The effects of lactate ions on the cardiorespiratory system in rainbow trout (*Oncorhynchus mykiss*)

Mikkel T. Thomsen[1*], Sjannie Lefevre[2], Göran E. Nilsson[2], Tobias Wang[1,3], Mark Bayley[1]

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Affiliation: [1]Department of Bioscience, Zoophysiology, Aarhus University, Aarhus, Denmark

[2]Department of Biosciences, Faculty of Mathematics and Natural Sciences, University of Oslo, Oslo, Norway

[3]Aarhus Institute of Advanced Studies, Aarhus University, Aarhus, Denmark

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***Author for correspondence:** Mikkel Thy Thomsen, Zoophysiology, C. F. Møllers Alle 3, 8000 Aarhus C, Denmark, e-mail: mikkel.thomsen@bios.au.dk

Abstract

Lactate ions are involved in several physiological processes including a direct stimulation of the carotid body causing increased ventilation in mammals. A similar mechanism eliciting ventilatory stimulation in other vertebrate classes has been demonstrated, but remains to be thoroughly investigated. Here, we investigated the effects of lactate ions on the cardiorespiratory system in swimming rainbow trout by manipulating the blood lactate concentration. Lactate elicited a vigorous, dose-dependent elevation of ventilation and bradycardia at physiologically relevant concentrations at constant pH. Following this initial confirmation, we examined the chiral specificity of the response and found that only L-lactate induced these effects. By removal of the afferent inputs from the 1st gill arch, the response was greatly attenuated and comparing responses to injections up- and downstream of the gills collectively demonstrated that the lactate response was initiated by branchial cells. Injection of specific receptor antagonists revealed that a blockade of serotonergic receptors, which are involved in the hypoxic ventilatory response, significantly reduced the lactate response. Finally, we identified two putative lactate receptors based on sequence homology, and found that both were expressed at substantially higher levels in the gills and that their expressions were very strongly correlated. We propose that lactate ions modulate ventilation by stimulating branchial oxygen sensing cells, thus eliciting a cardiorespiratory response through receptors likely to have originated early in vertebrate evolution.

Keywords: Hypoxic ventilatory response, chemosensing, gene expression, teleosts, *HCAR1*, *OR51E2*

Introduction

Increased ventilation is a ubiquitous vertebrate response to hypoxia (the hypoxic ventilatory response - HVR) which, in concert with a range of adjustments at all levels from altered gene expression to behavioral modifications, serves to protect tissue oxygen delivery (7, 58, 62). Despite extensive effort, the oxygen sensing mechanisms underlying the acute HVR remain as elusive as they are controversial. However, it seems likely that numerous mechanisms are involved (13, 41, 63, 65). One proposed mechanism involves receptor stimulation by lactate ions, the end-product of anaerobic metabolism in vertebrates. Lactate is continuously produced through the reversible enzymatic conversion from pyruvate by lactate dehydrogenase (L-LDH; D-LDH is not present in vertebrate cells). Lactate usually remains at quite stable, low concentrations because pyruvate drains into the TCA-cycle. With the onset of hypoxia, however, the oxidative phosphorylation pathway becomes limited, the TCA-cycle slows down, pyruvate concentrations increase, and the pyruvate-lactate equilibrium moves toward increased lactate concentrations. This central position of lactate in metabolism provides for a powerful signaling molecule in the regulation of cellular and physiological processes linked to inadequate oxygen availability and it is now known to be involved in a wide variety of processes (2, 26, 76).

The possible role of the lactate ion as a ventilatory stimulant was first observed as an increased ventilation following lactate infusions at constant pH in rats, thus separating the effect of lactate from the accompanying acidosis, long known to affect ventilation (28, 40). More recently, Chang et al. (2015) demonstrated that lactate ions directly stimulate the carotid body and evoke a HVR in mice (14). These authors further showed that an ectopic olfactory receptor (*Olfr78* also known as *OR51E2*) is expressed in the carotid body and responds to physiologically relevant lactate ion concentrations, and that knockout of this gene strongly reduces the ventilatory responses to both lactate ions and hypoxia. Following this discovery, a ventilatory response to lactate ions was identified in the air-breathing teleost

58 *Pangasianodon hypophthalmus* (80). These authors further demonstrated that denervation of the 1st gill
59 arch greatly attenuated the ventilatory responses to both lactate ions and NaCN, used as a general
60 stimulant of the HVR (80).

61 In fishes, the 1st gill arch is embryonically homologous to the mammalian carotid artery. This
62 has provided the basis for the argument that the neuroepithelial cells (NEC) of the gills, the putative oxygen
63 sensing cells, are likely homologous to the oxygen sensing cells of the carotid body, although recent
64 evidence questions this (31, 49). Nevertheless, it appears that NECs respond to a variety of stimuli,
65 including hypoxia (91). NECs, and the gills in general, are heavily innervated and contain a variety of
66 receptors, which have complicated the task of determining the pathway leading to hypoxic responses, but
67 evidence suggests an involvement of at least adrenergic, cholinergic, and serotonergic receptors (10, 11,
68 79, 91). Hence, it is likely that any lactate mediated ventilatory response as part of the HVR, also relies on
69 one or more of these receptor families.

70 There are two described lactate receptors in mammals, both belonging to the G-protein
71 coupled receptor family (GPCR): the hydroxycarboxylic acid receptor 1 (*HCAR1*, formerly known as *GPR81*),
72 which has been linked to a number of metabolic regulatory processes but not to ventilation (76) and
73 *OR51E2* (*Olfr78* in mice), whose function is less well understood but has been linked to blood pressure
74 regulation (57) and to ventilatory regulation through its expression in the carotid body (14). The function of
75 a teleost *OR51E2*-ortholog has not yet been established but the zebrafish *HCAR1*-ortholog responds to
76 lactate as in mammals (39). Since receptors can stimulate different functions, even across closely related
77 species, it is possible that *HCAR1* is involved in the teleost lactate ventilatory response.

78 The present study aimed at elucidating the possible role of the lactate ion in eliciting
79 cardiorespiratory responses in rainbow trout, a species where most of the genome is annotated and where
80 much is already known concerning its ventilatory and cardiovascular responses to hypoxia (54). These
81 experiments address several hypotheses that collectively serve to further the overall understanding of the

role of the lactate ion in ventilatory regulation in trout. The specific hypotheses are: 1) Lactate ions modulate ventilation in rainbow trout and are sensed in the branchial tissue, as suggested in *P. hypophthalmus*. 2) The lactate ventilatory response is receptor-mediated, demonstrating specificity towards the L-isomer of lactate. 3) The lactate ventilatory response is part of the HVR, thus pharmacological blockades reducing the lactate response should also reduce the HVR. 4) The mechanisms underlying the lactate ventilatory response are conserved between vertebrates, thus receptors homologous to the mammalian lactate receptors should be present in gill tissue.

Materials and methods

Experimental animals

Rainbow trout (*Oncorhynchus mykiss*, Walbaum, 1792) were purchased from a commercial fish farm (Funderholme Dambrug A/S, Silkeborg, Denmark) and kept at Aarhus University in a recirculating system in 1000 l tanks with normoxic water at 17-18°C for several weeks before experiments. Temperature and PO_2 of the water were continuously monitored and controlled. The fish were fed trout pellets to satiation daily and kept in a 12 h:12 h light cycle. All animals appeared healthy during the experiments.

Animal preparation

For experiments 1 and 2, the fish were fasted for at least 24 h prior to surgery (experimental protocols are described below). All fish were anesthetized in a 0.1 g l^{-1} benzocaine solution (pre-dissolved in a small volume of EtOH) until breathing movements ceased and transferred to a surgical setup in a supine position where the gills were irrigated with a 0.04 g l^{-1} benzocaine solution bubbled with pure oxygen. A PE50 catheter filled with heparinized saline (100 IU ml^{-1}) was inserted into the dorsal aorta (DA) through the roof of the mouth, extended through a hole in the cartilaginous tissue on the dorso-lateral side next to the rostrum, and secured with a cuff and a suture in the roof of the mouth (75). Another catheter (PE90) was

placed in the opercular cavity through a hole in the operculum and was secured by flattening the tip. Both catheters were brought to the dorsal side of the animal and secured tightly along the dorsal side with 3-4 sutures, the last placed in the proximal part of the dorsal fin. Additionally, 6 fish (experiment 1 - gill sectioning sub-experiment) had the 1st gill arch bilaterally ablated (these fish will be referred to as G1), by ligating tightly around the gill arch at both the ventral and dorsal ends, then removing the gill arch and cauterizing any bleeding occurring in the process (74). For experiment 2, an additional PE50 catheter with heparinized saline was inserted ventrally in the afferent branchial artery in the 3rd gill arch and advanced 2 cm downwards to reach the ventral aorta (VA). This catheter was secured with a ligature around the 3rd gill arch. After surgery, the fish were placed in a swim tunnel at low speed (<1 body length s^{-1}) in well-aerated water at 18°C. This approach was chosen to reduce the likelihood of the fish turning and thus tangling or kinking the catheters during the measurements. The setup was covered to avoid visual disturbance and the fish were left to recover for 24-48 h before starting the measurements. All procedures were conducted according to the guidelines of the Danish Law on Animal Experiments and were approved by the Danish Ministry of Food, Agriculture, and Fisheries (2016-15-0201-00865).

