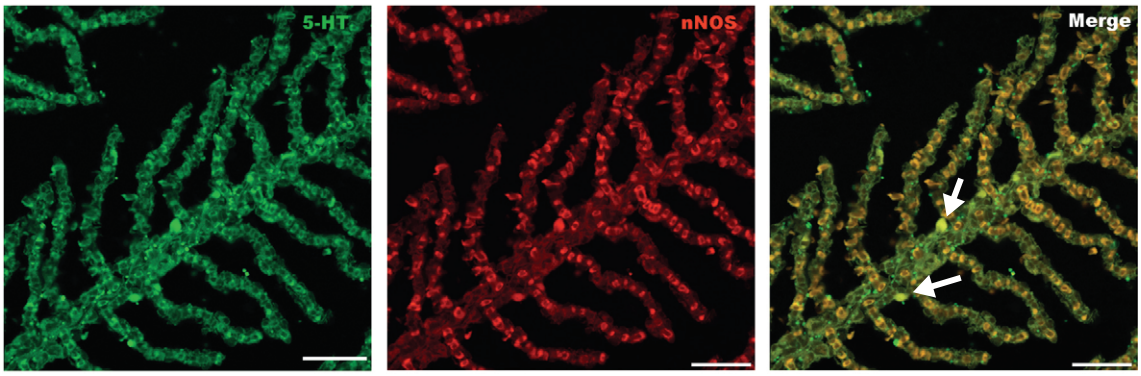

Fig. 3. Immunohistochemical labelling of neuroepithelial cells (NECs) in gill of adult zebrafish with nNOS and serotonin (5-HT). NECs contain nNOS, as indicated by the arrows. The merged image is an overlay of the two channels. Scale bars, 20 µm.

exposed to solutions containing 400 µmol l^{-1} SNP that had been prepared from stock solutions aerated and exposed to light (to eliminate NO; P=0.75, $F_{2,48}$=0.3), while those exposed to 400 µmol l^{-1} SNP without prior aeration and light exposure responded normally (Fig. 4B; P<0.005, $F_{2,27}$=8.6).

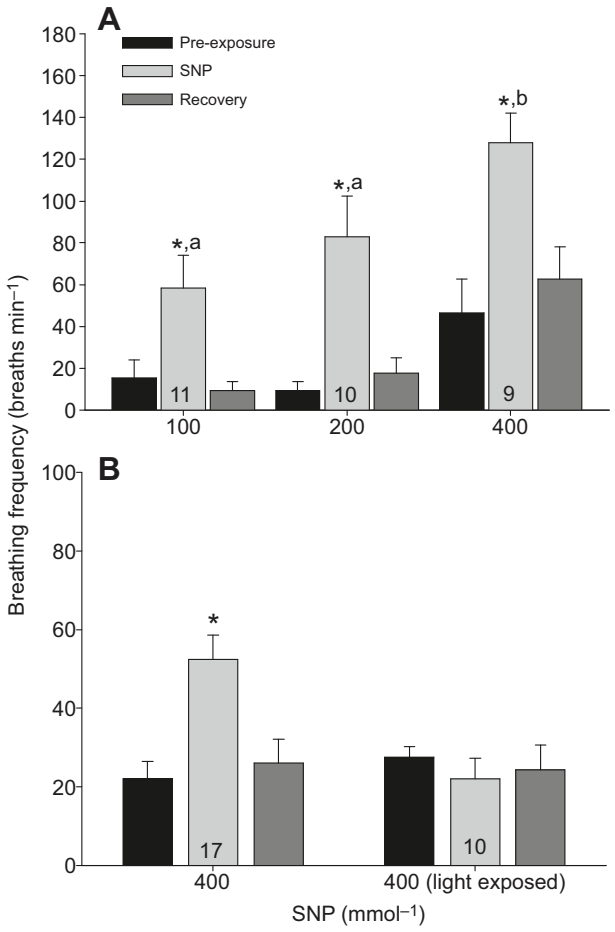

Fig. 4. Exogenous NO increases ventilation in zebrafish larvae (4 days post-fertilization, dpf) in a dose-dependent manner. (A) Breathing frequency increased in response to increasing concentrations of SNP, a NO donor. (B) Control larvae increased breathing frequency in response to 400 µmol l^{-1} SNP (although the response was slightly reduced – see Results). Larvae exposed to SNP that had been exposed to light and bubbled with air overnight to eliminate NO from the solution showed no hyperventilatory response. *Significant (P<0.05) difference from pre-exposure value. Bars not sharing the same letters are significantly different from one another. Values are means+s.e.m.; N is indicated within the bars.

