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Title:

A solution to Nature's haemoglobin knockout: a plasma-accessible carbonic anhydrase catalyses CO_2 excretion in Antarctic icefish gills

Running title:

CO_2 excretion without red blood cells

Harter T. S.[1*], Sackville M.[1], Wilson J. M.[2], Metzger D. C. H.[1], Egginton S.[3], Esbaugh A. J.[4], Farrell A. P.[1,5] and Brauner C. J[1].

[1]Department of Zoology, The University of British Columbia, Vancouver, BC V6T 1Z4, Canada

[2]Department of Biology, Wilfrid Laurier University, Waterloo, ON N2L 3C5, Canada

[3]Faculty of Biological Sciences, School of Biomedical Sciences, University of Leeds, LS2 9JT, UK

[4]Marine Science Institute, University of Texas at Austin, Port Aransas, TX 78373, USA

[5]Faculty of Land and Food Systems, The University of British Columbia, Vancouver, BC V6T 1Z4, Canada

[*]Corresponding author: harter@zoology.ubc.ca

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Summary Statement:

Haemoglobin-less Antarctic icefish express a membrane-bound carbonic anhydrase that catalyses CO_2 excretion at the gills, to compensate for the absence of the normal enzyme pool within red blood cells.

Abstract

In all vertebrates studied to date, CO_2 excretion depends on the enzyme carbonic anhydrase (CA) that catalyses the rapid conversion of HCO_3^- to CO_2 at the gas-exchange organs. The largest pool of CA is present within red blood cells (RBC) and, in some vertebrates, plasma-accessible CA (paCA) isoforms participate in CO_2 excretion. However, teleost fishes typically do not have paCA at the gills and CO_2 excretion is reliant entirely on RBC CA; a strategy that is not possible in icefishes. As the result of a natural knockout, Antarctic icefishes (Channichthyidae) are the only known vertebrates that do not express haemoglobin (Hb) as adults, and largely lack RBC in the circulation (haematocrit < 1%). Previous work has indicated the presence of high levels of membrane-bound CA activity in the gills of icefishes, but without determining its cellular orientation. Thus, we hypothesised that icefishes express a membrane-bound CA isoform at the gill that is accessible to the blood plasma. The CA distribution was compared in the gills of two closely-related notothenioid species, one with Hb and RBCs (*Notothenia rossii*) and one without (*Champsocephalus gunnari*). Molecular, biochemical and immunohistochemical markers indicate high levels of a Ca4 isoform in the gills of the icefish (but not the red-blooded *N. rossii*), in a plasma-accessible location that is consistent with a role in CO_2 excretion. Thus, in the absence of RBC CA, the icefish gill could exclusively provide the catalytic activity necessary for CO_2 excretion; a pathway that is unlike that of any other vertebrate.

41 Introduction

42 The first scientific investigation of an Antarctic icefish, less than a century ago (Ruud,
43 1954), overthrew the common perception that haemoglobin (Hb) was a necessity to sustain
44 vertebrate life. In fact, an entire family of teleosts, Channichthyidae within the suborder
45 Notothenioidei (Perciformes) and comprising 16 species, do not express Hb as adults (Ruud,
46 1954; Eastman, 1993) and largely lack red blood cells (RBC) in their circulation; residual
47 haematocrit (Hct) is typically < 1% (Egginton, 1994). The implications for cardiovascular gas
48 transport are tremendous. In the absence of Hb, icefish blood has a 10-fold lower O_2-carrying
49 capacity compared to red-blooded notothenioids (Holeton, 1970), and without RBCs icefish lack
50 the important pool of carbonic anhydrase (CA) that facilitates CO_2 transport and excretion in all
51 vertebrates (Tufts and Perry, 1998). Those adaptations that address the dramatic impairment of
52 O_2 transport in icefishes are largely known (Hemmingsen and Douglas, 1970; Holeton, 1970;
53 Hemmingsen and Douglas, 1972); however, those needed to resolve the associated problem of
54 CO_2 excretion are not.

55 Most vertebrates transport the majority of CO_2 that is produced in tissues as dissolved
56 HCO_3^- in the blood plasma. In this regard icefishes are no exception, as indicated by venous
57 blood pH and PCO_2 values (7.84 and 0.3 kPa in *Chaenocephalus aceratus*; Hemmingsen and
58 Douglas, 1972) that are in line with those found in other fishes. Under these conditions, and due
59 to the low apparent pK of the CO_2-HCO_3^- reaction of ~6.2 (Boutilier et al., 1984), blood plasma
60 is an effective sink for CO_2. While this greatly increases the capacitance for CO_2 transport in
61 blood (Tufts and Perry, 1998; Henry and Swenson, 2000), it also requires a rapid conversion of
62 CO_2 to HCO_3^- at the tissues and the reverse reaction at the gills for CO_2 excretion. However, the
63 spontaneous rates of these reactions are slow relative to the residence time of blood at the

respiratory surfaces and tissue capillaries, and these rates further slow with decreasing temperature. At physiological temperatures in icefish, around -1.9°C (Littlepage, 1965), the halftime of spontaneous HCO_3^- dehydration to CO_2 is ~300 s (Kern, 1960; Heming, 1984) and thus exceeds the residence time of blood at the gills (~1-3 s) by two orders of magnitude (Cameron and Polhemus, 1974; Hughes et al., 1981). Based on the arterial-venous differences in PCO_2 and pH in *C. aceratus* (Hemmingsen and Douglas, 1972), it can be estimated that in resting, normoxic icefish, about 68% of CO_2 excretion must depend on HCO_3^- dehydration at the gills, while the remainder is from physically dissolved CO_2 in the plasma. During aerobic exercise, where blood pH is largely maintained, HCO_3^- concentration may increase by 50% (Brauner et al., 2000) and the residence time at the gills will be reduced further, due to a higher cardiac output (Randall, 1982); an increase in cardiac output following exercise has recently been shown for *C. aceratus* (Joyce et al., 2018). Clearly, the uncatalysed rate of HCO_3^- dehydration is simply not rapid enough to support CO_2 excretion in any adult vertebrate, but in particular icefishes at these low temperatures.

The rate limitation of CO_2-HCO_3^- reactions in the blood of vertebrates is largely alleviated by the catalytic activity of CA. The major CA pools are: i) RBC intracellular CA (Maren, 1967), and plasma HCO_3^- has functional access to this CA pool via rapid Cl^-/HCO_3^- exchange across the RBC membrane (Romano and Passow, 1984); ii) soluble CA isoforms in the plasma (Henry et al., 1997b); and iii) plasma-accessible CA (paCA) isoforms that are anchored to the apical membranes of the endothelium (Henry and Swenson, 2000). At the tissue capillaries paCA is typically present and ensures a rapid conversion of CO_2 to HCO_3^- (Henry et al., 1997a). However, at the gas exchange surface, the contribution of different CA pools to CO_2 excretion varies largely among the major vertebrate groups. On one end of the spectrum are the basal

hagfishes (Esbaugh et al., 2009) and Chondrichthyes (Gilmour et al., 2002; Gilmour et al., 2007) that rely on RBC CA, soluble CA in the plasma and paCA at the gills for CO_2 excretion. All Euteleostomi lack soluble CA activity in the plasma, and thus most tetrapods rely on RBC CA, and to a lesser degree (< 10% of total CO_2 excretion) on paCA at the gas exchange surface (Bidani et al., 1983; Zhu and Sly, 1990; Stabenau and Heming, 2003). And finally, teleost fishes have also lost paCA activity at the gills (for review see Harter and Brauner, 2017), and thus HCO_3^- dehydration is shifted entirely into the RBC (1982; Wood et al., 1982; Desforges et al., 2001; Desforges et al., 2002; for review see Perry and Gilmour, 2002), creating a strong coupling between O_2 and CO_2 transport; a hallmark of teleost gas exchange (Brauner and Randall, 1996). This strategy is clearly not available to icefishes, which are teleosts, but lack RBCs. Thus, with a clear need to catalyse HCO_3^- dehydration, some other CA pool must be present in icefishes to compensate for the loss of RBC CA.

Previous studies on gill homogenates from icefishes have provided biochemical evidence for a higher activity of membrane-associated CA when compared to red-blooded notothenioids (Feller et al., 1981; Maffia et al., 2001). Tufts et al. (2002) further characterised the branchial CA isoform distribution of notothenioids and found biochemical markers for the presence of a membrane-bound Ca4 isoform in the gills of an icefish species, but surprisingly, also in the gills of a red-blooded notothenioid. A critical detail, the cellular orientation of putatively paCA isoforms remains unexplored and therefore the potential involvement of a Ca4 isoform in CO_2 excretion remains unresolved for icefishes. Building on these previous findings, we hypothesised that icefishes express a membrane-bound CA isoform at the gill that is accessible to the blood plasma where it would catalyse CO_2 excretion in the absence of RBC CA. To this end, biochemical, molecular and immunohistochemical techniques were used to compare the CA

isoform distribution in the gills of the icefish *Champsocephalus gunnari* and the red-blooded *Notothenia rossii*. The obtained results shed new light on a divergent strategy of CO_2 excretion in icefishes, unlike that found in any other adult vertebrate.