Blood analysis

For blood samples where arterial partial pressure of oxygen (P_aO_2), hematocrit (Hct), total plasma CO_2 concentration ($[CO_2]_{total}$), and hemoglobin concentration ($[Hb_4]$) were measured, 600 µl was drawn. For other blood samples, 200-300 µl was drawn. P_aO_2 was measured on a Radiometer oxygen electrode (Radiometer, Denmark), calibrated with pure N_2 and water-equilibrated atmospheric air between all measurements and kept at a constant temperature. Hematocrit was measured in duplicate as the fraction of red blood cells after separation at 12000 rpm for 3 min in a micro centrifuge (Biofuge 13, Heraeus, Brentwood, England). pH_a was measured using a GEM Premier 3500 automated blood gas analyzer (Instrumentation Laboratory, Bedford, MA, USA) using previously validated temperature compensation algorithms (42). Blood hemoglobin concentration was measured spectrophotometrically after conversion

to cyano-methemoglobin using Drabkin's reagent (18). $[CO_2]_{total}$ was determined as described by (12) and plasma $[HCO_3^-]$ was calculated by subtracting the dissolved CO_2 from the total CO_2:

$$[HCO_3^-]_{pl} = [CO_2]_{total} - \alpha CO_2 \cdot PCO_2 \text{ , (1)}$$

where αCO_2 is the temperature compensated solubility of CO_2 in trout plasma from (6), and PCO_2 was calculated by rearranging the Henderson-Hasselbalch equation:

$$PCO_2 = \frac{[CO_2]_{total}}{\alpha CO_2 \cdot (1+10^{pH-pK'})} \text{ , (2)}$$

where the pH compensated pK' was calculated from (6). [Lactate] was measured using a portable Accutrend-Plus device (Roche Diagnostics Limited, Rotkreuz, Switzerland) previously validated for fish blood (4). Values below the detection limit (0.8 mmol l^{-1}) were treated as 0.8 mmol l^{-1}, and values above the detection limit (22 mmol l^{-1}) were treated as 22 mmol l^{-1} in the data treatment, although values above detection limit very rarely occurred.

Experiment 1: Intra-arterial injections

Lactate dose-response and control injections

This experiment aimed at identifying a ventilatory response to specific lactate doses and controlling any potential confounding effects of lactate injections (Hypothesis 1). 6 fish were used in this experiment (mass = 459 ± 73 g, mean ± s.d.) and were prepared according to the surgical procedure described above. Following recovery, the opercular catheter was connected to a pressure transducer (PX600, Irvine, CA, USA) with minimal disturbance to the fish. Data from the pressure transducer was recorded at 200 Hz using a BIOPAC MP100 system (Biopac Systems Inc., CA, USA). Ventilation was recorded for 1-2 h for a stable baseline and a blood sample withdrawn to measure P_aO_2, Hct, $[CO_2]_{total}$, $[Hb_4]$, pH_a, and plasma [lactate] (see measurement details above). Then, an injection series was given through the DA catheter, which was

flushed between injections with heparinized (100 IU ml^{-1}) 0.9% saline. All injections were administered slowly (about 2 ml min^{-1}) and separated by at least 30 min. Ventilation was required to have returned to near pre-injection values (*i.e.* less than 10% deviation) before proceeding to the next injection. The injections were: 0.5 ml 0.9% saline (Merck, Kenilworth, NJ, USA), to exclude any effects of volemic changes or injections *per se*, 0.5 ml 1 mol l^{-1} NaCl, to exclude any effects of increased osmolality or $[Na^+]$, 0.5 ml 0.1 mol l^{-1} Na-pyruvate (Sigma-Aldrich Denmark A/S, Copenhagen, Denmark) to identify effects of pyruvate, and 0.25 ml 100 µg ml^{-1} NaCN (Merck) as a positive control to stimulate ventilation. The order of injections was randomized. Following these control injections, three lactate injections were given: 1 ml kg^{-1} 0.3 mol l^{-1} L-lactate (Sigma-Aldrich), <0.5 ml 0.6 mol l^{-1} L-lactate, <0.5 ml 1 mol l^{-1} L-lactate; all diluted in isotonic saline. The first L-lactate injection was corrected for fish mass and subsequent injections corrected according to the lactate level measured following the first injection, such that the [lactate] after each of the three doses were comparable between subjects. The doses were aimed at reaching approximately 5, 10, and 15 mmol l^{-1} plasma [lactate], respectively, which spans the range usually experienced following severe hypoxia or exercise (38, 44). Two minutes post-injection, small blood samples were taken and pH_a and [lactate] measured. The blood sample following the last injection was used to measure P_aO_2, $[Hb_4]$, $[CO_2]_{total}$, and Hct. The lactate solution was prepared by titrating Na L-lactate with lactic acid to a ratio resulting in a pH of 7.8 and a lactate ion concentration of 1 mol l^{-1}, which was then diluted with saline to the required concentrations for injection.

Gill sectioning

This experiment was designed to assess the role of the putative receptors in the 1st pair of gill arches in the lactate response (N = 6, mass = 564 ± 100 g, mean ± s.d.) (Hypothesis 1). The 1st pair of gill arches were ablated as described above (74). The opercular catheter was connected to a pressure transducer and the fish left for 1-2 h for a stable baseline, after which a dose of NaCN and a series of lactate-doses were

injected, identical to the doses used above. Blood samples were withdrawn prior to injections and two minutes post-injection, where pH and plasma [lactate] were measured. In the first blood sample, P_aO_2, $[Hb_4]$, $[CO_2]_{total}$, and Hct were also measured as well as in the blood sampled following the highest lactate dose.

Time course of [lactate] following injections

This experiment investigated the course of the plasma [lactate] following injection and the concurrent ventilatory changes (N = 6, mass = 637 ± 93 g, mean ± s.d.). The setup was similar to the sub-experiments above, but the experiments were split to avoid anemia due to serial blood sampling, which is required in all experiments. After recovery, the opercular catheter was connected to a pressure transducer. Gill ventilation was measured for 1-2 h for a baseline, followed by a 1 ml kg^{-1} 1 mol l^{-1} Na L-lactate injection through the DA catheter. Blood samples were drawn at times -1 (before injection), 2.5, 5, 7.5, 10, 15, 30, 45 and 60 min after injection and [lactate] was measured as described above.

D-lactate injections

This experiment tested the specificity of the response by comparing the effects of the two enantiomers of lactate (N = 6, mass = 523 ± 46 g, mean ± s.d.) (Hypothesis 2). The opercular catheter was connected and data sampled as above. Baseline ventilation was measured for 1-2 h, followed by a 1 ml kg^{-1} 1 mol l^{-1} Na L-lactate injection and a 1 ml kg^{-1} 1 mol l^{-1} Na D-lactate through the DA catheter in random order. Lactate injections were temporally separated by at least 30 min to allow a return to baseline. Pre-injection and 2 min post-injection blood samples were drawn and pH and [L-lactate] measured. D-lactate concentration was not determined but was expected to reach the same initial plasma concentrations as the doses were identical, although the time course of clearance from plasma likely differed (83).

Experiment 2: Pharmacological blockade

This experiment investigates potential receptors and neural pathways associated with the lactate ventilatory response and the HVR (Hypothesis 3). Further, to pursue the hypothesis that a lactate receptor is located in the gills, the injections were given in the VA, which also allowed for continuous measurements of heart rate (f_H) and mean arterial blood pressure (MAP) through the DA catheter, and hence an investigation of the cardiovascular response. A total of 16 fish were used in this experiment (704 ± 146 g, mean ± s.d.). The DA catheter and the opercular catheter were connected post-recovery to pressure transducers (PX600, Irvine, CA, USA) and left for 1-2 h for baseline measurements. The fish were then divided into three groups: A control group (N = 4), a serotonin receptor (5-HT_3)-blockade group (N = 6), and a double blockade (DB, muscarinic cholinergic and β-adrenergic) group (N = 6). All groups received the following injection series into the VA in randomized order (pre-blockade): 1 ml kg^{-1} 0.9% saline to identify any effects of injections *per se*, 0.8 ml kg^{-1} 0.1 mol l^{-1} Na L-lactate, and 100 µl kg^{-1} 250 µg ml^{-1} NaCN. NaCN is a known hypoxic mimicking stimulant and is additionally used as a positive control of correct catheter positioning (67). In addition to these injections shared across groups, a group-specific agonist was given into the VA: saline for control; 100 µl kg^{-1} 10^{-3} mol l^{-1} serotonin (Sigma-Aldrich) for the 5-HT_3-blockade-group; and both 100 µl kg^{-1} 10^{-3} mol l^{-1} noradrenaline (NA) and 100 µl kg^{-1} 10^{-3} mol l^{-1} acetylcholine (ACh) separately for the DB group. Following these injections, a specific receptor antagonist was injected: saline for the control group; 1 ml kg^{-1} 1 mg ml^{-1} tropanyl 3,5-Dichlorobenzoate (MDL 72222; 5-HT_3 receptor antagonist; Abcam, Cambridge, UK) for the serotonin-group; and 1.5 mg kg^{-1}; 1 mg ml^{-1} atropine (muscarinic cholinergic receptor antagonist, Sigma-Aldrich) followed by 1.5 mg kg^{-1}; 1 mg ml^{-1} propranolol (β-adrenergic receptor antagonist) together with an additional dose of atropine (1 mg kg^{-1}; 1 mg ml^{-1}) for the DB group. Following each antagonist injection, lactate, NaCN, and agonists were injected (same doses as above) in random order. The control group and the DB group were thus given the injection series three times, whereas the serotonin receptor-blockade group was given the injection series twice. All injections were at least 15 min apart, and a return to pre-injection values (less than 10% deviation) for all measured

variables were required before proceeding with the next injection. Some antagonistic injections moved the baseline (see results), and in those cases, a return to these post-antagonist values were required before continuing. Only on rare occasions did it take longer than 15 min for the variables to return to baseline, and these instances may have been due to spontaneous, random increased activity by the fish. All injections were diluted in saline and pH-regulated to 7.8 prior to injection. Prior to the first injection and prior to all lactate injections, a blood sample was taken (through the DA-catheter) and pH, Hct, and [lactate] were measured as described above, and [lactate] was required to be < 1 mmol l^{-1} before continuing.