Effect of inhibiting endogenous NO production on ventilation

Zebrafish larvae responded to hypoxia by increasing ventilation rate from 15±5 to 75±11 breaths min^{-1} (Fig. 5A; P<0.001, $F_{6,60}$=16.5). The inhibition of NO synthesis by knockdown of nNOS resulted in a 23–45% inhibition of the hypoxic ventilatory response compared with sham-injected larvae (Fig. 5A; P<0.05, $F_{1,60}$=7.1). Larvae subjected to morpholino injection showed strong labelling of NECs (5-HT) but negligible labelling with nNOS (Fig. S1), confirming effective knock down of nNOS. Similar to larvae experiencing nNOS knock down, inhibition of NO synthesis with L-NAME inhibited the hypoxic ventilatory response by 34–44% compared with controls (Fig. 5B; P<0.05, $F_{1,51}$=5.1).

Immunohistochemistry of NECs in larvae

NECs found in the eye and tail of zebrafish larvae exhibited co-localization of serotonin (5-HT) and nNOS (Fig. 6A); similar results were obtained for NECs on the yolk sac epithelium (data not shown). The labelling of nNOS typically was confined to discrete regions of NECs near the plasma membrane in the vicinity of neural innervation (Fig. 6B, arrowheads) as demonstrated by triple labelling with the neuronal marker zn-12, 5-HT and nNOS (Fig. 6B).

DISCUSSION

Previous studies on fish have focused on the presence of the NO metabolites nitrite and nitrate in the plasma and tissues of hypoxic goldfish (Hansen and Jensen, 2010) and anoxic carp (Sandvik et al., 2012). Exposure of goldfish to 2 days of hypoxia (around or below their critical oxygen tension, P_{crit}) decreased nitrite and nitrate levels in plasma but not in tissues. It was suggested (Hansen and Jensen, 2010) that these results indicated the transfer of nitrite from plasma to tissues, allowing nitrite to be used as a source for maintaining NO levels in the tissues. Furthermore, in anoxic carp NO seems to have a protective role on the heart during re-oxygenation by scavenging reactive oxygen species (ROS; Sandvik et al., 2012). However, the role of NO in the control of breathing in fish was unknown prior to the present study.

To determine whether exogenous NO affected breathing frequency, adult zebrafish were exposed to SNP, a NO donor. Although previous studies used slightly lower concentrations of SNP (10–50 µmol l^{-1}) to produce NO, SNP was applied directly to tissues (Alcayaga et al., 1999) while in the present study SNP (100–

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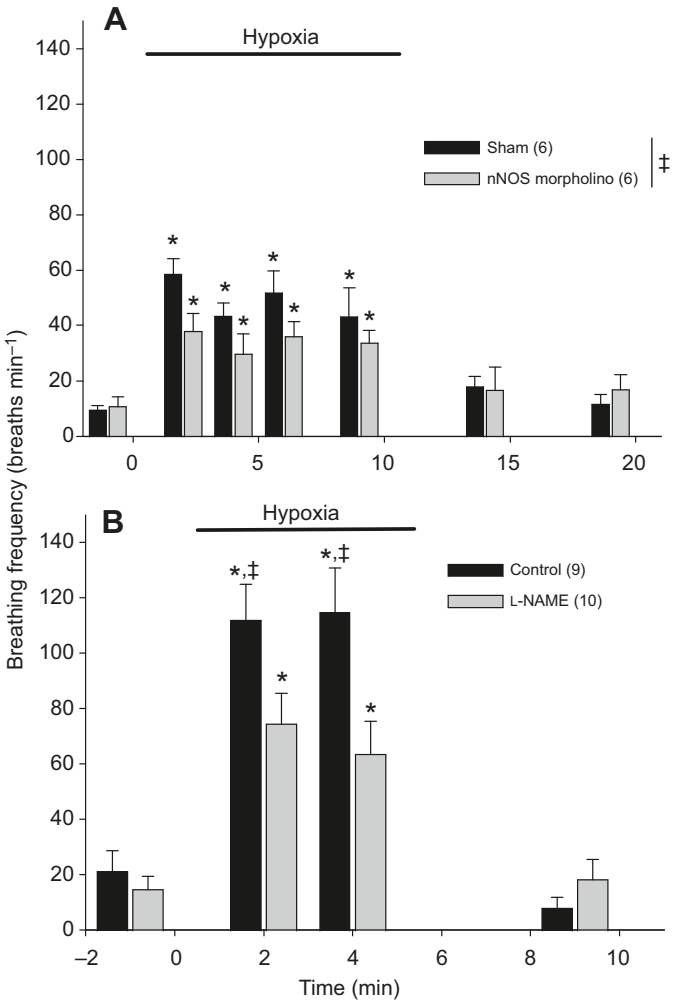