Materials and Methods

Sample collection

Specimens of *Notothenia rossii* and *Champsocephalus gunnari* (average mass 343.4±17.2 and 644.3±70.1 g, and length 38.8±0.7 and 37.7±1.1 cm) were captured using otter trawls or baited pot traps deployed from the *U.S. ARSV Laurence M. Gould* at Low Island (63° 30' S, 62° 37' W) and North Dallmann Bay (63° 55' S, 62° 43' W), Antarctica. Animals were stunned by a sharp blow to the head. Blood was drawn from the caudal vein and mixed with 3.2% sodium citrate (9:1 for *N. rossii* and 4:1 *C. gunnari*). All samples were centrifuged at 5,300 x g for 10 min and plasma was decanted. Blood cells and plasma were frozen in liquid nitrogen and stored at -70°C. After blood sampling, animals were euthanised by severing the spinal cord and brain pithing. Gills and hearts were perfused with notothenioid Ringer (in mM: 260 NaCl, 2.5 $MgCl_2$, 5 KCl, 2.5 $NaHCO_3$, 5 NaH_2PO_4, at pH 8.0) and tissues were frozen at -70°C or fixed in 10% buffered formalin for 24 h and then transferred to 70% EtOH. Fixed tissues were shipped on ice and frozen tissues were shipped on dry ice, to The University of British Columbia (UBC), in Vancouver. All samples were collected opportunistically and in strict compliance with the guidelines of The Institutional Animal Care and Use Committee (IACUC Protocol no. 14-L-004, Ohio University).

Biochemical analysis of CA activity

Approximately 2 g of gill lamellae were homogenised (Polytron PT1200, Luzern, Switzerland) in 8 mL of assay buffer on ice (in mM: 225 mannitol, 75 sucrose, 10 TRIS base, and adjusted to pH 7.4 with 10% phosphoric acid). Differential centrifugation was at 4°C according to (Henry, 1988; Henry et al., 1993): i) 800 x g for 20 min; ii) 8500 x g for 20 min (Allegra 64R, Beckman Coulter, Brea, CA); iii) 100 000 x g for 90 min (Beckman L8-70M) to

produce a microsomal pellet containing plasma membranes and a supernatant containing the cytosolic fraction. Pellets were re-suspended in 3 mL of assay buffer, by vortexing and mild sonication (5 W for 3 s). Protein concentration was measured spectrophotometrically at 595 nm using the Bradford assay (Sigma B6916, St. Louis, MO) and bovine serum albumin standards (BioRad Quickstart 5000206; Hercules, CA).

The activity of CA in cellular fractions was measured using the electrometric ΔpH assay (Henry, 1991). Reactions were in 6 mL of assay buffer in a thermostatted vessel at 4°C using 100 µL CO_2 saturated water as a substrate. The reaction kinetics were assessed as the time for a 0.15 unit pH change, with a GK2401C electrode and PHM84 meter (Radiometer, Copenhagen, Denmark). Uncatalysed reaction rates (without sample addition) were subtracted from the enzymatic rates and absolute enzyme catalytic rates were calculated from the buffer curve of the assay buffer over the tested pH range (determined in separate titrations).

Membrane pellets were washed by an additional step of ultracentrifugation (100 000 x g for 90 min) and re-suspended in 3 mL of fresh buffer. Washed pellets were incubated with 1 I. U. phosphatidylinositol-specific phospholipase C (PI-PLC; Invitrogen P6466, Carlsbad, CA), an enzyme that cleaves the common glycosylphosphatidylinositol (GPI) membrane anchor, or with assay buffer as a control, for 90 min at 21°C. CA inhibition kinetics were assessed by: i) adding 0.005% sodium dodecyl sulfate (SDS) to the assay buffer; ii) titrations with 0.6-6 nM acetazolamide (Az) according to (Easson and Stedman, 1936; Dixon, 1953); and iii) adding 100 µL of plasma from either *C. gunnari* or *N. rossii* to the assay buffer. RBC lysates were produced from 50 µL packed RBCs from *N. rossii*, diluted 50-fold in distilled water and frozen in liquid nitrogen twice; CA activity was measured on 5 µL of lysate.

158 *Plasma characteristics*

159 Plasma protein concentration was measured in both species as described above. In
160 addition, Hb concentration in plasma samples from *N. rossii* was measured
161 spectrophotometrically at 540 nm using the cyanomethaemoglobin method with human Hb
162 dilutions as standards (Sigma H7379). The concentration of protein from Hb was then subtracted
163 from total protein concentration measured in the plasma. The plasma non-bicarbonate buffer
164 capacity (β_{plasma}) was measured with an automated titrator (TIM865, Radiometer, Copenhagen,
165 Denmark). Plasma aliquots of 200 µL were added to 4.5 mL of deionised water in a magnetically
166 stirred glass titration vessel (4°C) that was continuously sparged with N_2. All results represent
167 upward titrations from pH 4 to 9 with 0.01 M NaOH. The plasma non-bicarbonate buffer
168 capacity (β_{plasma}) was calculated from the change in pH that corresponded to individual steps of
169 base addition (10 µL) over the physiologically relevant pH range in notothenioids of pH 7.4 to
170 8.2 (Acierno et al., 1997). β_{plasma} was then calculated as the mean value over the tested pH range.
171 *Immunohistochemistry*

172 Localisation of Ca4 in the gills of *C. gunnari* and *N. rossii* was with a custom rabbit
173 polyclonal antibody raised against rainbow trout (*Oncorhynchus mykiss*) Ca4, which has been
174 described in detail (Gilmour et al., 2007) and has been successfully used in rainbow trout and
175 spiny dogfish (*Squalus acanthias*). The antigenic sequence (TRRTLPDERLTPFTFTGY)
176 corresponds to amino acids 57–74 of the rainbow trout Ca4 (GenBank AAR99330), which is
177 73% conserved in *N. corriceps*. The immunohistochemical results were later replicated using a
178 custom chicken polyclonal antibody raised against the Ca4 of three Chondrichthyes (*Squalus*
179 *acanthias*, DQ092628.1; *Rhincodon typus*, XM_020514262.1; *Callorhinchus milii*,
180 XP_007894777.1). The antigenic peptide sequence for *S. acanthias* Ca4 was

GSEHTIDGEQYPMELHIVH (aa125-144), and the sequence in the notothenioid Ca4 is 100% conserved. Other sections were immunolabeled with a rabbit anti-Ca2 antibody (ab191343, Abcam, Cambridge, UK); the cytosolic Ca2-like isoform in fishes was recently reclassified as Ca17 (Ferreira-Martins et al., 2016). Ca4 and Ca2 antibodies were tested by western blot analysis using cytosolic and microsomal fractions of gill homogenates from both species. Subsamples containing 20 µg of protein were separated by SDS-page using 10% polyacrylamide gels (with 4% stacking gel). Proteins were then wet-transferred onto 0.2 µm PVDF membranes (Immun Blot, BioRad), rinsed and air dried. Transfer was assessed using total protein staining with 0.5% Ponceau S in 1% acetic acid and then imaged. Blots were rinsed with TTBS (Tris Buffered Saline with 0.05% tween 20, pH 7.4) and blocked with 5% blotto in TTBS overnight at 4°C. Thereafter, one membrane was probed with a 1:1,000 dilution of the rtCa4 and the other with a 1:2,500 dilution of the Ca2 antibody, overnight at room temperature on a rotisserie (Lab QuakeII, Thermo). Protein size was determined using a Precision Plus Protein Dual Color ladder (BioRad 1610374). All membranes were rinsed three times with TTBS and incubated with a 1:25,000 dilution of a goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (HRP; Genscript Piscataway, NJ), for 1 h at room temperature. Finally membranes were rinsed with TTBS and proteins were visualised using a chemiluminescent HRP substrate (Clarity, BioRad). Images were acquired using the Azure C300 imaging system and provided software (Azure Biosystems, Dublin, CA).

To localise Ca4 and Ca17 in the gills, fixed tissues were stepwise dehydrated in EtOH, cleared in xylene and embedded in paraffin. Thin sections (5 µm) were cut on a microtome (Leica RM2500, Wetzlar, Germany) and mounted on aminopropylsilane (APS) coated microscope slides. A hydrophobic barrier (SuperPAP, Sigma) was created around the sections

that were incubated in a blocking buffer (BLØK, Millipore, Burlington, MA) for 15 min. Incubation with the primary antibody (rtCa4 or Ca2, 1:200) in blocking buffer was overnight at 4°C in a humidified chamber. Negative controls were incubated with blocking buffer alone, or with normal rabbit serum. Detection of the primary antibody was done with a goat anti-rabbit IgG conjugated to Alexa 488 (Jackson Immunoresearch, West Grove, PA). Sections were then rinsed three times with 0.1 M phosphate buffered saline (PBS) for 5, 10 and 15 min and incubated with secondary antibody in a humidified chamber for 1 h, at 37°C. DAPI was added to the second wash step to visualise cell nuclei. Coverslips were mounted with 1:1 PBS glycerol containing 0.1% NaN_3 and imaging was done with a fluorescence photomicroscope (Leica DM5500; Orca Flash 4, Hamamatsu, Japan).