Experiment 3: Gene-expression experiment

Tissue sampling

The following measurements were performed to investigate expression of receptors homologous to the mammalian lactate receptors (Hypothesis 4). The experimental animals (N = 8, mass = 131 ± 26 g, mean ± s.d.) were stunned with a blow to the head and euthanized by decapitation. The liver, heart (atrium + ventricle), white muscle, adipose tissue, individual gill arches, and brain (the medulla was separated from the anterior parts) were rapidly harvested under a surgical microscope and immediately submerged in >5x volume of RNAlater (Life Technologies, Carlsbad, CA, USA) and stored at -20 until total RNA extraction.

Total RNA extraction, DNase treatment, and cDNA synthesis

Total RNA was extracted with TRIzol® (Life Technologies) according to the manufacturer's protocol. An external RNA standard was added to all samples (20 pg mw2060 standard per mg of tissue) (20). DNase treatment was carried out with the DNA-free™ DNA removal kit (Life Technologies). RNA quantity was measured with a Nanodrop 2000 (Thermofischer Scientific Inc., Waltham, MA, USA) and RNA quality was

analyzed on a denaturing agarose gel stained with Ethidium Bromide. cDNA synthesis was carried out in duplicate with superscript® III First-Strand Synthesis System for RT (Life Technologies) according to the manufacturer's protocol.

Partial cloning and sequencing of *OR51E2*-like gene

Partial cloning was performed to obtain the gene sequence of an *OR51E2*-like gene based on a BLASTn analysis in the NCBI database of the mouse *OR51E2* (also known as *Olfr78*) gene sequence (NCBI database reference number NM_001168503.1). A number of similar nucleotide sequences within the teleosts were found, including in the close relative *Salmo salar* (NCBI database reference number XM_014215437.1). Primers for partial cloning were designed using the primer3 online utility (68) based on the sequence of the *OR51E2*-like gene found in *S. salar* and other teleosts in the NCBI database. Several primer pairs were designed (Table 1), and they were synthesized by Sigma-Aldrich (Sigma-Aldrich® Norway A/S, Oslo, Norway). PCR (Eppendorf Mastercycler gradient, Eppendorf AG, Hamburg, Germany) was carried out on cDNA extracted from all gill arches using Platinum® Taq DNA polymerase and dNTP mix (Life Technologies) according to the manufacturer's protocol. The following PCR program was used: (1) 95°C for 10 min, (2) 95°C for 30 s, (3) 48°C for 1 min, (4) 68°C for 1 min; steps 2-4 were repeated 39 times, followed by (5) 68°C for 10 min and (6) Hold 4°C. The PCR products were run on a 1% agarose gel with ethidium bromide (Sigma-Aldrich), 10x BlueJuice™ Gel Loading Buffer (Life Technologies) and 1 kb+ DNA ladder (Life Technologies). Fragments of the expected size were ligated into vectors using the pGEM easy-vector System I kit (Promega, Fitchburg, WI, USA) and subsequently transformed into $CaCl_2$-competent *E. coli* cells (produced from stock at the University of Oslo) by heat-shock treatment. The bacteria were then grown on lysogeny broth (LB) plates with ampicillin and a mix of iso-propyl β-D-1-thiogalactopyranoside (IPTG, Thermo Scientific) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, Promega). Colony PCR with M13F2 and M13R primers (Life Technologies) on successfully transformed colonies was carried out with the

following PCR program: (1) 94°C for 10 min, (2) 94°C for 30 s, (3) 55°C for 1 min, (4) 72°C for 1 min, steps 2-4 were repeated 35 times, followed by (5) 72°C for 10 min, and (6) hold 4°C. The PCR products were purified with ExoSAP (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's protocol and sequenced with T7 promoter primer by GATC Biotech (Konstanz, Germany).

Quantitative real-time PCR of *OR51E2*-like and *HCAR1* gene

Based on the sequence obtained from partial cloning of the *OR51E2*-like gene qPCR primers were designed using the Primer3 online utility (68). For the *HCAR1* gene, the primers were designed from the trout sequence XP_021478519.1, which share high similarity with the known lactate receptor *HCAR1-1* (and its paralog *HCAR1-2*) described in zebrafish (NM_001163292.1) (39). The primer pairs used only showed one melting curve with the LightCycler 480 SYBR green I Master (Roche) detection (Table 1). The qPCR products were then cloned and sequenced as described above to verify that the correct sequence was obtained from the qPCR. The qPCR was performed using a LightCycler 480 Real-Time PCR System with the LightCycler 480 SYBR Green Master I according to the manufacturer's protocol. All samples were run in triplicate (with duplicate cDNA synthesis resulting in 6 technical replicates tissue^{-1} fish^{-1}) with the *OR51E2*-like primer pair, the *HCAR1*-like primer pair, and the mw2060 (external standard) primer pair, respectively (Table 1). The following qPCR program was used: (1) pre-incubation (95°C for 10 min), (2) three-step amplification (95°C 10 s, 54°C 10 s, 72°C 13 s), this step was repeated 42 times, followed by (3) melting (95°C 5 s, 65°C 10 s, 97°C 1 s) and (4) cooling (40°C 10 s)**.** Primer efficiency was calculated using the LinRegPCR software (version 2017.0) (69) and an average primer efficiency for each primer pair was used in the final calculation of gene expression levels. All data were then normalized to the expression of mw2060 (external standard) using the second derivative maximum method to calculate the expression level for each gene (20):

$$Normalized\ mRNA\ expression\ level = \frac{E_{target\ gene}^{-CP_{target\ gene}}}{E_{reference\ gene}^{-CP_{reference\ gene}}}, \quad (1)$$

where E is the priming efficiency and CP is the crossing point.

Data collection and analysis

Data from the pressure transducers were collected with a BIOPAC MP100 system at 200 Hz coupled with the associated program Acqknowledge (v. 3.9.1). From these signals, the ventilation frequency (f_G) and heart rate (f_H) were determined as the rate of the pressure fluctuation of each channel. Mean arterial pressure (MAP) was determined as the average pressure in the DA, and the absolute value was confirmed by a daily two-point calibration against a static water column. Ventilatory amplitude (V_{amp}) was determined as the peak-to-peak pressure difference in the opercular pressure signal for each breath. Each value presented in the results and figures for all of these variables is averaged over 45-60 s. Periods with high background noise levels were excluded from the analysis because reliable V_{amp} values cannot be established at these times. These periods were always transient (<20 s; insert in Fig. 1).

Statistics

All data are presented as mean ± s.e.m. All data were examined for variance homoscedasticity using a residual plot and normality from a normal probability plot. The data were analyzed using a one-way repeated measures analysis of variance (RM ANOVA) using SigmaPlot (v. 12.1) followed by a Dunnett's (experiments 1 and 2) or Tukey's (experiment 3) *post hoc* test. The gene expression data were $\log_{10}$-transformed to achieve normality before the RM ANOVA was performed. A few cardiorespiratory traces were rank-transformed to achieve homoscedasticity, namely the data used for Figs. 4F, and 5FH; the rest of the data did not require transformation. All figures were prepared using SigmaPlot (v. 12.1). Differences were considered significant when $p < 0.05$.

Results

Intra-arterial injections

Control injections and lactate doses

Intra-arterial (DA) injections of Na L-lactate elicited a rapid, transient, dose-dependent, and significant increase in V_{amp} (Figs. 1 and 2A; p = 0.088; 0.011; <0.001 for the three doses, respectively; RM ANOVA, N = 6) and no change in f_G (Fig. 2C; p = 0.38; 0.41; 0.74, for the low, medium and high doses, respectively). There was considerable variance in the ventilatory response to lactate (Fig. 2A), with V_{amp} elevations ranging from +34 to +291% peak changes (mean = 107 ± 35%) following the highest dose of lactate, which could not be attributed to individual dosing differences, size differences or baseline ventilation differences.

Isosmotic saline or 1 mol l^{-1} NaCl had no effect on gill ventilation (Fig. 3), confirming that the ventilatory responses did not stem from pressure or volemic effects of injections *per se*, or from the small changes in plasma [Na^+] or osmolality. Pyruvate as well did not induce any effect on gill ventilation, indicating that the response was unlikely to be a secondary effect of lactate conversion to pyruvate. Following D-lactate injections only 1 out of 6 fish showed a minor ventilatory response (46% increase in V_{amp} at peak level), while none of the other fish showed any increase at all (Fig. 3).

Over the course of the experiment (control series and lactate doses), Hct and [Hb_4] dropped slightly (Table 2; p = 0.024; p = 0.018, respectively, paired t-test with Bonferroni p-value adjustment, N = 6) likely due to serial blood sampling. pH_a remained constant throughout (pH_a = 7.85 ± 0.06; p = 0.49, RM ANOVA, N = 6). P_aO_2 and [HCO_3^-] did not differ between resting conditions and the highest lactate dose (Table 2; p = 0.23 and p = 0.97, respectively), which were the only points measured, due to the relatively large blood volume needed for reliable P_aO_2 measurements.