Fig. 5. Effects of inhibiting nNOS on the hypoxic ventilatory responses of zebrafish larvae. The production of NO was inhibited either by nNOS knockdown (A) or by using the nNOS inhibitor L-NAME (B). Both methods resulted in an inhibition of the hypoxic (3.5 kPa) ventilatory response. *Significantly different (P<0.05) from pre-exposure (normoxia) value; ‡significant difference (P<0.05) from controls. Values are means+s.e.m.; N is indicated in parentheses.

400 µmol l^{-1}) was added to the surrounding water. The resulting concentrations of NO released from SNP are difficult to measure and will decrease with time, making it challenging to determine the exact dose of NO that fish are being exposed to over the course of an experiment. Regardless, exposure to SNP (and thus exogenous NO) inhibited ventilation in adult zebrafish (Fig. 1B), which clearly revealed the potential for endogenous NO to modulate breathing. Consistent with our findings, SNP inhibited the response of the carotid sinus nerve to sodium cyanide (Alcayaga et al., 1997) and to ACh (Alcayaga et al., 1999) in cats.

In adult zebrafish, the extent of inhibition of ventilation by SNP was related to the pre-existing ventilatory drive; no effect was observed when ventilatory drive was low (hyperoxia) and greatest inhibition was observed when ventilatory drive was high (hypoxia) (Fig. 1C). It is difficult to distinguish the influence of respiratory drive per se from the effects of oxygenation status (which was altered to modify ventilatory drive) as the cause of the widely different responses to SNP. Because oxygen is required for the production of NO from arginine, the endogenous levels of NO may vary directly with increasing oxygenation. Thus, endogenous levels of NO are expected to be highest under conditions of hyperoxia, intermediate during normoxia and low during hypoxia. Consequently, the scope for exogenous NO to influence breathing will be highest when endogenous levels are low (hypoxia) and lowest when endogenous levels of NO are high (hyperoxia). Moreover, independently from (or in addition to) the effects of O_2 levels on NO production, the extent of tonic inhibition of ventilation by endogenous NO is likely to be highest during hyperoxia and lowest during hypoxia.

To further determine the role of NO in the control of breathing in adult zebrafish, endogenous production of NO was impaired using the NOS inhibitor L-NAME. In fish treated with L-NAME, the hyperoxic ventilatory response was attenuated (Fig. 2), revealing that NO contributes, at least in part, to hyperoxic hypoventilation. Therefore, in adult zebrafish, NO reduces ventilatory drive as it does in mammals. In cats, the inhibition of NO production with L-NAME increased the sensitivity of the carotid body to ACh, electrical stimulation and hypoxia (Alcayaga et al., 1999; Wang et al., 1995b). Additionally, in mice, exogenous NO inhibited the response of the carotid body to hypoxia just like in our study; inhibition of NO production with L-NAME reversed this response in mice (Wang et al., 1995a). Therefore, in adult zebrafish, NO reduces ventilatory drive just as it does in mammals.

Immunohistochemistry was used to determine whether the putative O_2 chemoreceptors of zebrafish, the NECs, contain nNOS. NECs were identified using an antibody against 5-HT as in previous studies (Jonz et al., 2004). NECs found either in the filament or on the lamellae contained nNOS (Fig. 3). In fish, nNOS is localized in the NECs, nerve fibres surrounding the efferent lamellar arterioles of the gill and the vascular walls of the air bladder of some catfish species (Zaccone et al., 2003). In mammals, nNOS is found in the carotid body nerve, petrosal ganglion and the glossopharyngeal nerve, which are all efferent nerves, but not in the glomus cells (Campanucci and Nurse, 2007). In mammals, ACh and ATP are the primary neurotransmitters released from glomus cells during acute hypoxia (Zapata, 2007). ACh and ATP act on the afferent nerves to stimulate breathing but also act on the nearby glossopharyngeal nerve, which in turn release NO and inhibit neurotransmitter release from glomus cells (Campanucci and Nurse, 2007). Thus, NO provides negative feedback control on mammalian carotid body chemoreceptors to control breathing. In adult zebrafish, nNOS is present in NECs and thus NO might act in an autocrine fashion to inhibit neurotransmitter release and modulate ventilatory responses to altered levels of ambient or internal O_2. However, although inhibition of NOS activity attenuated the hyperoxic hypoventilatory response, it did not modify the hyperventilatory response to hypoxia, suggesting that, unlike in mammals, NO does not contribute to negative feedback during hypoxia but rather may function exclusively under conditions of hyperoxia.