Sequencing and expression of ca4

Total RNA was extracted from approximately 100 mg of gill and ventricle tissue in 1 mL of Trizol, following the manufacturer's protocol (Invitrogen 15596018, Carlsbad, CA). Ventricles were used as a control tissue, in which the presence of Ca4 has been confirmed in several teleost species (Georgalis et al., 2006; Alderman et al., 2016). Tissues were homogenised with a Bullet Blender 24 with ~10 zirconium oxide beads (Next Advance, Averill Park, NY). The resulting RNA samples were treated with DNAse I (Thermo Scientific EN0521, Waltham, MA). RNA concentrations were measured using a nanodrop ND-2000 spectrophotometer (Thermo Scientific). First strand cDNA was synthesised from 2 µg of RNA using a high capacity reverse transcription kit (Applied Biosystems 4368814, Foster City, CA) and the cDNA product was diluted three-fold with molecular grade DEPC treated deionised water (Invitrogen 46-2224).

Degenerate PCR primers were designed by aligning available fish *ca4* sequences using the Clustal Omega web service (http://www.clustal.org), and identifying conserved sections

among the sequences (primer sequences were F 5'-GGA GAG CAG TAY CCC ATG G-3' and R 5'-TGG GCT TCT CAA ACA MRG TCC-3'). PCR products (40 cycles; 94°C for 2 min, 94°C for 30 s, 72°C for 1 min) were purified on a 1% agarose gel with a 1 kb ladder. Sections of the gel containing the 323 bp PCR product were cut out of the gel and purified using a GeneJet gel extraction kit (Thermo Scientific K0691). Purified PCR products were ligated into Topo2.1 plasmids and transformed in One-Shot Topo10 competent cells (Invitrogen C404010) following the manufacturer's protocol. Plasmids were extracted from ten different bacterial colonies with a GeneJet MiniPrep plasmid kit (Thermo Scientific K0503). Purified plasmids were sequenced at the UBC Nucleic Acid and Protein Service core facility (NAPS, Vancouver, Canada).

Primers for real-time quantitative PCR (RT-qPCR) analysis were designed by aligning the obtained partial coding sequences (CDS) for *C. gunnari ca4a* with the *N. corriceps ca4a*-like mRNA sequence (XM_010775657.1). The generated primers were used on gill and ventricle tissues of both species (primer sequences were F 5'-GGG AAG CAG AGA AGT GTT GC -3' and R 5'-TTT CAG ACG CAG AGG GAG TT-3'). Primers for the *ef1α* control gene were those reported by Urschel and O'Brien (2008), designed for three notothenioid species, and all results are reported relative to the expression of *ef1α*. RT-qPCR amplifications were with the SybrGreen kit (Applied Biosystems 4309155) on a Biorad CFX96 RT-PCR Detection System (Hercules, CA) with the following cycling conditions: 40 cycles, 95°C for 10 min, 95°C for 15 s, 55°C for 1 min; melt curve over 65-95°C at 0.5°C s^{-1}. No-amplification controls (no reverse transcriptase in the cDNA synthesis reaction) were run for each sample and showed no detectable amplification. Standard curves were run on each plate by serially diluting (1:5) pooled sample cDNA with molecular grade water in five steps. Primer pair efficiencies were within 100-120% and $R^2 > 0.99$ for all samples. To confirm the identity of the amplified products, RT-qPCR products were

processed with a GeneJet PCR purification kit (Thermo Scientific K0701). The purified RT-qPCR products were cloned and plasmids were extracted as described above. Purified plasmids from ten colonies were sequenced using M13 forward and reverse primers (UBC NAPS).

Data analysis and statistics

All data were analysed in RStudio v1.1.383 (RStudioTeam, 2016) with R v3.4.1 (RCoreTeam, 2017) and figures were generated with the ggplot2 v.2.2.1 package (Wickham, 2009). Normality of distribution was tested with the Shapiro-Wilk test ($P < 0.05$) and by visually confirming the distribution of the residuals in quantile-quantile (q-q) plots (for dependent samples t-tests, normality was tested on the differences between dependent scores). Homogeneity of variances was tested with the Levene's test ($P < 0.05$). To assess the CA inhibition kinetics of Az, titrations were carried out according to Dixon (1953) and the inhibition constant k_i was calculated as the slope of:

$$\frac{I_o}{i} = \frac{k_i}{1 - i} + E_0$$

where I_0 is the concentration of inhibitor, E_0 is the concentration of free enzyme and i is the fractional inhibition of enzyme activity at a given inhibitor concentration (Easson and Stedman, 1936). The effects of SDS and plasma on CA activity were tested with a dependent samples t-test against control measurements without these inhibitors, and the results are expressed as % inhibition. The effects of washing and Az on CA activity were tested using dependent samples t-tests, and an independent samples t-test for the effect of PI-PLC (due to an imbalance in replicates). Differences in relative gene expression between tissues, and plasma protein concentration between species, were tested using an independent samples t-test ($P < 0.05$).

Differences in plasma buffering capacity between species were tested by linear regression analysis, and analysis of variance on the combined regression model (ANOVA, $P < 0.05$). All data are presented as means±s.e.m., with $N = 6$ unless indicated otherwise.

Results

Immunohistochemistry

The results for the immunohistochemical localisation of Ca4 and Ca17 protein in the gills of *C. gunnari* and *N. rossii* are shown in Figure 1. In *C. gunnari*, reactivity for Ca4 protein was observed as a clear ring, lining the entire blood space of the secondary lamellae (marked with *). This staining pattern was associated with the apical membrane of pillar cells and the basolateral membrane of lamellar epithelial cells (panel a). In contrast, in *N. rossii*, reactivity for the Ca4 antibody was a diffuse staining pattern associated with the intracellular space of lamellar pillar cells and epithelial cells and absent from the lamellar blood space (panel b). Nonetheless, reactivity for the cytosolic Ca2 antibody showed a similar pattern for both species, with staining confined to the cytosol of all lamellar cell types and RBCs that remained in un-perfused areas within the lamellae (marked with * in panels c and d). The specificity of the antibodies was confirmed by the western blotting results. Probing of immunoblots from *C. gunnari* microsomal pellets with the Ca4 antibody revealed one band at ~37.5 kDa that was not observed in the pellets of *N. rossii* or the supernatants of either species. The Ca2 antibody showed immunoreactivity against a band at ~25 kDa in the cytosolic fractions from both species, but not in the pellets.

Gene expression

Homology cloning yielded a CDS for *C. gunnari ca4a* of 323 bp (uploaded to Genbank: MG561387) that was blasted against the stickleback (*Gasterosteus aculeatus*) and cod (*Gadus morhua*) genomes from the Ensembl genome browser (http://www.ensembl.org). BLAST results returned a ~90% sequence homology with the *ca4a* gene in both stickleback and cod (E-values were $1e^{-29}$ and $4e^{-9}$, respectively). The CDS of *C. gunnari* codes for a deduced protein of 103

297 amino acids, most closely resembling Ca4 and sharing 95% identity with *N. corriceps* Ca4
298 (XM_010775657.1) and 67% with *O. mykiss* Ca4 (XP_021479942.1).

299 Control gene expression of *ef1α* did not differ between ventricles and gills of *C. gunnari*
300 ($P = 0.338$) or *N. rossii* ($P = 0.203$), and the expression of *ca4a* is reported relative to that of the
301 control gene in Figure 2. The relative expression of *ca4a* mRNA in the gills of *C. gunnari* was
302 not different from that of the ventricle ($P = 0.610$), a tissue in which *ca4* expression has been
303 reported in other teleosts (Georgalis et al., 2006; Alderman et al., 2016), and expression values
304 were comparable to the expression of the control gene. Likewise, in the ventricle of *N. rossii*,
305 *ca4a* was expressed at levels comparable to the control gene; however, expression in the gills
306 was significantly lower compared to the ventricle ($P = 0.044$).

307 *Biochemical analysis of CA activity*

308 All cellular fractions obtained by differential centrifugation of gill homogenates showed
309 significant CA activity. In both species, CA activity was highest in the supernatant containing the
310 cytosolic fraction, compared to microsomal pellets that contain plasma membranes, and averaged
311 over species values were 529 ± 50 and 99 ± 28 µmol H^+ mg^{-1} min^{-1}, respectively. The effects of
312 washing on microsomal CA activity for both notothenioids are shown in Figure 3. Washing
313 significantly increased CA activity in pellets of *C. gunnari* (when expressed per unit protein),
314 from 17 ± 1 to 109 ± 9 µmol H^+ mg^{-1} min^{-1} ($P < 0.001$). Whereas washing significantly
315 decreased CA activity in pellets of *N. rossi*, from 181 ± 27 to 31 ± 2 µmol H^+ mg^{-1} min^{-1} ($P =$
316 0.002). However, washing significantly reduced total CA activity in both species (when
317 expressed per volume of fraction) from 244 ± 17 to 149 ± 11 µmol H^+ mL^{-1} min^{-1} in *C. gunnari*
318 ($P < 0.001$), and from 453 ± 69 to 94 ± 9 µmol H^+ mL^{-1} min^{-1} in *N rossii* ($P = 0.003$).