Time course of ventilatory responses and lactate concentration

Lactate injections into the DA caused a transient elevation of V_{amp} starting 30-45 s after injection, peaking at 78 ± 3 s and subsiding over the course of the following 5 min (Fig. 1). Plasma [lactate] was statistically elevated above pre-injection levels for the first 7.5 min post-injection with a half-time in the blood of 4.8 ± 0.3 min (Fig. 1; RM ANOVA with Dunnett's *post hoc* test, N = 6). The injections of lactate into the VA initiated an immediate response with ventilatory increases initiated within 5 s, peaking after 41 ± 3 s (Figs. 4BD and 5BD; N = 16).

Gill-sectioning

Removing the input from receptors located on the 1st gill arch increased the baseline f_G slightly, but had no apparent effect on V_{amp}. As a result of the inter-individual variation in the absolute V_{amp} values, the relative changes are plotted to better visualize the observed responses (Fig. 2B). Ablation of the 1st gill arch reduced the response following lactate injections on V_{amp}, but did not abolish it completely at the highest dose (Fig. 2B; p = 0.87; 0.14; 0.003, for the three doses, respectively). Further, the return to baseline level took slightly longer compared to fish with intact gills (Fig. 2AB). As in the intact fish, f_G did not increase following lactate injections (Fig. 2D).

Pharmacological blockade

Injections of lactate into the VA initiated an immediate ventilatory response (Figs. 4BD and 5BD) and in many subjects a transient, strong hypotension that recovered within 1-2 min, often followed by hypertension (Figs. 4F and 5F). This response was highly variable, and even though it was present in most subjects, the MAP changes were not significant (Figs. 4F and 5F, p = 0.18, N = 12, RM ANOVA). For the control series (3x NaCN and 3x lactate doses, randomized), the responses were similar within individuals,

i.e. previous injections did not affect the response observed (supplementary Fig. S1). Supplementary figures are available at https://figshare.com/s/ced195f2f4288fb2880a.

5-HT_3 receptor blockade

Serotonin injections caused a strong hypotension, a moderate bradycardia as well as a brief period of reduced ventilation followed by strong elevation of V_{amp} similar to (11) (supplementary Fig. S2). MDL 72222 decreased all responses to serotonin slightly, but did not abolish them (supplementary Fig. S2).

Injections of the 5-HT_3 receptor antagonist MDL 72222 reduced the V_{amp}-response to both NaCN (Fig. 4A; p = 0.030, RM ANOVA with Dunnett's *post hoc* test, N = 6) and lactate (Fig. 4B; p = 0.008); for lactate the V_{amp} increase was decreased to just below the significance threshold following the blockade (p = 0.052). The bradycardia following both NaCN and lactate was also decreased following MDL 72222-injections, however for lactate, not significantly (Figs. 4GH and 5GH; NaCN: p = 0.016; lactate: p = 0.097).

Muscarinic and β-adrenergic blockade

Atropine increased the baseline f_H (Fig. 5GH), but almost abolished the response to ACh for all four variables (increased V_{amp} and f_G as well as bradycardia and hypotension; supplementary Fig. S3). Noradrenaline caused a pronounced hypertension without affecting the ventilatory variables or f_H, possibly due to a fast clearance and injection far from the heart (the VA; supplementary Fig. S3). The peak hypertension following NA was reduced slightly by DB (supplementary Fig. S3). DB increased the V_{amp} baseline slightly and lowered the MAP and f_H (Fig. 5).

Atropine alone caused minor, non-significant effects on the V_{amp} response (Fig. 4AB; NaCN: p = 0.077; lactate: p = 0.27). The addition of the propranolol, for the double blockade, reduced the V_{amp} response to both injections (Fig. 4AB; NaCN: p = 0.023; lactate: p <0.001), and the lactate response was

almost abolished. Small but non-significant effects of lactate were apparent, hardly recognizable from the main figure in 5B due to the variance in baseline and time to peak, but apparent in an analysis of peak values (see insert in Fig. 5B). Following the NaCN injections, DB elicited no further decrease in the V_{amp} response than atropine alone (Fig. 5A, p = 0.006). Atropine abolished the bradycardia following both NaCN and lactate injections (Fig. 5GH; p = 0.012; p = 0.002, respectively, RM ANOVA with Dunnet's *post hoc* test, N = 6). However, there were no significant differences between atropine and propranolol on the other variables measured, apart from the changed baseline of MAP and f_H, as described above (Fig. 5E-H).

Gene-expression

The sequence of the partial cloning of the *OR51E2*-like gene in trout revealed a 55.5% similarity at the amino acid level between this and *OR51E2* in mice (assessed with an EMBOSS Needle Pairwise Sequence Alignment). Shortly after conducting these experiments, the full predicted sequence of this gene in *O. mykiss* became available in the NCBI database (NCBI reference XM_021581623.1). The partial cloning mRNA products we sequenced were identical to a part of this sequence (NCBI reference MG812383). The mRNA of the *OR51E2*-like gene had a widespread expression, but with significantly higher expression levels in the gills (especially the 1st gill arch) (Fig. 6A) ($p < 0.001$; RM ANOVA, N = 8). In all tissues, the expression levels of the *OR51E2*-like gene were, however, low, but this is typical for G-protein coupled receptors (24).

The *HCAR1* gene from trout shared 84.6 % similarity in amino acid sequence with the zebrafish lactate receptor (*HCAR1-1*, assessed with an EMBOSS Needle Pairwise Sequence Alignment) and apart from the paralog, which is also sensitive to lactate (*HCAR1*-2) (39), no other genes showing a high similarity to *HCAR1* appeared in a BLAST analysis. The expression pattern was strikingly similar to that of the *OR51E2*-like gene (Fig. 6AB). Thus, *HCAR1* had significantly higher expression levels in the gills (especially the 1st gill arch) (Fig. 6B) ($p < 0.001$, RM ANOVA, N = 8).

Discussion

Elevation of plasma lactate concentration strongly stimulated ventilation in trout in the absence of any changes in pH_a, P_aO_2, or plasma [HCO_3^-] (Figs. 1 and 2, Table 2). We can rule out any role of pyruvate, osmotic/volumetric changes, or altered [Na^+] concentration caused by the injections (Fig. 3). Overall, the ventilatory responses at specific plasma [lactate] were similar in trout and the air-breathing *P. hypophthalmus*. However, the amplitude of the response differed slightly between the two species at equal lactate concentrations, where *P. hypophthalmus* demonstrated a stronger response at lower lactate levels than trout, possibly reflecting differences in concentrations naturally occurring in plasma (80). This identification of lactate mediated ventilatory responsiveness in the phylogenetically distant trout therefore lends further support to its being a general teleost (or vertebrate) trait. At the same time, this new finding allows for more in-depth investigation of the properties of the response in fish because of the much greater knowledge of both the physiology and genetic code of this species as well as the accessibility of its ventral aorta. D-Lactate injected at the highest L-Lactate dose failed to stimulate ventilation, strongly indicating that the responses are receptor-mediated and that this receptor is specific to the L-isomer, thus supporting hypothesis 2. Further, based on the decreased response following sectioning of the 1st gill arch (Figs. 2) and the reduced time lag following injections in the VA compared to DA (Figs. 1 and 2 vs. Figs. 4 and 5), we conclude that the receptor probably resides in the gills. Both the ventilatory and cardiovascular responses to lactate were similar to those elicited by NaCN injections (Figs. 4 and 5) and qualitatively typical of the trout HVR, where ventilation primarily increases by elevation of V_{amp} with only modest changes in f_G, accompanied by bradycardia and hypertension (22, 32, 53, 54). There was a clear dose-dependency in the response (Fig. 2), and there was strong correspondence between the lactate clearance from the blood and the recovery of normal ventilation (Fig. 1). Overall, these data support hypothesis 1 that the cardiorespiratory system of rainbow trout responds to increased plasma [lactate], as demonstrated in mammals and the distantly related teleost *P. hypophthalmus* (14, 28, 80).

Location of a putative lactate sensor

Based on previous investigations of the chemosensors coupled to ventilation in fishes, we hypothesized that a putative lactate receptor was likely to reside in gill tissue as suggested in *P. hypophthalmus* (80) (hypothesis 1). The gills are the main chemosensory organs and despite much variability between species, the 1st gill arch typically dominates, whereas the role of the other gill arches and extra-branchial locations varies across species (21, 23, 48). In rainbow trout, chemoreceptors affecting heart rate seem to reside only in the 1st gill arch, whereas sub-sets of the receptors affecting ventilation are also found on other arches and elsewhere (16, 55, 74, 92). After removal of the afferent inputs from the 1st gill arch, a clear reduction in the ventilatory responses to both NaCN and lactate were observed (Fig. 2AB). Further, when lactate was injected into the DA, the ventilatory responses were delayed by about 78 s (time to peak), which fits well with the blood transit time from the DA to the gills, *i.e.* almost a full circuit, which has been proposed to average between 43-130 s depending on level of activity (29). Conversely, with injections into the VA, the responses were initiated within 5 s (peaking after about 41 s). The most thoroughly studied (and possibly the only) chemosensing cells in the gills are the NECs, which have been demonstrated to be multimodal sensors (91). They respond to a variety of stimuli and house neurotransmitter-containing vesicles (mainly serotonin, 5-HT), which seem to release their content when stimulated (19, 35). Both the 5-HT$_2$ and 5-HT$_3$ receptors elicit cardioventilatory responses in fishes. However, in adult trout the 5-HT$_2$ receptor does not seem to be the primary receptor involved in ventilatory stimulation (although it is clearly involved in branchial vascular regulation) (25, 34, 35, 52, 78). Conversely, blockade of the 5-HT$_3$ receptor decreased the ventilatory response to NaCN and lactate (Fig. 4A-D) indicating a serotonergic involvement in the signal transduction, possibly through activation of the NECs. Further, two putative lactate receptors are expressed in gill tissue (Fig. 6), thus supporting hypothesis 4. Collectively, these data strongly support the presence of a lactate receptor in trout gill tissue and fit well with the notion of NEC involvement in the lactate ventilatory response.