As opposed to its effects in adults, SNP stimulated ventilation in a dose-dependent manner in zebrafish larvae (Fig. 4). SNP breaks down to produce NO, with cyanide as a by-product (Bates et al., 1991). Therefore, we ran control experiments using aerated and light-exposed SNP to ensure that the increases in ventilation in larvae were caused by NO and not the cyanide released from the breakdown of SNP or any other by-product of this reaction. In these control experiments, larvae failed to exhibit hyperventilation when exposed to SNP solutions from which the NO was removed by photolysis. Therefore, the increase in ventilation was probably caused specifically by the NO and not another chemical by-product. Furthermore, knocking down nNOS or inhibiting nNOS production with L-NAME (Fig. 5) blunted the hypoxic ventilatory response of

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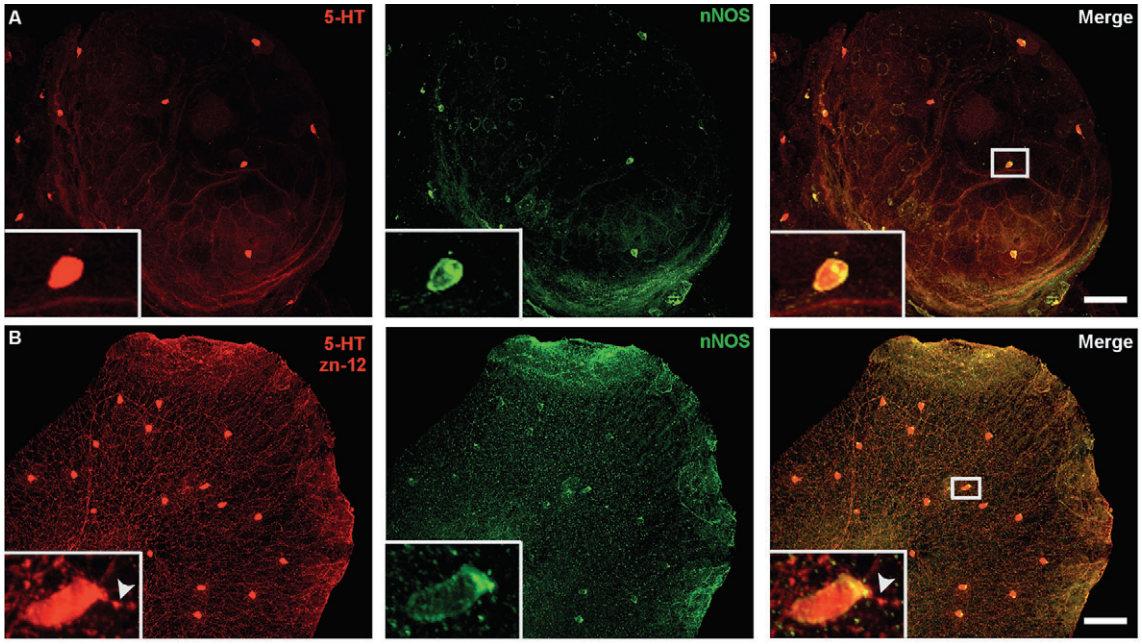

Fig. 6 . Immunohistochemical labelling of NECs of 4 dpf zebrafish larvae with 5-HT and nNOS. (A) Double labelling of NECs with 5-HT and nNOS in the eye. (B) Triple labelling of NECs with serotonin (5-HT), a neuronal marker (zn-12) and nNOS in the tail of a zebrafish larvae. Insets show a magnified view of the cell in the box. Arrowheads show the presence of nNOS in the innervation of NECs. Scale bars, 50 µm.

larvae. These responses were different from those in adult zebrafish (see Results) in which L-NAME treatment did not alter the hypoxic ventilatory response. The blunted hypoxic ventilatory response associated with nNOS knockdown in zebrafish larvae also was in stark contrast to the enhanced hypoxic ventilatory response and increased carotid sinus nerve activity in nNOS knockout mice (Kline et al., 1998). It is possible that the role of NO in the control of breathing changes with developmental age in zebrafish, being excitatory in peripheral chemoreceptors during early development, when ventilatory drive is low, and inhibitory in adults, when ventilatory drive is higher.