To assess whether microsomal CA isoforms were membrane-bound by a GPI anchor, membrane pellets were incubated at 21°C for 90 min, in the absence (Ctrl) or presence or of PI-PLC. In the Ctrl incubations of both species, CA activities per unit of protein were reduced by about half compared to initial values (67 ± 9 and 15 ± 1 2 µmol H^+ mg^{-1} min^{-1} for *C. gunnari* and *N. rossii*, respectively). The effects of PI-PLC on microsomal CA activity of both notothenioids are shown in Figure 4. Treatment of *C. gunnari* pellets with PI-PLC significantly decreased CA activity compared to Ctrl values, to 20 ± 1.5 µmol H^+ mg^{-1} min^{-1} (P = 0.031), and a corresponding increase was observed in the CA activity of the supernatant (P = 0.002). Likewise, a significant effect of PI-PLC was detected on CA activity in pellets of *N. rossii* that decreased to 10 ± 1 µmol H^+ mg^{-1} min^{-1} (P < 0.001); however, no significant change in CA activity was observed in the supernatant (P = 0.450).

Figure 5 shows the inhibitory effect of SDS, a surfactant, on the CA activity in cellular fractions of both notothenioids. In the pellets of *C. gunnari*, CA activity was unaffected by SDS (0.7 ± 2.5% inhibition; P = 0.813), whereas CA activity in the supernatant was significantly inhibited by 55.1 ± 6.6% (P = 0.003). In contrast, in *N. rossii*, SDS significantly inhibited CA activity in the pellets by 38.5 ± 4.4% (P = 0.003), and in the supernatant by 44.8 ± 3.9% (P = 0.001). In addition, titrations with increasing concentrations of Az resulted in inhibition constants (k_i) that were significantly different (P = 0.032) between CA isoforms derived from microsomal pellets or supernatants of *C. gunnari* gills; and the average k_i were 0.74 ± 0.11 and 1.18 ± 0.13 nM, respectively.

As expected, RBC lysates from *N. rossii* had a high CA activity of, on average, 39 ± 1 µmol H^+ mg^{-1} min^{-1} (despite the high non-CA protein content of this fraction) and the inhibitory effects of 100 µL of plasma from either species are shown in Figure 6. The addition of plasma

342 from *N. rossii* significantly inhibited CA activity in the RBC lysate, by 92.3 ± 2.8% ($P < 0.001$).
343 Likewise, the addition of plasma from *C. gunnari* significantly inhibited CA activity in the RBC
344 lysates of *N. rossii*, by 81.7 ± 3.7% ($P < 0.001$). However, the CA activity in microsomal pellets
345 of *C. gunnari* gills was unaffected in the presence of endogenous plasma (0.9 ± 1.1% inhibition;
346 $P = 0.809$); whereas, CA activity in pellets of *N. rossii* was significantly inhibited in the presence
347 of endogenous plasma, by 77.2 ± 4.3% ($P < 0.001$). In both species, CA activity in the
348 supernatant was significantly inhibited by the addition of endogenous plasma; by 73.1 ± 6.3% in
349 *C. gunnari* ($P < 0.001$) and by 90.2 ± 3.5% in *N. rossii* ($P < 0.001$).
350 *Plasma characteristics*
351 Plasma protein concentration was significantly higher ($P = 0.001$) in *C. gunnari*
352 compared to *N. rossii* (18.3 ± 1.4 and 10.8 ± 0.3 mg mL^{-1}, respectively). Figure 7 shows β_{plasma}
353 for both notothenioids over the physiological pH range. A significant species effect in the
354 regression analysis indicated that β_{plasma} of *C. gunnari* was significantly ($P < 0.001$) higher than
355 that of *N. rossii*, and average values were 5.19 ± 0.26 and 4.32 ± 0.15 mmol L pH^{-1}, respectively.
356 Plasma protein concentrations in *N. rossii* were corrected for Hb protein from RBC lysis. Hb
357 concentration was typically low in all samples and close to the detection limit of the assay (25 µg
358 mL^{-1}). However, a single sample had an elevated Hb concentration of 5.49 mg mL^{-1}, and this
359 sample was excluded from the analysis of β_{plasma} (open symbols in Fig. 7).

Discussion

Taking advantage of the natural Hb-knockout model provided by Antarctic icefishes, we tested the hypothesis that, in the absence of RBC CA, icefish gills express a paCA isoform that can provide the catalytic activity necessary for CO_2 excretion. To determine the cellular orientation of the putatively plasma-accessible Ca4 isoform in the gills of notothenioids (Tufts et al., 2002), gill sections of the icefish *C. gunnari* and the red-blooded *N. rossii* were immuno-labelled with an antibody raised against rainbow trout Ca4 (Gilmour et al., 2007). In gills of *C. gunnari*, a clear immunohistochemical signal (Fig. 1a) placed Ca4 protein in association with the apical plasma membranes of pillar cells and the basolateral membrane of some lamellar epithelial cells. Thus, Ca4 appears to line the entire lamellar blood space (marked with *), a pattern that is consistent with a plasma-accessible orientation of the enzyme, and which has not been observed previously in a teleost. A similar pattern has been described in the gills of dogfish, an elasmobranch (Gilmour et al., 2007), where the presence of Ca4 has been linked to functional measurements that infer a role of the enzyme in CO_2 excretion (Gilmour et al., 2001). Western analysis revealed a Ca4 protein of ~37.5 kDa in *C. gunnari* that matches closely the size of dogfish Ca4, of ~40 kDa (Gilmour et al., 2007). Our immunohistochemical finding was corroborated by the pattern of gene expression in the gills of *C. gunnari*, where expression of *ca4a* was detected at high levels, comparable to those in the ventricle (Fig. 2). This is unlike the situation in other teleosts, such as rainbow trout, where *ca4* is expressed in the ventricle but not in the gills (Georgalis et al., 2006). Surprisingly, the gills of *N. rossii* also showed detectable expression of *ca4a*, albeit at a significantly lower level compared to those in the ventricle (Fig. 2), and without a corresponding immunohistochemical signal. Reactivity for the Ca4 antibody was clearly absent in the lamellar blood space of *N. rossii* (marked with *; Fig. 1b), but some

intracellular reactivity was detected. These immunohistochemical results were later confirmed using a second antibody, raised against Ca4 in three Chondrichthyes, for which the antigenic peptide sequence of the notothenioid Ca4 was 100% conserved (data not shown). It is possible that the low expression of *ca4a* mRNA in gills of *N. rossii* is translated into a small pool of protein that is anchored to intracellular membranes and does not undergo the post-translational modifications required for export to a plasma-accessible location (Waheed et al., 1996). This issue was clarified by the biochemical characterisation of this CA pool as follows.

To characterise the CA-isoform distribution in gills of *C. gunnari* and *N. rossii*, gill homogenates were fractionated by differential centrifugation and CA activity was measured in the supernatant, comprising the cytosolic fraction, and in microsomal pellets that contain plasma membranes. In both species CA activity was highest in the supernatant, compared to microsomal pellets (averaged over species 529±50 and 99±28 µmol H^+ mg^{-1} min^{-1}, respectively). This result is in line with immunohistochemical data showing reactivity for a soluble Ca17 protein in the gills of both species that is clearly confined to the cytosol of both pillar- and lamellar epithelial cells, although more abundant in the latter (Fig. 1c and d). This prevalence of cytosolic over membrane-associated CA activity is consistent with previous findings on the CA isoform distribution in the gills of notothenioids (Maffia et al., 2001) and other fish species (Harter and Brauner, 2017) and highlights the importance of this CA pool for iono- and acid-base regulation and the sensing of CO_2 and pH in neuro-epithelial cells (for review see Gilmour, 2012). However, this soluble cytoplasmic CA is not plasma-accessible and thus cannot participate in plasma HCO_3^- dehydration and CO_2 excretion.

Membrane-bound Ca4 isoforms were identified by using four common biochemical markers: i) resistance to washing of microsomal pellets, ii) liberation of CA by PI-PLC, iii)

406 resistance to SDS and iv) resistance to plasma CA inhibitors. Washing significantly reduced the
407 CA activity in the pellets of *N. rossii*, but, when expressed per unit of protein, washing increased
408 CA activity in the pellets of *C. gunnari* (Fig. 3). This was likely due to the washout of non-CA
409 proteins from the microsomal fraction, and washing significantly reduced total CA activity in the
410 pellets of both species. Importantly, after washing, the pellets of *C. gunnari* retained a three-fold
411 higher CA activity, compared to *N. rossii* (109±9 and 31±2 μmol H^+ mg^{-1} min^{-1}, respectively).
412 PI-PLC treatment significantly reduced CA activity in the microsomal pellet of *C. gunnari*,
413 releasing CA activity into the supernatant (Fig. 4); this is a clear indication for the presence of a
414 GPI membrane-bound Ca4 and/or Ca15 isoform in the icefish. A statistically significant, but
415 numerically small effect of PI-PLC was also detected on CA activity in the pellets of *N. rossii*,
416 however, without a corresponding increase in supernatant CA activity. This is in line with the
417 data of Tufts et al. (2002), who found a significant effect of PI-PLC on CA activity in
418 microsomal pellets of *C. aceratus* and *N. coriiceps*, while other studies have found no effect of
419 PI-PLC in non-notothenioid teleosts (Gilmour et al., 2001; Gilmour et al., 2002). In combination,
420 these results corroborate the finding of a CA isoform that is linked to membranes by a GPI
421 anchor in the gills of the icefishes *C. gunnari* and *C. aceratus*, and provide equivocal indications
422 for the presence of a similar, but perhaps less abundant, isoform in red-blooded notothenioids,
423 that may be restricted to intracellular membranes or may be associated with epithelial cells; and
424 thus, is membrane-associated, but not plasma-accessible.
425 To further determine whether the observed CA activity in the gills of *C. gunnari* was
426 derived from Ca4 protein, microsomal pellets were treated with SDS. Mammalian studies show
427 that CA4 isoforms have two additional disulfide bonds that stabilise the enzyme against
428 denaturation by SDS (Waheed et al., 1996) and thus, SDS-resistant CA activity is often

described as Ca4-like in fishes and other non-mammalian vertebrates (Gervais and Tufts, 1998; Gilmour et al., 2002; Stabenau and Heming, 2003; Gilmour et al., 2007; Esbaugh et al., 2009). CA activity in the pellet of *C. gunnari* was unaffected by SDS (Fig. 5), while CA activity was significantly reduced in pellets of *N. rossii* (by 38.5±4.4%). As expected, cytosolic CA activity in the supernatant of *C. gunnari* and *N. rossii*, which are typically SDS-sensitive, soluble CA isoforms, was significantly reduced in the presence of SDS (by 55.1±6.6 and 44.8±3.9%, respectively). These findings corroborate previous data that indicate Ca4-like enzyme activity in gill membranes of the icefish *C. aceratus*, but not in those of *N. coriiceps* or in the supernatants of either species (Tufts et al., 2002).