Involvement of serotonergic, cholinergic, and β-adrenergic receptors

Overall, the responses to NaCN and lactate injections were similar. Injection of either into the VA stimulated a V_{amp} increase and bradycardia within a few seconds (Figs. 4ABGH and 5ABGH). Following 5-HT_3 receptor blockade, these ventilatory responses were strongly attenuated (Fig. 4AB), thereby supporting hypothesis 3. Further, atropine abolished the bradycardia but not the MAP or ventilatory responses (Fig. 5E-H). However, there were also differences between the stimulants, with a faster recovery (10-15 s) from the initial transient post-injection hypotension following NaCN than after lactate (30-120 s). The reason for this difference is not clear, however the pressure drop may be an acute hypoxic response, as hypoxia increases branchial resistance (which may cause a transient drop in DA blood pressure until the cardiac output increases), but may also be indicative of a difference in the strength of the two stimuli (51, 79). This hypoxia-induced increase in branchial resistance is mainly regulated by constriction of the proximal part of efferent filamental arteries (77, 79), and likely causes hypertension upstream in the gills where baroreceptors are located (8, 71, 88). Stimulation of these receptors may explain the source of this transient bradycardia, but does not explain the difference between the two stimulants. The concentration of the lactate injections used here is quite high but the dilution in blood during injections is unknown. The other difference in the responses to NaCN and lactate is the large decrease in the V_{amp} response to lactate but not to NaCN following double blockade. The lactate transduction pathway is not identical to the NaCN pathway. Lactate receptors are likely associated with the branchial oxygen sensitive cells whose signaling pathway is significantly blocked by propranolol (Fig. 5B) (9, 10), whereas NaCN asserts its hypoxia-mimicking effect through inhibition of mitochondrial energy metabolism, not by receptor stimulation, thus exerting a broader effect (89).

Since elevated plasma lactate is also associated with a prolonged elevation in metabolism, which is reduced by adrenergic antagonists, it has been suggested to initiate a general stress response

481 rather than a more specific HVR (61). Whether lactate is specifically linked to a stress response can be
482 difficult to determine, as hypoxia may also evoke a stress response, especially in severe hypoxia (5, 66). In
483 the present study, some transient agitation was seen in some but not all fish following lactate injections
484 (insert in Fig. 1). However, if lactate stimulation initiated hyperventilation through a stress response,
485 tachycardia associated with catecholamines would have been expected and certainly not bradycardia as
486 seen here (Figs. 4H and 5H) (11, 87). Further, the reduction in ventilatory responses following 5-HT_3
487 receptor blockade (Fig. 4B) is difficult to explain if the role of lactate is a stress-inducing molecule.

488

489 ## Linking of the hypoxic response and the lactate response in trout

490 If the lactate ventilatory response is part of the acute hypoxic response, local lactate concentrations near
491 the branchial chemosensing cells must increase swiftly in response to hypoxia – much earlier than
492 circulating systemic levels. This possibility remains to be investigated and requires identification of the
493 appropriate cell populations. However, as the gills have a remarkably high mass-specific metabolic rate it is
494 not implausible that specific branchial cells could produce lactate earlier than the systemic [lactate]
495 increases (50, 56, 86). If this is the case, there are two possibilities: Either lactate stimulation is a part of the
496 hypoxic signaling chain (*i.e.* lactate production is required for initiation of the HVR) or lactate provides one
497 of several initiation mechanisms leading to a hypoxic response (*i.e.* several modalities stimulate the same
498 downstream biochemical cascade leading to a HVR). The marked reduction of the ventilatory response to
499 lactate, but not to NaCN, following double blockade suggests that lactate did not stimulate all cells involved
500 in hypoxic responses, supporting the latter possibility (Fig. 5AB). As in mammals, the specific oxygen
501 sensing mechanisms in gills remain to be definitely confirmed, however, several substances may stimulate
502 or modulate the NECs and the ventilation (1, 51, 59, 60, 64, 82, 90, 92). How these different pathways
503 interact and how lactate fits into this group of hypoxia sensing modalities are not clear. In mammals, both
504 identified lactate receptors (*OR51E2* and *HCAR1*) are stimulated by extracellular lactate, thus transport

505 from intracellular compartments must precede any response. If this is also the case in fishes is yet to be
506 determined, *e.g.* by blockade of the lactate transporters (83). From an evolutionary point of view, the
507 sensing of oxygen level is key to survival for all aerobic organisms, particularly aquatic organisms where it
508 provides the primary ventilatory drive, making it perfectly feasible that redundancy by virtue of several
509 independent mechanisms provides for a failsafe in case of a malfunction of one of the mechanisms.
510 Another possibility for the ventilatory role of lactate in trout is that it is not directly involved in the natural
511 initiation of the HVR, but rather functions as a modulator of ventilation when the systemic lactate levels
512 increase. Principally, there are two main causes of increased systemic lactate levels: severe hypoxia or
513 intense exercise. Exposure to either causes plasma [lactate] to rise, often reaching 10-20 mmol l^{-1} in trout
514 (5, 38, 46), similar to the concentration observed after the medium and high doses used in Fig. 2.

515 Increased plasma [lactate] is typically first observed in the post-exercise/hypoxic phase, as a
516 result of its slow release from the white muscles (83). In trout, peak plasma [lactate] is attained about 2
517 hours post-exercise/hypoxia and then slowly returns to pre-exercise/hypoxia levels over 6-12 h (17, 38, 43,
518 44, 46, 47, 72, 81). Oxygen consumption and ventilation, however, peak during or immediately after
519 cessation of exercise, and remain elevated for 4-6 h (45, 73, 85). Circulating lactate is therefore clearly not
520 the sole contributor to ventilatory changes in animals, and the difference in the time of the peaks must be
521 due to other stimulants. Further, there is considerable inter-study variation where only one of these
522 variables has been measured making a meta-analysis of the relationship between plasma [lactate] and
523 ventilation in fishes difficult. However, the available data indicate that ventilation returns to resting values
524 (*i.e.* non-significantly elevated) earlier than [lactate], which is also the case in other vertebrate classes (27).
525 This indicates that although circulating lactate ions provide a dose-dependent, acute stimulatory effect on
526 ventilation (Figs. 1 and 2), some short-term adaptation occurs post-exercise, or alternatively that the
527 ventilatory increases caused by the low lactate levels observed >6 hours post-exercise are too small to be
528 detected by the methods used.

In contrast to the natural condition where plasma [lactate] is elevated for several hours, injections of lactate are cleared rapidly from the blood (Fig. 1) (45, 81). Plasma lactate is cleared by three routes: 1) oxidation to CO_2 and water, 2) gluconeogenesis restoring glucose and glycogen stores, or 3) conversion to other biomolecules such as amino acids and fatty acids. Depending on species, the proportion of lactate cleared through each route differs markedly but in trout, the majority of exercise produced lactate (80-85%) seems to remain in the white musculature for *in situ* post-exercise gluconeogenesis and oxidation (27, 33, 36, 43, 44, 47, 81). Following lactate injections in resting fish, oxidation is the main fate of lactate, and interestingly, a significant proportion is still taken up by the white muscles (43). The short half-life in plasma observed following injections provides an explanation of why ventilation returns to near normal levels so rapidly post-injection in the present study, but conversely also suggests that the plasma lactate concentration *per se* may stimulate ventilation directly. In this scenario, the present fish data would then be directly similar to the observations in mammals (14, 28). The dose-dependent relationship between [lactate] and relative ventilation change observed lends further support for the putative direct role of lactate in ventilatory regulation (Fig. 2).

Putative lactate receptors in trout

In mice, *OR51E2* has been suggested as the carotid body receptor responding to increased lactate and thereby providing a link between lactate and hyperventilation (14). The trout gene with the highest similarity to this (55.5% amino acid sequence similarity) was found to be expressed in gill tissue in trout (Fig. 6A). The sequence similarity might seem unimpressive, but due to the phylogenetic distance between mice and rainbow trout, all but the genes for the most important cellular functions have undergone substantial mutations since the separation of the lineages 450 million years ago (30). This does, however, make it difficult to establish whether the function of the gene is retained, especially as the ligand interaction with residues within the binding site of olfactory receptors is not fully understood (37). *OR51E2*

553 is one of the most widely expressed ectopic olfactory receptors in mammals as is also the case here with
554 the *OR51E2*-like gene in trout, found at low expression levels in all tissues tested (Fig. 6A), indicative of an
555 important function. Determining the evolutionary relationship between genes across vertebrate classes can
556 be done by establishing a phylogeny of the genes within the gene family. For olfactory receptors this is not
557 an easy task as there has been a large expansion in the number of genes present in mice (and mammals in
558 general) which apparently originate from only few (ca. 8) ancestral olfactory receptor families in the last
559 common ancestor of teleosts and tetrapods (3). Thus, a phylogenetic analysis gives little insight regarding
560 preserved functions. Consequently, until the *OR51E2*-like teleost gene is actually cloned and the response
561 to lactate investigated in cell lines, its role as a lactate receptor will remain putative.