An alternative explanation for the differences in the response to NO in adults and larvae is a shift in the distribution of NECs. At the gill, the NECs appear at 5 dpf and become innervated only at 7 dpf (Jonz and Nurse, 2005). Before the maturation of the gill NECs, zebrafish larvae are thought to sense changes in O_2 via NECs located on the skin (Coccimiglio and Jonz, 2012). It is unclear whether the two populations of NECs (skin and gill) function and respond to NO in a similar manner although they share many characteristics (Jonz and Nurse, 2006). Therefore, it might be that in skin NECs, NO has an excitatory role in oxygen chemoreception, while in gill NECs, NO might be inhibitory.

Additionally, the differences in the response to NO in adults and larvae may reflect the effect of NO at multiple sites along the respiratory reflex pathway. Previous studies using *in situ* hybridization of zebrafish larvae revealed that by 55 h post-fertilization all cell populations that express nNOS in the adult brain are present in zebrafish larvae (Holmqvist et al., 2000, 2004), indicating that NO is present during early development in the brain of zebrafish larvae. In both adults and larvae, information from peripheral chemoreceptors is sent to the CNS via afferent nerves in the nucleus tractus solitarius (NTS), where chemoreceptor information is integrated. The role of NO in the NTS is unclear, with one study finding that it promotes respiratory frequency in awake rats (Granjeiro and Machado, 2009), while a more recent study found that NO did not play a role in the acute hypoxic response of awake rats (Pamenter et al., 2015). Information from the NTS is then transmitted to the respiratory rhythm generation sites of the CNS; in mammals, NO is excitatory and promotes rhythm generation (Pierrefiche et al., 2002). Therefore, NO is acting at multiple sites and the balance between the role of peripheral and central sites might be changing through development.

Moreover, it is possible that the role of NO centrally changes from inhibitory to excitatory in the rhythm-generating sites during development, as shown in amphibians (Hedrick et al., 2005). In pre-metamorphic frogs, NO inhibits CNS fictive gill and lung breathing but becomes excitatory to lung breathing post-metamorphosis (Hedrick et al., 2005). Therefore, it is possible that NO might be excitatory centrally in larvae, promoting the hypoxic ventilatory response during early development, but might become inhibitory, therefore reducing ventilation in adult zebrafish. Here, we show that either the specific effects of NO at any receptor site, or the balance of effects of NO at multiple sites, must change during development; however, further studies are necessary to determine the exact role of NO in peripheral chemoreception versus central integration and rhythm generation in the control of breathing.

Lastly, NO has been described as a 'meta-modulator' of neural activity (McLean and Sillar, 2004), and in frogs the role of NO changes through development. NO inhibits swimming via indirect glycinergic and direct GABAergic inhibition (McLean and Sillar, 2002, 2004); however, in frogs, endogenous production of NO might not be fully developed at earlier stages of development (McLean and Sillar, 2004). Therefore, it is possible that modulation of NO in zebrafish larvae is also not fully developed at 4 dpf owing to a lack of inhibitory targets.

Conclusions

We provide the first evidence that NO is involved in the control of breathing in fish and that its role changes during development. Specifically, we demonstrated that NO has an inhibitory effect on the hypoxic ventilatory response of adult zebrafish, but an excitatory effect on the hypoxic ventilatory response of larvae. Despite the

different roles of NO in regulating ventilation as a function of development, the NECs of both adult and larvae contained nNOS, indicating they are both capable of producing NO endogenously. We propose that the different effects of NO in adults and larvae either is due to differences in ventilatory drive or reflects changes in the role of NO in peripheral and central control of breathing during zebrafish development, or both.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
S.F.P., C.S.P., Y.K., S.J.A., J.P. and W.K.M. designed and conceptualized the experiments; S.F.P., C.S.P., V.T., S.J.A., J.P., R.W.M.K., G.Z., E.R.L. and Y.K. performed the research; C.S.P., S.J.A., J.P., R.W.M.K. and S.F.P. analysed the data; S.F.P. and C.S.P. wrote the paper; and all authors contributed feedback to various drafts of the manuscript. We dedicate this paper to the memory of Dr Yusuke Kumai (1985–2015).

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