The inhibition characteristics for Az, a common sulfonamide CA inhibitor are well studied in mammals and allow further differentiation among CA isoforms (Baird et al., 1997). Tufts et al. (2002) found no difference between the inhibition constant (k_i) for Az in pellets and supernatants of *C. aceratus*, indicating similar CA isoforms in both fractions. However, here, in *C. gunnari*, the k_i for Az was 0.74±0.11 nM in gill microsomal pellets, compared to 1.18±0.13 nM in the supernatant; a significant difference, indicating that different CA isoforms are present in the two fractions. The discrepancy with previous data may indicate the presence of two isoforms with similar k_i in *C. aceratus*, or perhaps that a low number of replicates in the earlier study (N = 4; Tufts et al., 2002), was insufficient to resolve the small numerical difference observed here.

An intriguing finding was the discovery of a CA inhibitor in the plasma of the icefish, *C. gunnari*. In fact, the CA activity in RBC lysates from *N. rossii* was significantly reduced in the presence of 100 µl of plasma from either *N. rossii* (by 92.3±2.8%; Fig. 6) or *C. gunnari* (by 81.7±3.7%), providing strong evidence that both species possess a CA inhibitor in their plasma.

452 The putative role of plasma CA inhibitors is to either inactivate or recycle CA from RBC lysis
453 (Henry and Heming, 1998), but neither role would be relevant for icefishes that largely lack
454 RBCs. A plasma CA inhibitor has also been described in the icefish *C. aceratus* (Tufts et al.,
455 2002) and because the phylogenetic distance between *C. aceratus* and *C. gunnari* spans nearly
456 the entire clade of Channichthyidae (Near et al., 2003) it is plausible that plasma inhibitors of CA
457 are present in all icefishes. Whether the plasma CA inhibitor in icefishes is an evolutionary relic
458 from a red-blooded ancestry, or whether its role should include the scavenging of cytoplasmic
459 CA shed by the lysis of other cell types, remains unclear. Regardless, the presence of an
460 endogenous plasma CA inhibitor can be used as a powerful diagnostic for Ca4 that, in mammals,
461 is largely unaffected by the inhibitor, and this safeguards its function in plasma-accessible
462 locations (Hill, 1986; Heming et al., 1993). A critical finding, thus, was that CA activity in
463 pellets of *C. gunnari* was unaffected by the presence of endogenous plasma (Fig. 6), whereas CA
464 activity in pellets of *N. rossii* was significantly inhibited by 77.2±4.3%. Further, the supernatant
465 of both species was significantly inhibited by plasma addition (in *C. gunnari* by 73.1±6.3% and
466 in *N. rossii* by 90.2±3.5%). Thus, CA activity in membranes of *C. gunnari* displays Ca4-like
467 characteristics that are not seen in membranes of *N. rossii* or in those fractions containing soluble
468 CA isoforms.

469 Four biochemical criteria are commonly used to characterise membrane-bound Ca4: i)
470 resistance to washing of pellets, ii) liberation by PI-PLC, iii) resistance to SDS and iv) resistance
471 to plasma CA inhibitors. CA activity in the pellets of *C. gunnari* conformed to all four criteria
472 and this was supported by the expression of *ca4a* mRNA at the gills and the
473 immunohistochemical detection of Ca4 protein of the predicted size, in a subcellular location that
474 indicates a plasma-accessible orientation. CA activity in the pellets of *N. rossii* was largely

removed by washing and inhibited by SDS. A significant effect of PI-PLC and low levels of *ca4a* expression may indicate the presence of some Ca4 protein that appears to be localised to intracellular membranes. Regardless of the isoform identity, the fact that microsomal CA activity in *N. rossii* was susceptible to the plasma CA inhibitor prohibits this CA pool from participating in HCO_3^- dehydration in the plasma. In combination, these data support the hypothesis that *C. gunnari* possess plasma-accessible Ca4 at the gills that should catalyse CO_2 excretion, while gills of *N. rossii* appear to lack a CA pool that could participate in this role. Thus, in the absence of RBC CA, icefish may be the only adult vertebrate in which CO_2 excretion is driven exclusively by the paCA activity provided by the gill.

Why most other teleosts lack paCA activity at the gills, despite its potential benefit for CO_2 excretion is still debated. One powerful argument relates to the evolution of highly pH-sensitive Hbs that required the active regulation of RBC intracellular pH to safeguard branchial O_2 uptake during a blood acidosis (Nikinmaa et al., 1984; Berenbrink et al., 2005); this protective mechanism requires an absence of CA activity in the plasma (Jacobs and Stewart, 1942; Motais et al., 1989; Rummer and Brauner, 2011). If the presence of paCA at the teleost gill was functionally constrained by the characteristics of teleost Hb and RBC function, perhaps these constraints were released in icefishes, which lack both. Assessing the presence of paCA in the gills of the closest red-blooded relatives of the Channichthyidae (the Bathydraconidae; Near et al., 2004) and confirming the absence of paCA in other notothenioid families, would strengthen the functional link between the loss of Hb and the expression of paCA. In addition, teleost plasma is an unfavourable medium to support high CA activities, mainly due to its low buffer capacity (β_{plasma}), as HCO_3^- dehydration requires equimolar amounts of H^+ (Bidani and Heming, 1991; Gilmour et al., 2002; Szebedinszky and Gilmour, 2002). In the presence of RBCs

498 with fast Cl^-/HCO_3^- exchange, an abundant pool of CA and buffers on Hb, paCA activity may be
499 largely inconsequential for CO_2 excretion in teleosts (Desforges et al., 2001). Notably, it is those
500 fishes with the highest β_{plasma} that also have paCA activity; conditions that, to varying degrees,
501 contribute to CO_2 excretion in *Squalus acanthias* (Lenfant and Johansen, 1966; Graham et al.,
502 1990; Gilmour et al., 2001) and *Eptatretus stoutii* (Esbaugh et al., 2009).
503 The β_{plasma} in *C. gunnari* was 5.19±0.26 mmol L pH^{-1} and significantly higher compared
504 to that in *N. rossii*, of 4.32±0.15 mmol L pH^{-1} (Fig. 7). Previous studies that measured β_{plasma} in
505 other icefish species over the same pH range reported 3.4±0.2 mmol L pH^{-1} in *Pagetopsis*
506 *macropterus* (Wells et al., 1988) and 9.7±0.9 mM L pH^{-1} in *Chionodraco hamatus* (Acierno et
507 al., 1997). While these values vary largely between studies and species, the β_{plasma} reported here
508 exceed, by about two-fold, typical teleost values (2-3 mmol L pH^{-1}; Tufts and Perry, 1998),
509 perhaps with the exception of some catfishes (Cameron and Kormanik, 1982; Szebedinszky and
510 Gilmour, 2002). Plasma proteins in *Channichthys rhinoceratus*, another icefish, are rich in
511 imidazole-based histidines, a residue capable of reversibly binding H^+, which likely contribute to
512 the high β_{plasma} in this species (Feller et al., 1994). Similarly, histidine-rich proteins (in this case
513 albumins) appear to underlie the unusually high β_{plasma} in *Ameiurus nebulosus*, a catfish
514 (Szebedinszky and Gilmour, 2002). And in fact, plasma protein concentration in *C. gunnari* was
515 18.3±1.4 mg mL^{-1} and significantly higher compared to that in *N. rossii*, of 10.8±0.3 mg mL^{-1}; a
516 finding that may correlate with the higher β_{plasma} in the icefish. The protein concentration in *N.*
517 *rossii* plasma conforms with the range typically reported in teleosts (Acierno et al., 1997),
518 however values in *C. gunnari* are lower compared to other icefishes studied (Egginton, 1994;
519 Acierno et al., 1997; Feller and Gerday, 1997); the reason for this discrepancy is unknown.

