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RESEARCH ARTICLE

Carbonic anhydrase expression in the branchial ionocytes of rainbow trout

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ABSTRACT

Rainbow trout (*Oncorhynchus mykiss*) exposed to acid–base challenges activate branchial mechanisms for the excretion of acid–base equivalents. Current models of branchial acid–base excretion in freshwater rainbow trout propose two main ionocyte types: the peanut lectin agglutinin-positive (PNA^+) mitochondrion-rich cell or ionocyte is believed to secrete HCO_3^- in exchange for Cl^-, whereas H^+ secretion is thought to occur across PNA^- ionocytes in exchange for Na^+. Both HCO_3^- and H^+ are supplied by intracellular hydration of CO_2 catalysed by cytosolic carbonic anhydrase (CAc). Immunohistochemical approaches revealed that under control conditions, CAc was detectable in 92.3±1.0% (*N*=11) of PNA^- ionocytes, and the abundance of PNA^- ionocytes increased in response to systemic acidosis elicited by 72 h exposure to water of low pH (nominally pH 4.5), hypercapnia (1% CO_2, nominally 7.6 Torr) or hyperoxia (achieved by gassing water with pure O_2), as did the abundance of PNA^- ionocytes that exhibited immunofluorescence for CAc. However, just 4.3±0.6% (*N*=11) of PNA^+ ionocytes expressed detectable CAc under control conditions. Marked increases in the abundance of CAc-positive PNA^+ ionocytes were detected following exposure of trout to a base load via recovery from hypercapnia or base infusion (72 h infusion with 140 mmol l^{-1} $NaHCO_3$). The percentage of CAc-positive PNA^+ ionocytes also was increased in trout treated with cortisol (10 mg kg^{-1} hydrocortisone 21-hemisuccinate daily for 7 days). These results suggest that regulation of CA within PNA^+ ionocytes and/or the abundance of CAc-positive PNA^+ ionocytes plays a role in activating base secretion in response to systemic alkalosis.

KEY WORDS: *Oncorhynchus mykiss*, Gill, Ionic regulation, Acid–base, Cortisol

INTRODUCTION

Acid–base regulation in teleost fish relies primarily on the direct transfer of the acid–base equivalents H^+ and HCO_3^- across the gill (reviewed by Claiborne et al., 2002; Perry et al., 2003; Evans et al., 2005; Perry and Gilmour, 2006). For example, compensation for a systemic alkalosis is achieved by increasing branchial HCO_3^- excretion to lower plasma HCO_3^- levels (Goss and Wood, 1990b; Goss and Perry, 1994). In a systemic acidosis, in contrast, branchial net acid excretion is increased through increased H^+ excretion and/or reduced HCO_3^- excretion, resulting in the accumulation of HCO_3^- ions in the plasma (Hyde and Perry, 1989; Goss and Wood, 1991). Thus, systemic pH is regulated by adjusting plasma HCO_3^- levels through the differential regulation of branchial H^+ and HCO_3^- effluxes, with these effluxes being coupled to the influx of Na^+ and Cl^-, respectively (Claiborne et al., 2002; Perry et al., 2003; Evans et al., 2005). The exchange of H^+ for Na^+ and HCO_3^- for Cl^-, which functionally couple acid–base regulation in teleost fish with ionic regulation, are carried out by specialized ion-transporting cells or ionocytes.