562 In zebrafish, the *HCAR1* receptor has been identified as a lactate sensor, although an
563 involvement in ventilatory responses has never been examined. The high similarity, as well as its lack of
564 similarity to other genes, is strongly suggestive of its role as a lactate responsive receptor in trout (39). The
565 higher level of mRNA expression of both these receptors in gill tissue compared to other tissues make them
566 prime candidates as lactate sensing receptors in the trout, but it can, of course, not be regarded as
567 conclusive evidence for an involvement of one or the other, or both, in the cardiorespiratory lactate
568 responses. Determining the spatial expression of these receptors relative to chemosensory cells, as well as
569 examining if the *OR51E2*-like receptor is also sensitive to lactate in fish, will be important next steps in
570 understanding the molecular background for the lactate response.

571

572 ## Perspectives and significance

573 Collectively, the data demonstrate a direct, dose-dependent stimulation by lactate of receptors eliciting
574 cardiorespiratory responses in rainbow trout. The lactate receptor(s) eliciting these responses is mainly
575 branchial and since serotonin signaling is demonstrated, it is likely housed in the NECs. The lactate
576 ventilatory response is now documented in mammals and in two distantly related teleosts (14, 28, 80, 84).

Thus, it is tempting to suggest that receptor-based lactate sensing is a general vertebrate trait, but clearly more species need investigating. Whether the response is part of the HVR or is simply utilized to modulate ventilation as systemic lactate levels increase remains unclear, but the ventilatory stimulation by NaCN, when the lactate response is abolished following double blockade, indicates that lactate ions are not a necessity for the initiation of the HVR. Clearly, future studies are required to determine their cellular role and the precise localization of the receptors. These could involve receptor studies in isolated cells opening up for the use of specific pharmacological blockers that are unsuitable for use in whole animals and thus allow determination of the role of lactate in the cellular transduction pathway in NECs preceding the HVR.

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803

804 Figures

805 **Figure 1: The ventilatory response to lactate and the course of plasma [lactate] following Na L-lactate**
806 **injections.** The injections in the dorsal aorta were initiated at time 0, indicated by the gray vertical bars.
807 The insert shows a representative data trace of the opercular pressure following lactate injections, with a
808 response starting ca. 30 s after the injection, followed by some movement (the few high-amplitude spikes)
809 and an increased ventilatory amplitude. Data are presented as means ± s.e.m. Asterisks indicate a
810 significant difference from the pre-injection value. N = 6.

811 **Figure 2: Dose-response of the ventilatory variables following increasing doses of lactate in intact and 1st**
812 **gill arch sectioned (G1) fish.** The intact responses of lactate injections in the dorsal aorta are on the left
813 panels (A and C) and the G1-fish are visualized on the right panels (B and D). The inserts in the figures are
814 the subsampled peak changes, *i.e.* the increase/decrease relative to the measured variable at time -1 min.
815 The peak values were usually at either time point 0.5 or 1 min. 'Low', 'med', and 'high' refer to the three
816 doses of lactate injected. Data are presented as means ± s.e.m. Asterisks indicate a significant difference

from the pre-injection value and # indicate a significant difference in the peak change compared to the saline injections. N = 6 for both groups.

Figure 3: Effects on ventilation of negative and positive control injections and D-lactate. The injections were administered into the dorsal aorta and the concentrations used were: saline: 0.9% NaCl; NaCl: 0.5 ml 1 mol l^{-1}; pyruvate: 0.5 ml 0.1 mol l^{-1}; NaCN: 0.25 ml 100 µg ml^{-1}; D- and L-lactate: 1 ml kg^{-1} 1 mol l^{-1}. The peak change in ventilatory amplitude is plotted as relative change from pre-injection level. Data are presented as means ± s.e.m. Asterisks indicate significant differences from pre-injection levels. N = 6 for all. Note that D-lactate injections were done in different fish than the other injections (see text).

Figure 4: Ventilatory and cardiovascular effects of NaCN and Na L-lactate before and after blocking serotonergic 5-HT_3 receptors. The left panels show the response to NaCN (A, C, E, G) and the right panels show the response to lactate (B, D, E, H) injected into the ventral aorta. The inserts in the figures are the subsampled peak changes, *i.e.* the increase/decrease relative to the measured variable at time -1 min. The peak values were usually at either time point 0.5 or 1 min except for in panel F. Data are presented as means ± s.e.m. Significant differences from the pre-injection level (time -1 min) are indicated by * for pre-blockade, and † for 5-HT_3-blockade. # indicates a significant difference from the pre-blockade response in the peak change. N = 6.

Figure 5: Ventilatory and cardiovascular effects of NaCN and Na L-lactate injections before and after muscarinic and β-adrenergic blockade. The left panels show the response to NaCN (A, C, E, G) and the right panels show the response to lactate (B, D, E, H) injected into the ventral aorta. The inserts in the figures are the subsampled peak changes, *i.e.* the increase/decrease relative to the measured variable at time -1 min. The peak values were usually at either time point 0.5 or 1 min except for in panel F. Data are presented as means ± s.e.m. Significant differences from the pre-injection level (time -1 min) are indicated by * for pre-blockade, † for atropine, and § for DB. # indicates a significant difference from the pre-blockade response in the peak change. N = 6.

Figure 6: Expression profiles of two putative lactate receptors in different tissues. The top panels show the expression levels of the OR51E2-like gene (A), and HCAR1 (B) in the tissues examined. Data are presented as means ± s.e.m. Tissues sharing a letter are not significant different from each other. N = 8, except for 4th gill arch and adipose tissue where N = 7.

Tables

Table 1: Primers used for partial cloning and quantitative real-time PCR.

Gene	Direction	Primer sequence (5' → 3')
Partial cloning		
OR51E2-like	F	TAATATGTGGTTTGGCGGCG
	R	TCACAGCCAATCCTATCGCA
qPCR		
OR51E2-like	F	ATGGCTCTGGACCGCTATG
	R	GCGAGCGCAACTAGTACAAAC
HCAR1	F	ACGAATGCCGGTATTTCCAAGA
	R	CAGCTTTAGGAAGGTCCCGT
mw2060	F	CTGACCATCCGAGCGATAAT
	R	AGCAAGCTGTTCGGGTAAAA

F, forward primer; R, reverse primer. Several primers were designed for both partial cloning, but only the primer pairs that resulted in the correct sequence are shown in the table. For qPCR, the primer pairs chosen were based on the lowest crossing point (CP), best efficiency, and the primers that gave only one melting curve with SYBR Green I detection.

852

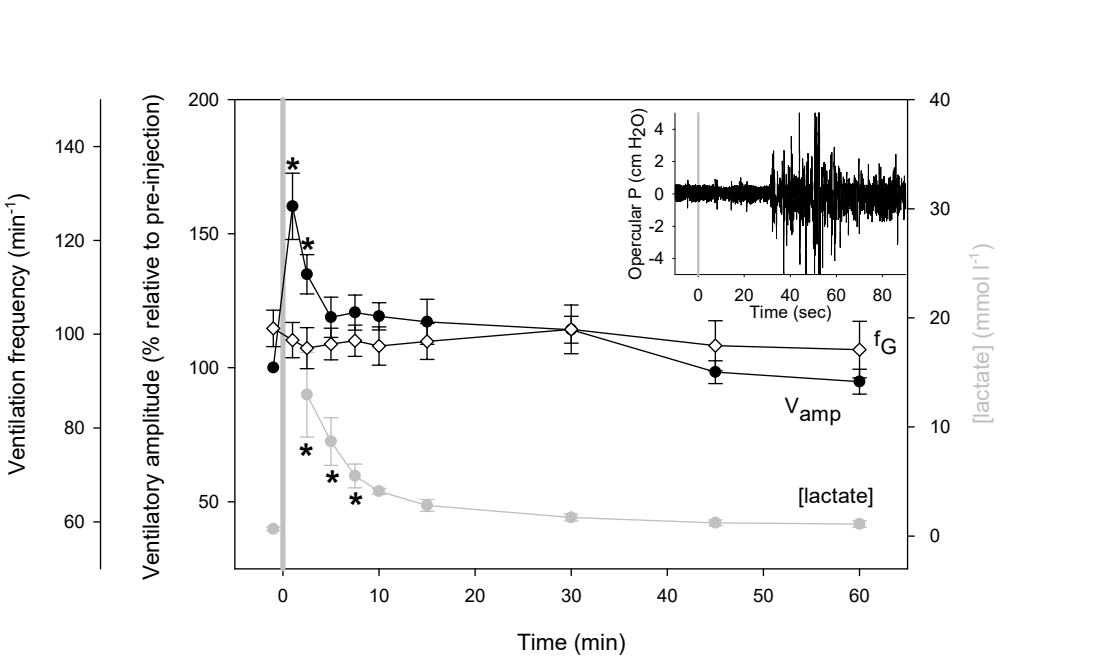
853 **Table 2: Changes in blood variables following the injection series.**