Due to the large blood volume of icefishes, about 7.6% of body weight (Hemmingsen and Douglas, 1970) compared to 2-3% in other teleosts (Thorson, 1961; Houston and DeWilde, 1969), and their low Hct (< 1% compared to > 25% in other teleosts; Holeton, 1970), the total volume of plasma in icefishes is at least three-times higher than in most teleosts (Feller et al., 1994). The plasma of red-blooded teleosts contributes 20-40% to whole blood buffer capacity, typically < 10 mmol L pH^{-1}, which is largely determined by the buffer capacity of Hb (Wood et al., 1982; Tufts and Perry, 1998; Gilmour et al., 2002; Szebedinszky and Gilmour, 2002). Although the measured β_{plasma} in *C. gunnari* is only half that of typical teleost whole blood, this is clearly overcompensated by the three-fold higher plasma volume of icefishes. Thus, per unit of animal mass, icefishes have a greater capacity to buffer metabolically produced H^+ in their blood compared to most teleosts, despite lacking Hb. In combination with a low metabolic rate (Hemmingsen et al., 1969), hence a lower release of CO_2 and H^+ to the plasma, β_{plasma} in icefishes would seem adequate to sustain arterial-venous pH homeostasis and HCO_3^- dehydration (and this is supported by experimental data; Hemmingsen and Douglas, 1972), which is catalysed by the paCa4 isoform at the gill.

The evolutionary time-course over which RBCs were lost from the circulation in the common ancestor of Channichthyidae, and whether this coincided with the loss of transcriptionally active Hb genes, is presently unknown. However, Hb is the largest H^+ buffer within the RBC cytosol (in teleosts largely through the Bohr-Haldane effect) and the absence of Hb will have severely restricted the functional significance of RBC CA. Thus, the time-course over which icefishes had to acquire paCA at the gill, to compensate for the reduction of RBC CA function, may have corresponded closely to the loss of Hb. The molecular mechanism by which icefishes catalyse HCO_3^- dehydration in the plasma is analogous to that in all other non-teleost

vertebrates, where CA4 is GPI-anchored to the apical membrane at the gas exchange organs. Therefore, it seems likely that paCA was never "lost" at the teleost gill, but functional constraints related to the pH-sensitivity of teleost Hb prevented a significant expression of the trait, until the loss of Hb in icefishes simultaneously released functional constrains and created a need to catalyse HCO_3^- dehydration in the plasma. Possible scenarios may include: i) natural selection favoured phenotypes with higher paCA activity at the gill, which requires that there was standing variation in this trait in the common ancestor of Channichthyidae; ii) phenotypic plasticity induced an up-regulation of *ca4a* gene expression at the icefish gill, which may be supported by the presence of the transcript in *N. rossii*; or iii) neoteny allowed for branchial paCA to be retained throughout icefish ontogeny, a mechanism that underlies other adult characters in notothenioids (Montgomery and Clements, 2000), and which would place branchial paCA as an embryonic trait in teleosts; a scenario that could be tested experimentally.

In conclusion, the natural knockout of Hb in Antarctic icefishes had profound consequences for cardiovascular O_2 transport and resulted in fascinating adaptations that compensate for the reduction in O_2-carrying capacity of the blood. In addition, results from the present study show that the reduction of RBCs and the associated loss of CA catalytic activity in the blood of icefishes led to a divergent strategy of CO_2 excretion. While paCA is functionally absent at the gills of teleosts, icefishes may have re-acquired this trait, and unlike the situation in any other vertebrate studied to date, in icefishes, the CA catalytic activity required for CO_2 excretion is provided exclusively by the gills. Therefore, the study of Antarctic icefishes may reveal a previously unidentified evolutionary plasticity in the vertebrate CO_2 excretion pathway and perhaps provide a framework to address more general questions on the evolutionary dynamics of vertebrate gas exchange.

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Figure Legends

Figure 1 Immunohistochemical localisation of membrane-bound Ca4 and cytosolic Ca17 protein in the gills of the icefish *C. gunnari* and the red-blooded *N. rossii*. All CA antibodies were labelled with a green secondary antibody and nuclei were stained blue with DAPI (4',6'-diamidino-2-phenylindole). In *C. gunnari*, immunoreactivity for Ca4 resulted in a circular staining pattern associated with the apical membrane of pillar cells, lining the entire lamellar blood space (marked with * in panel a), consistent with a plasma-accessible orientation of the enzyme (white scale bar = 25 µm). No immunoreactivity for Ca4 was detected in the lamellar blood space of *N. rossii* gills (marked with * in panel b), but some intracellular reactivity was detected that does not appear to be plasma-accessible. Ca17 protein was detected in the cytosol of pillar and epithelial cells of both notothenioid species (panels c and d). The lower panel (e) shows representative western blots for crude gill homogenates (H), supernatants (S, cytosolic fraction) and pellets (P, membranes fraction), obtained by differential centrifugation from two individuals of *C. gunnari* and *N. rossii*, respectively. Probing with the Ca4 antibody produced a strong band at ~37.5 kDa in the pellets of *C. gunnari* that was not observed in the supernatants or in any fraction of *N. rossii*. Probing for Ca17 protein produced bands at ~25 kDa in the supernatants but not the pellets, of both species.

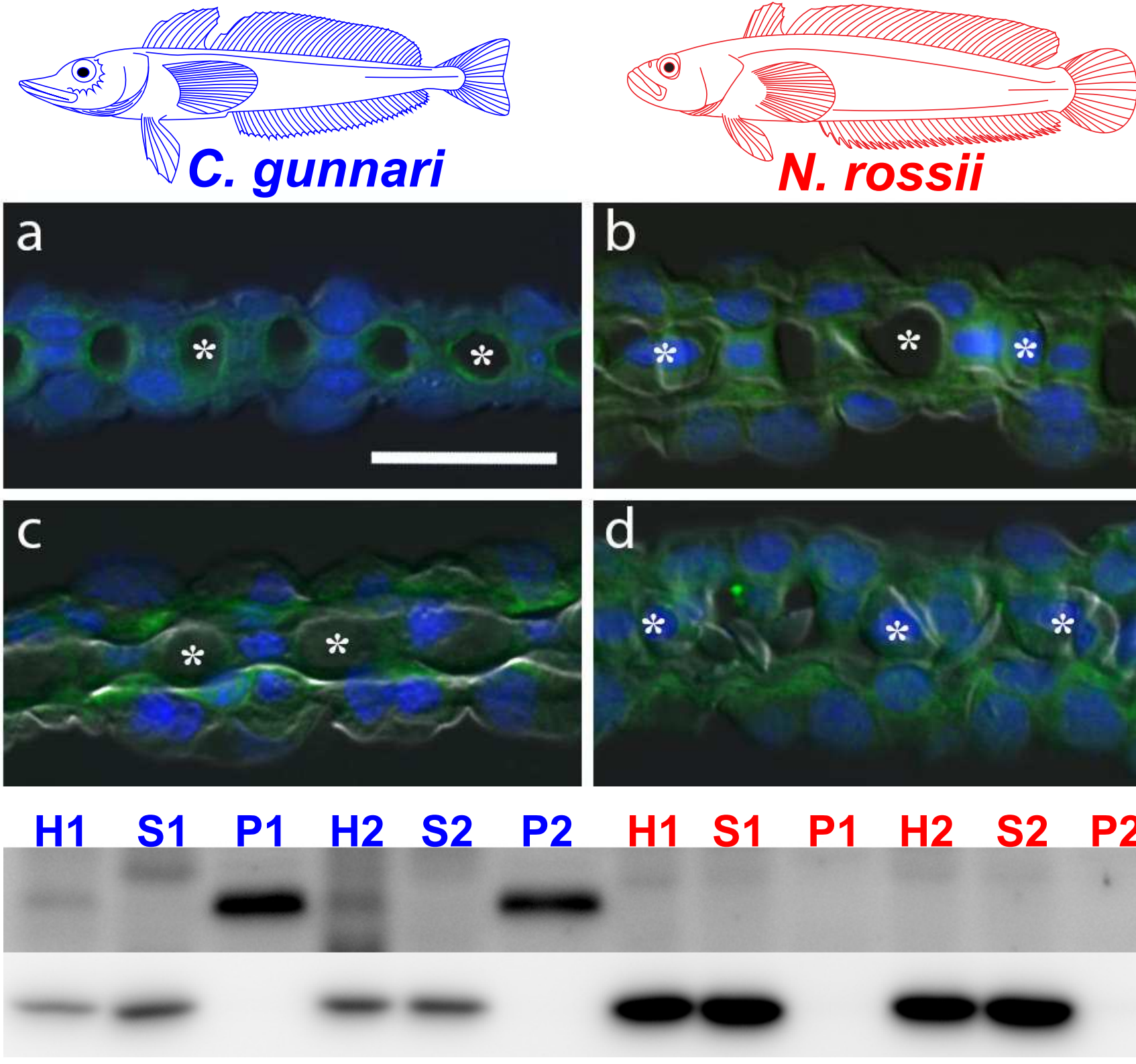
Figure 2 Relative expression of *ca4a* mRNA in ventricle and gill homogenates of *N. rossii* and *C. gunnari*. Measurements were by real-time quantitative PCR (RT-qPCR) and all expression levels are standardised to that of *ef1α*. Differences in relative gene expression between tissues, were assessed with independent t-tests within species ($N = 5$, except for *C.*

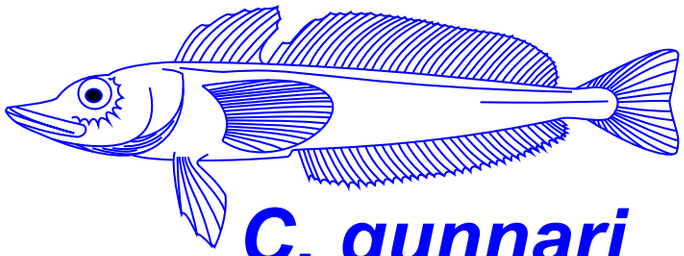
807 *gunnari* ventricle where $N = 2$; $P < 0.05$) and are indicated as: $P < 0.05$*; 0.01**; 0.001***; or
808 "ns" for non-significant. All data are mean±s.e.m..
809
810 Figure 3 Carbonic anhydrase (CA) activity (μmol H^+ mg $protein^{-1}$ min^{-1}) in the
811 microsomal fraction of gill homogenates from the icefish *C. gunnari* and the red-blooded *N.*
812 *rossii*. Membrane pellets were obtained by differential centrifugation and a final step of
813 ultracentrifugation. Samples were measured before (Pre) and after (Post) a washing step with
814 fresh assay buffer and measurements were with the electrometric ΔpH assay (Henry, 1991). The
815 effects of washing on CA activity were assessed with a depend t-test within species ($P < 0.05$, N
816 = 6) and are indicated as: $P < 0.05$*; 0.01**; 0.001***; or "ns" for non-significant. All data are
817 mean±s.e.m..
818
819 Figure 4 Carbonic anhydrase (CA) activity (μmol H^+ mg $protein^{-1}$ min^{-1}) in cellular
820 fractions of gill homogenates from the icefish *C. gunnari* and the red-blooded *N. rossii*.
821 Membrane pellets and supernatants (Super) were obtained by differential centrifugation and a
822 final step of ultracentrifugation. Measurements were with the electrometric ΔpH assay (Henry,
823 1991) after incubation of samples with saline (Ctrl) or phosphatidylinositol-specific
824 phospholipase C (PI-PLC), an enzyme that cleaves the membrane anchors of CA4 and CA15.
825 The effect of PI-PLC on CA activity was assessed with an independent t-tests for each cellular
826 fraction within species ($P < 0.05$, $N = 6$, except for *C. gunnari* Ctrl where $N = 3$). Significant
827 differences are indicated as: $P < 0.05$*; 0.01**; 0.001***; or "ns" for non-significant. All data
828 are mean±s.e.m..
829

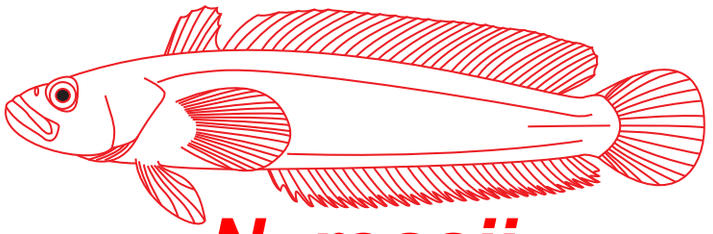
Figure 5 Inhibition of carbonic anhydrase (CA) activity (in %), by sodium dodecyl sulfate (SDS) in cellular fractions of gill homogenates from two notothenioid species, the icefish *C. gunnari* and the red-blooded *N. rossii*. Membrane pellets and supernatants (Super) were obtained by differential centrifugation and a final step of ultracentrifugation, and CA activity was measured with the electrometric ΔpH assay (Henry, 1991), in the absence (Ctrl) or presence of 0.005% SDS, a surfactant that inhibits CA activity, but is less potent for CA4 isoforms. The effect of SDS on CA activity was assessed with a dependent t-tests comparing Ctrl and SDS treated samples, for each cellular fraction within species ($P < 0.05$, $N = 6$). Inhibition of CA activity that is significantly different from zero is indicated as: $P < 0.05$*; 0.01**; 0.001***; or "ns" for non-significant. All data are mean±s.e.m..

Figure 6 Inhibition of carbonic anhydrase (CA) activity (in %), by blood plasma, in cellular fractions of gill homogenates from two notothenioid species, the icefish *C. gunnari* and the red-blooded *N. rossii*. Membrane pellets and supernatants (Super) were obtained by differential centrifugation and a final step of ultracentrifugation. Red blood cell (RBC) lysates were obtained from *N. rossii* and CA activity was measured with the electrometric ΔpH assay (Henry, 1991), in the absence (Ctrl) or presence of 100 µL plasma from either *N. rossii* or *C. gunnari*. The effect of plasma on CA activity was assessed with a dependent t-tests comparing Ctrl and plasma treated samples, for each cellular fraction within species ($P < 0.05$, $N = 6$). Inhibition of CA activity that is significantly different from zero is indicated as: $P < 0.05$*; 0.01**; 0.001***; or "ns" for non-significant. All data are mean±s.e.m..

Figure 7 Plasma buffer capacity (β_{plasma}; mmol H^+ L $sample^{-1}$ pH^{-1}) of two notothenioid species, the icefish *C. gunnari* (blue) and the red-blooded *N. rossii* (red). Measurements were performed with an automated titrator over a pH range of 7.4-8.2. Linear regression models were fitted to the data and 95% confidence intervals are indicated by shaded areas. Haemoglobin (Hb) concentration was measured in all samples from *N. rossii* and was low (around the detection limit of the assay; 25 µg mL^{-1}) except in one individual where 5.49 mg mL^{-1} Hb were detected; this individual was excluded from the analysis (open symbols). (B) Raw values of β_{plasma} are plotted for both species and the mean±s.e.m. are indicated by the error bars. Analysis of variance (ANOVA) detected a significant effect of species on β_{plasma} in the combined regression model (*N. rossii*, $N = 4$; *C. gunnari*, $N = 6$, $P < 0.001$ indicated as ***).


C. gunnari
N. rossii
a
b
c
d
Ca4
Ca17
(e)
H1
S1
P1
H2
S2
P2
H1
S1
P1
H2
S2
P2
Ca4
Ca17





C. gunnari

N. rossii

rel. *ca4a* expression (mRNA *ca4a ef1α*$^{-1}$)