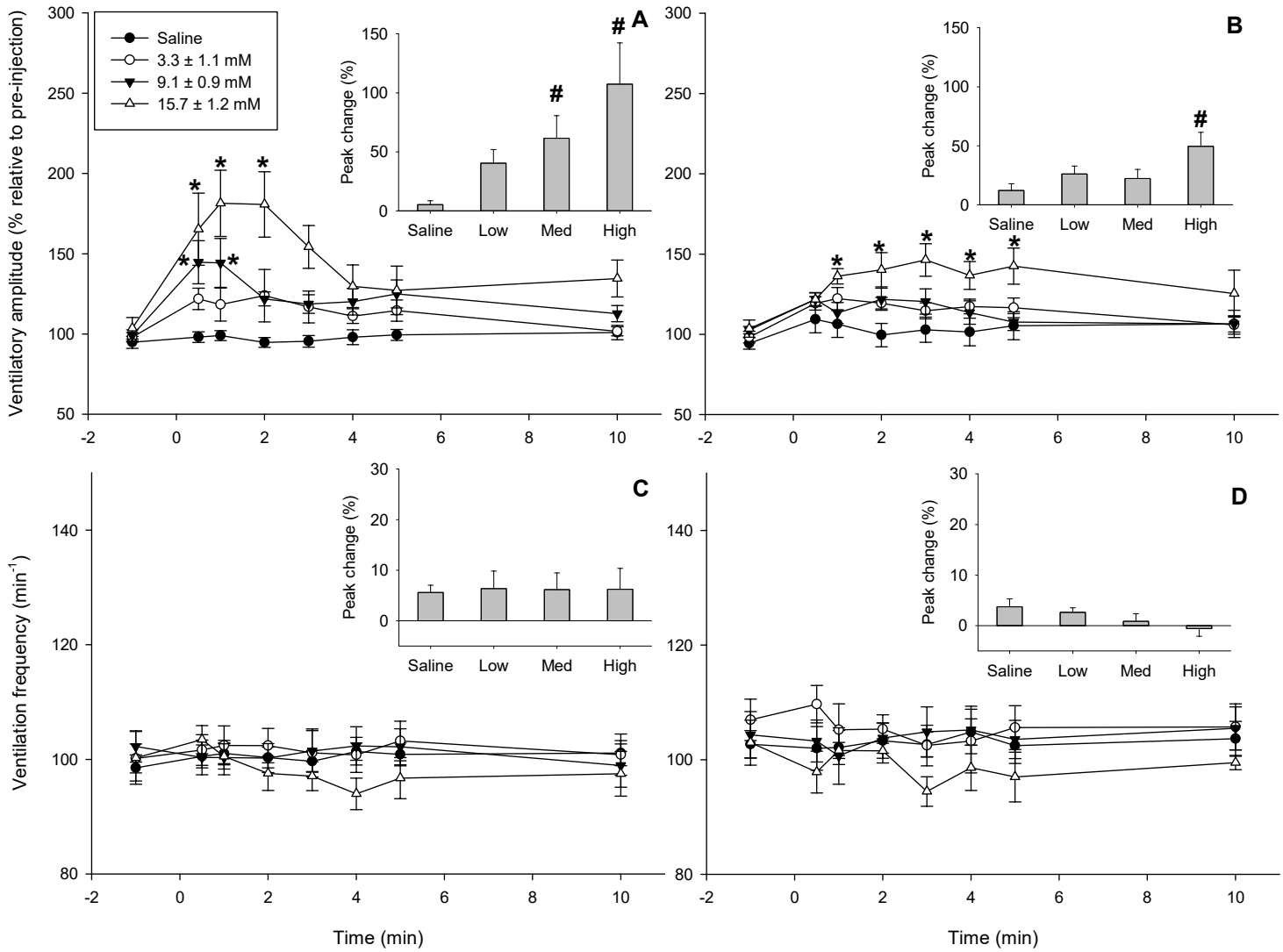
	P_aO_2 (mmHg)	Hematocrit (%)	$[Hb_4]$ (mmol l^{-1})	$[HCO_3^-]_{pl}$ (mmol l^{-1})
Pre-injection	84.3 ± 7.2	19.7 ± 1.1	0.90 ± 0.07	8.38 ± 0.78
Post-injection	79.1 ± 4.5	16.1 ± 0.9 *	0.70 ± 0.07 *	8.52 ± 0.60

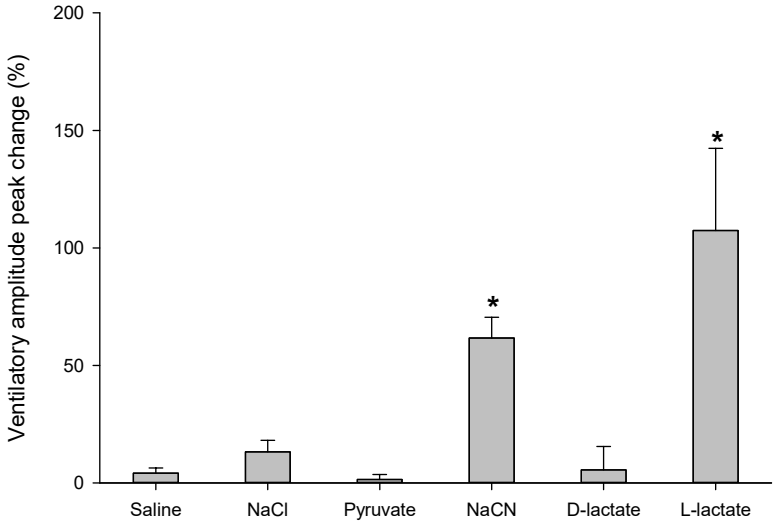
854 The post-injection sample was withdrawn 2 min after the last lactate injection. Asterisks indicate significant

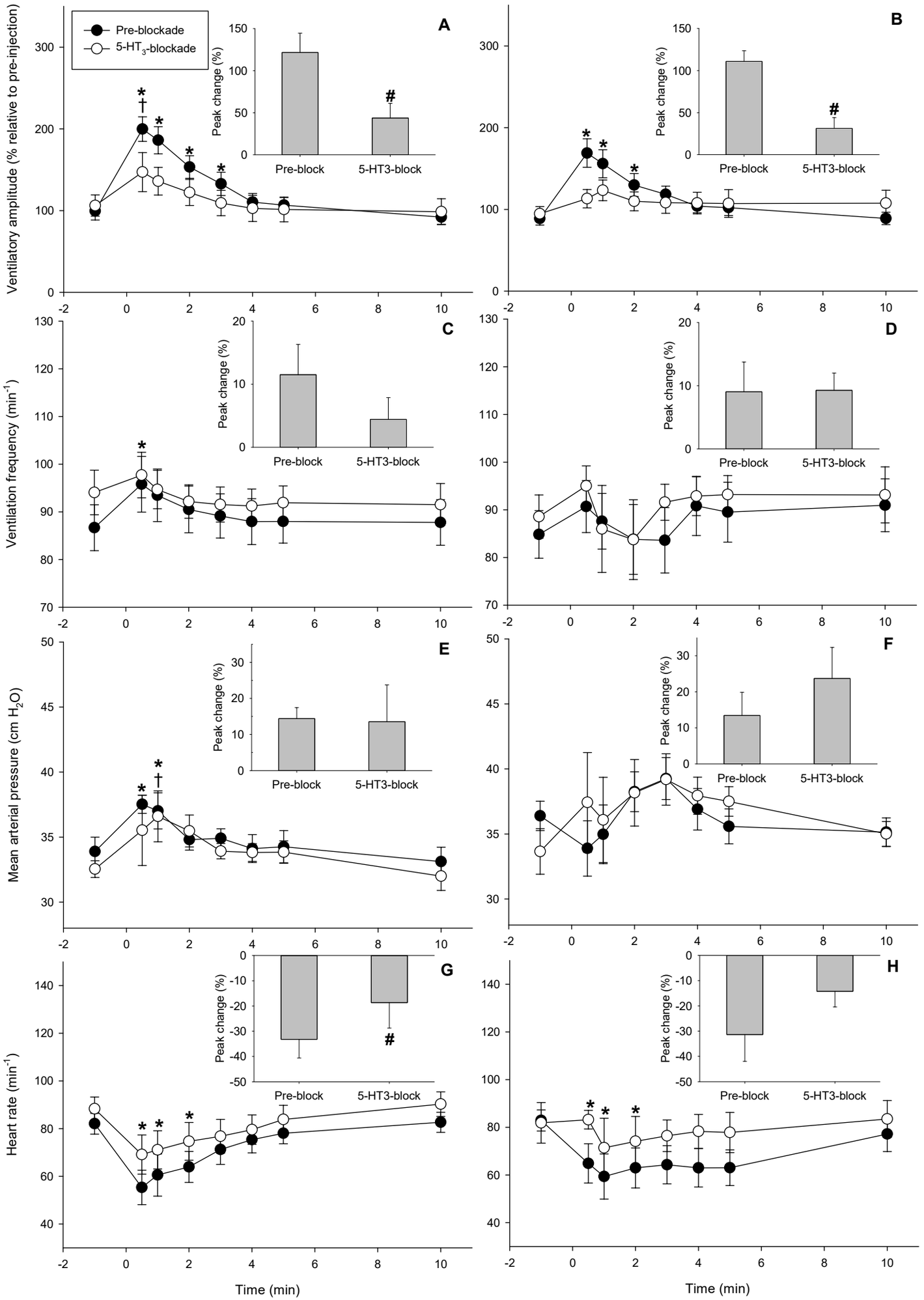
855 changes from pre-injection values (N = 6; two-tailed paired t-test with Bonferroni p-value adjustment).