2.0

1.5

1.0

0.5

0.0

ns

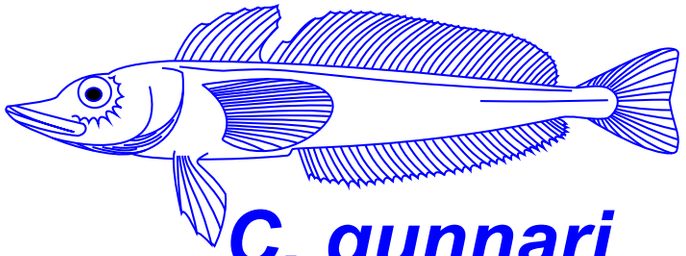
*

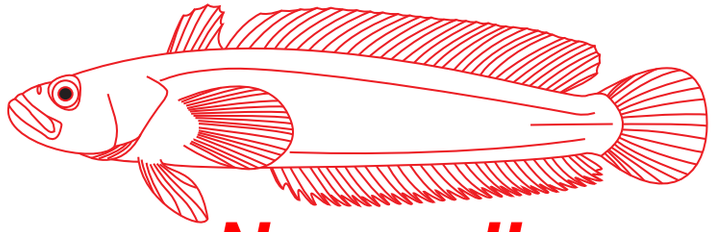
Ventricle

Gill

Ventricle

Gill





C. gunnari

N. rossii

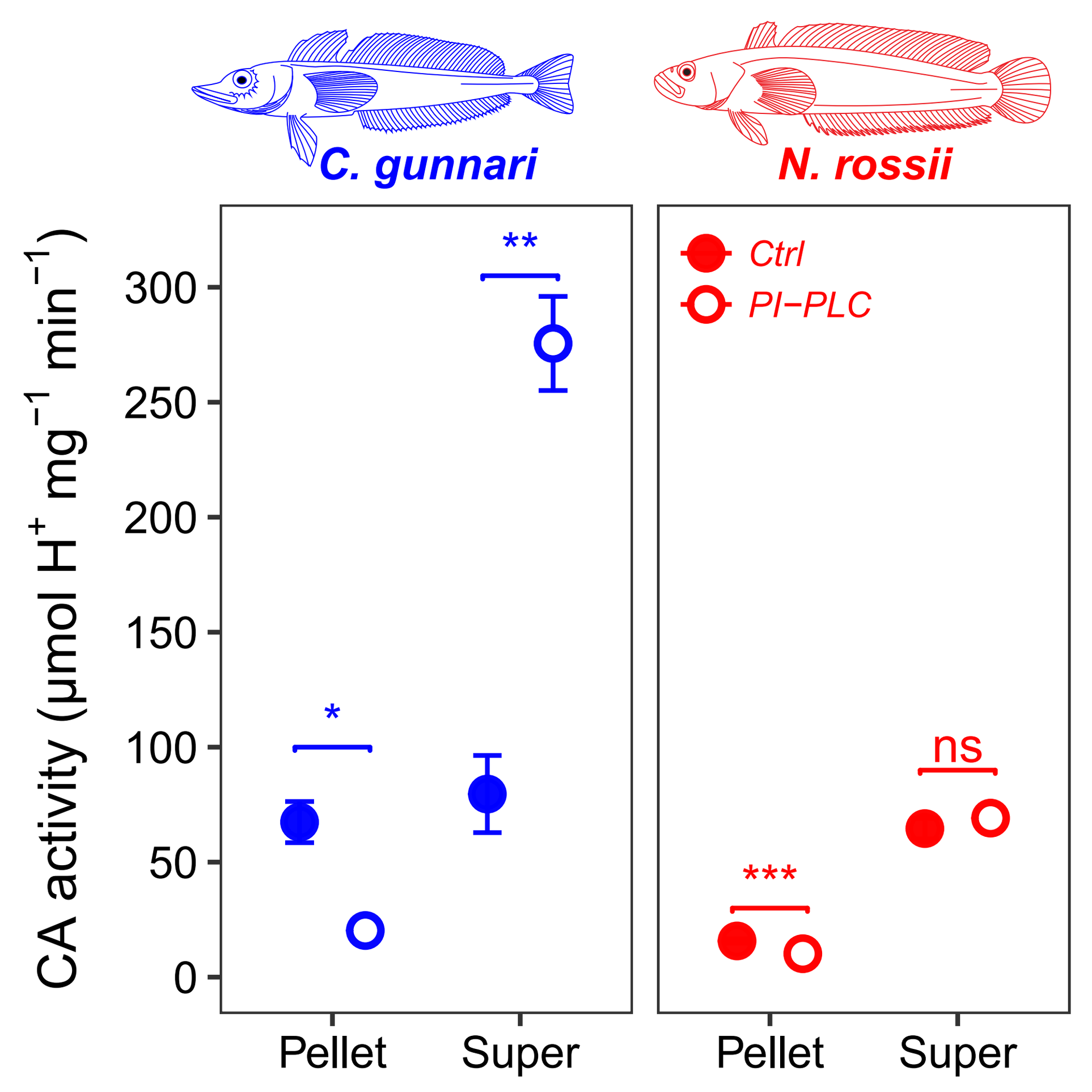
CA activity (μmol H^+ mg^{-1} min^{-1})

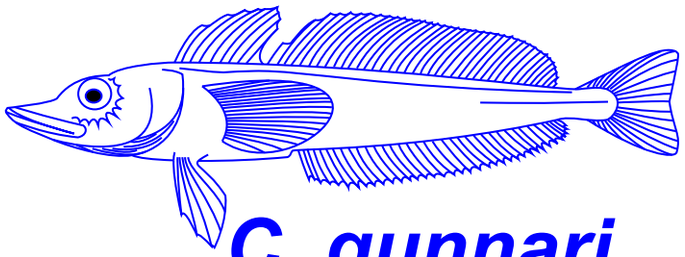
250
200
150
100
50
0

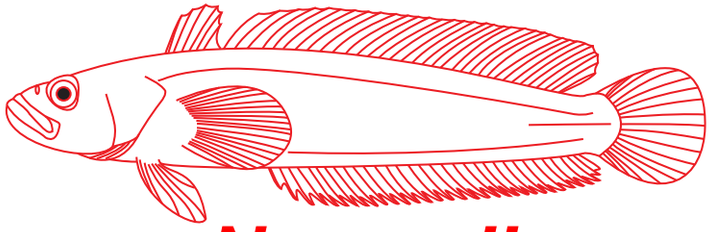
***

**

Pre
Post
Pre
Post


C. gunnari
N. rossii
CA activity (µmol H+ mg−1 min−1)
300
250
200
150
100
50
0
**
*
***
ns
Ctrl
PI−PLC
Pellet
Super
Pellet
Super





C. gunnari

N. rossii

inhibition by SDS (%)

100

80

60

40

20

0

ns

**

**

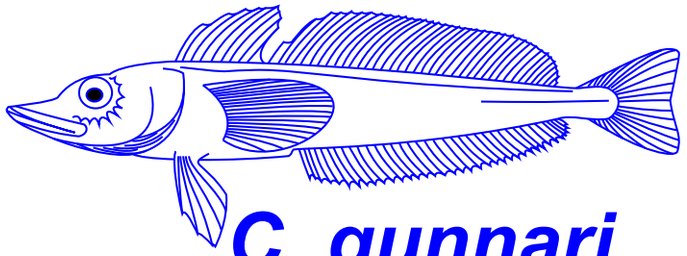
***

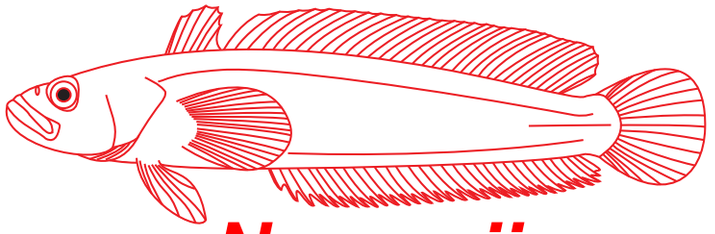
Pellet

Super

Pellet

Super





C. gunnari

N. rossii

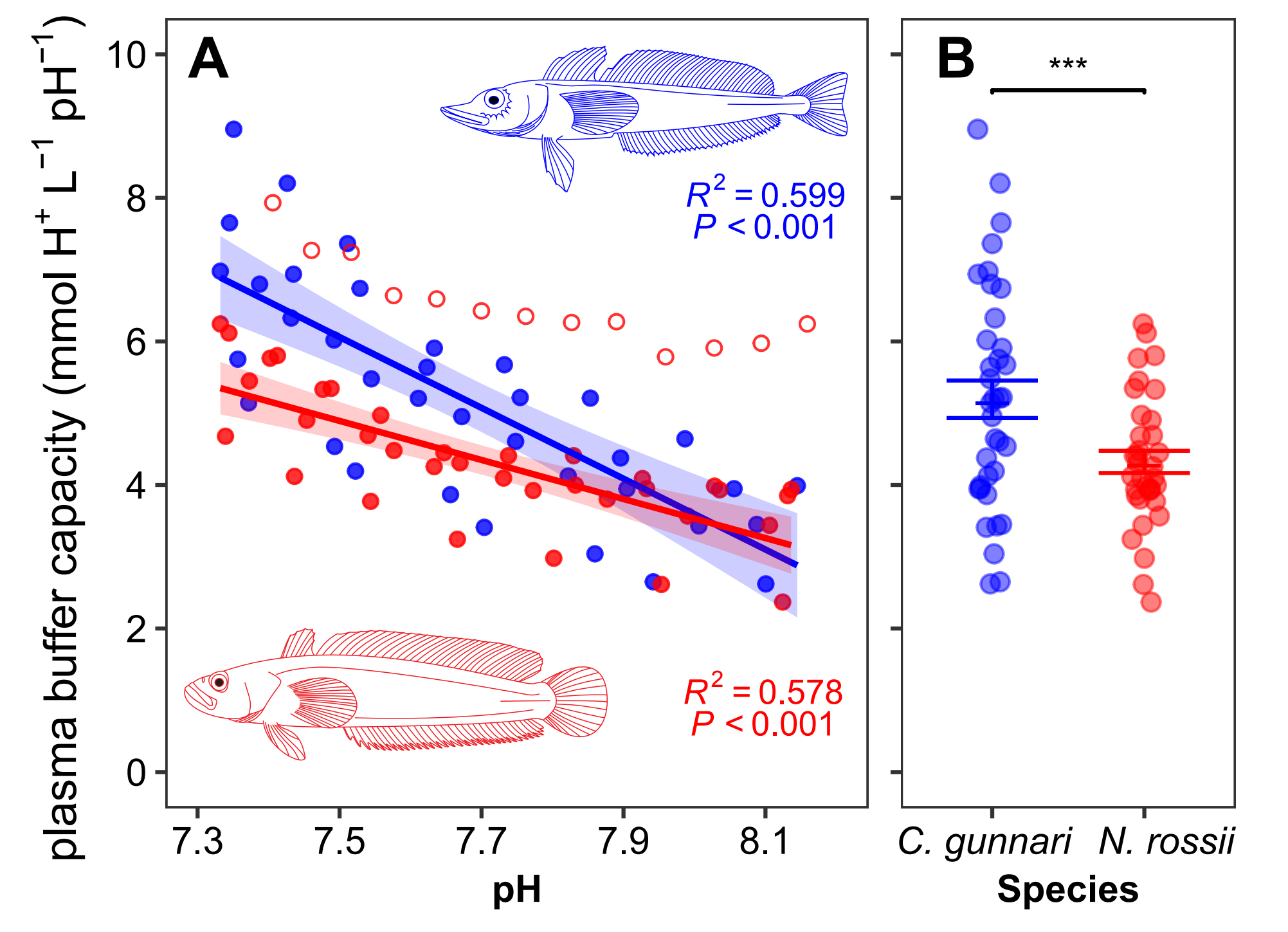
inhibition by plasma (%)

100
80
60
40
20
0

***
***
ns
***
***
***

RBC Pellet Super

RBC Pellet Super


A
B
plasma buffer capacity (mmol H+ L−1 pH−1)
10
8
6
4
2
0
7.3
7.5
7.7
7.9
8.1
pH
R2 = 0.599
P < 0.001
R2 = 0.578
P < 0.001
***
C. gunnari
N. rossii
Species