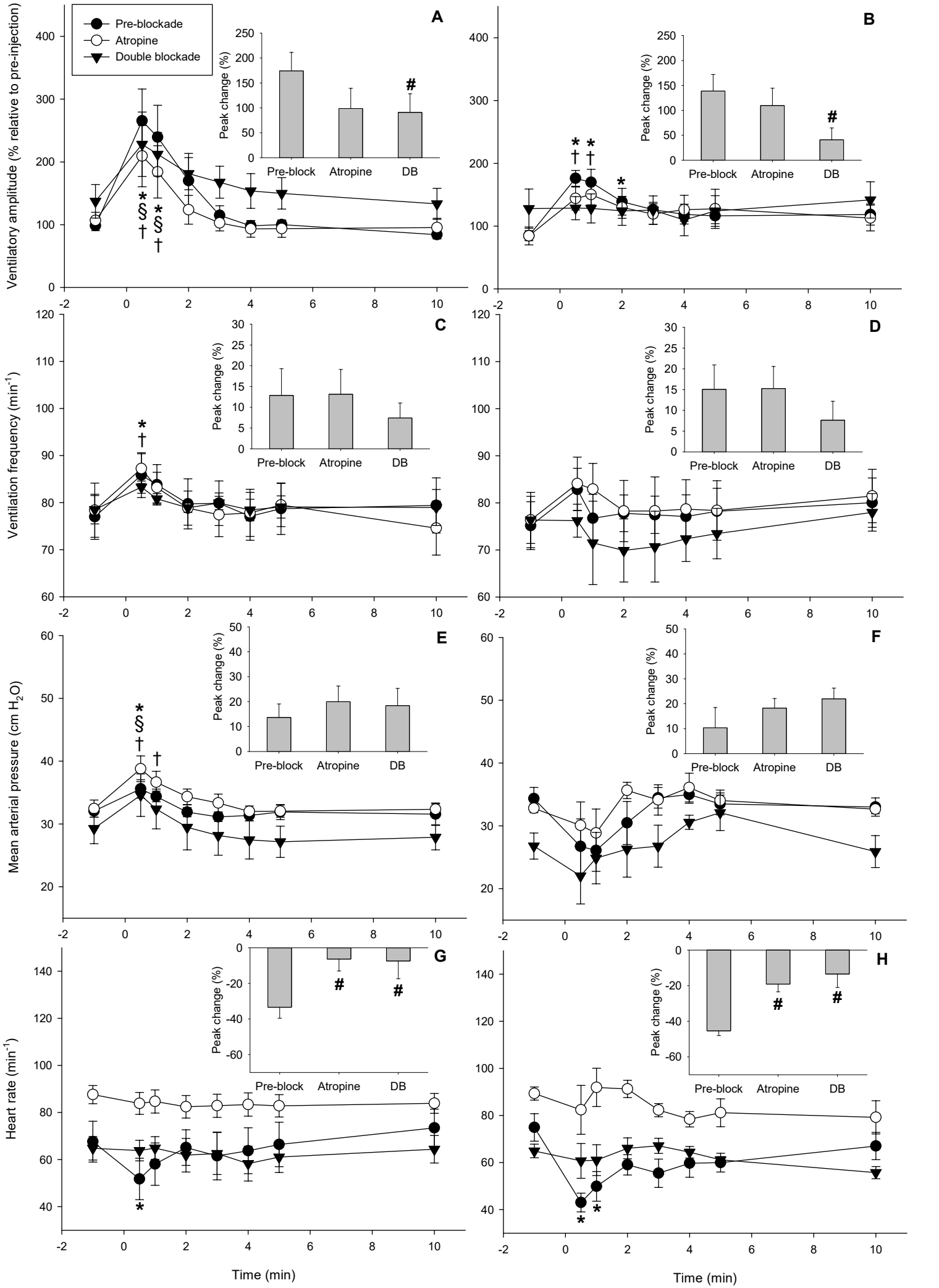
856

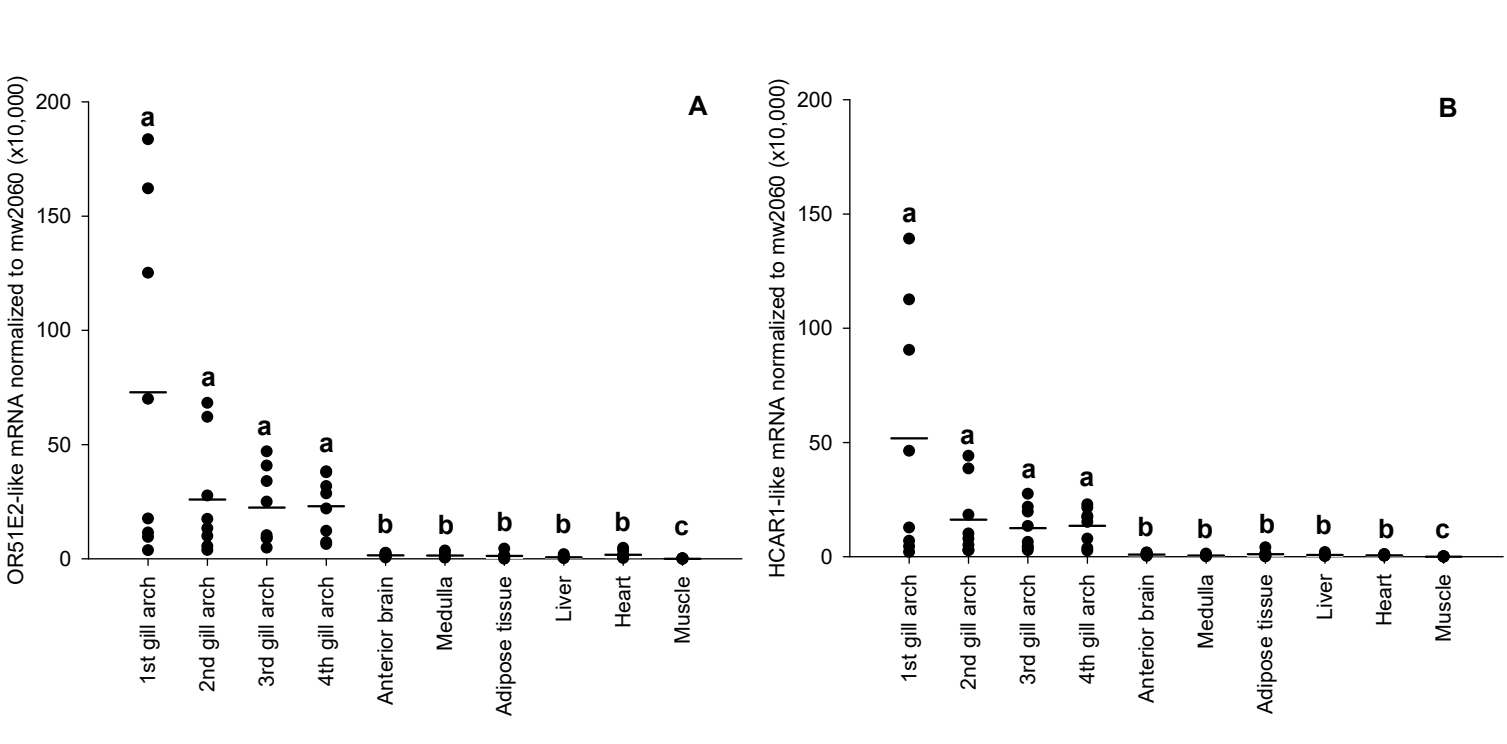

Ventilation frequency (min-1)
Ventilatory amplitude (% relative to pre-injection)
[lactate] (mmol l-1)
Time (min)
Opercular P (cm H2O)
Time (sec)
fG
Vamp
[lactate]
*


A
B
C
D
Saline
3.3 ± 1.1 mM
9.1 ± 0.9 mM
15.7 ± 1.2 mM
Ventilatory amplitude (% relative to pre-injection)
Ventilation frequency (min-1)
Peak change (%)
Saline
Low
Med
High
Time (min)


Ventilatory amplitude peak change (%)
200
150
100
50
0
*
*
Saline
NaCl
Pyruvate
NaCN
D-lactate
L-lactate


Pre-blockade
5-HT3-blockade
A
B
C
D
E
F
G
H
Ventilatory amplitude (% relative to pre-injection)
Ventilation frequency (min-1)
Mean arterial pressure (cm H2O)
Heart rate (min-1)
Peak change (%)
Pre-block
5-HT3-block
Time (min)


Pre-blockade
Atropine
Double blockade
A
B
C
D
E
F
G
H
Ventilatory amplitude (% relative to pre-injection)
Ventilation frequency (min-1)
Mean arterial pressure (cm H2O)
Heart rate (min-1)
Peak change (%)
Pre-block
Atropine
DB
Time (min)


A
OR51E2-like mRNA normalized to mw2060 (x10,000)
200
150
100
50
0
a
a
a
a
b
b
b
b
b
c
1st gill arch
2nd gill arch
3rd gill arch
4th gill arch
Anterior brain
Medulla
Adipose tissue
Liver
Heart
Muscle
B
HCAR1-like mRNA normalized to mw2060 (x10,000)
200
150
100
50
0
a
a
a
a
b
b
b
b
b
c
1st gill arch
2nd gill arch
3rd gill arch
4th gill arch
Anterior brain
Medulla
Adipose tissue
Liver
Heart
Muscle