Ionocytes are mitochondrion-rich cells of the fish gill epithelium that express a complement of ion transporters and channels that is specific to their function (reviewed by Hwang et al., 2011; Dymowska et al., 2012; Guh et al., 2015). Based on the handful of species in which ionocytes have to date been investigated, the number and function of ionocyte types is species specific (Dymowska et al., 2012; Hsu et al., 2014). Current models for rainbow trout (*Oncorhynchus mykiss*) posit two main ionocyte types that are distinguished by their ability to bind peanut lectin agglutinin (PNA; Goss et al., 2001; Galvez et al., 2002). The PNA^+ ionocyte, by analogy with the PNA-binding β-intercalated cell of the mammalian kidney, is proposed to be a base-secreting cell (Galvez et al., 2002). It is enriched in Na^+/K^+-ATPase (NKA), exhibits an extensive tubular network of basolateral membranes, and would be expected to express an apical Cl^-/HCO_3^- exchanger (Goss et al., 2001; Galvez et al., 2002), although the molecular identity and cellular localization of the Cl^- uptake/base excretion mechanism in rainbow trout have yet to be confirmed (Dymowska et al., 2012). The PNA^- ionocyte exhibits acid-stimulated Na^+ uptake (Reid et al., 2003). This ionocyte type expresses V-type H^+-ATPase that increases in abundance in cells isolated from trout exposed to hypercapnic acidosis (Galvez et al., 2002), and the acid-stimulated Na^+ uptake is bafilomycin sensitive, implicating the proton pump in the Na^+-uptake mechanism (Reid et al., 2003; Goss et al., 2011). Thus, the PNA^- ionocyte is a strong candidate for the acid-secreting cell of the trout gill epithelium.

If the PNA^+ and PNA^- ionocytes of the trout gill epithelium serve as base-secreting cells that take up Cl^- and acid-secreting cells that take up Na^+, respectively, then both ionocyte subtypes would be predicted to express carbonic anhydrase (CA) (reviewed by Perry and Gilmour, 2006; Gilmour and Perry, 2009). More specifically, these ionocytes would be predicted to express CAc, the cytoplasmic CA isoform that, in the branchial epithelium of trout, would catalyse the hydration of CO_2 that is diffusing across the gill to yield H^+ and HCO_3^- for use in acid–base compensation and NaCl uptake (Rahim et al., 1988; Esbaugh et al., 2005; reviewed by Gilmour and Perry, 2009). Although CA has been localized by immunohistochemistry to both ionocytes and pavement cells of the trout gill epithelium (Rahim et al., 1988; Georgalis et al., 2006), direct confirmation of CAc in PNA^+ and PNA^- ionocytes is lacking. The presence of CA in PNA^- ionocytes is supported by functional data, such as the reduction in branchial net acid excretion (Kerstetter et al., 1970; Georgalis et al., 2006) and reduction in Na^+ uptake (Kerstetter et al., 1970; Payan et al., 1975) detected in trout treated with the CA

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inhibitor acetazolamide, and the inhibition of both acid-stimulated Na^+ uptake and CA activity in PNA^- ionocytes from trout exposed to silver (Goss et al., 2011). Functional data implicating CA in Cl^- uptake and/or base excretion, the proposed functions of the PNA^+ ionocyte, appear to be limited to studies in which Cl^- uptake has been measured following CA inhibition and have yielded mixed results, with Cl^- uptake being reduced by inhibition of CA in larval (Boyle et al., 2015) but not adult (Kerstetter and Kirschner, 1972) trout. Thus, one objective of the present study was to localize CAc to PNA^- and PNA^+ ionocytes of the trout gill epithelium by immunohistochemistry.

Previous work has demonstrated a morphological component to acid–base responses in the trout gill (reviewed by Goss et al., 1995). In particular, the surface area of the gill epithelium occupied by ionocytes decreased in trout experiencing systemic acidosis elicited by exposure to hypercapnia or hyperoxia, and increased in trout recovering from hypercapnia or hypoxia or infused with $NaHCO_3$, conditions that cause systemic alkalosis (Goss and Perry, 1993, 1994; Goss et al., 1994). At the time these experiments were carried out, trout ionocytes were considered to be base-secreting cells responsible for Cl^- uptake (and called 'chloride cells'). Therefore, these morphological changes were consistent with requirements for compensatory responses to the acid–base disturbances because decreased chloride cell surface area would decrease HCO_3^- loss during acidosis while increased chloride cell surface area would increase base excretion during alkalosis (Goss et al., 1995). Puzzlingly, however, acid-infused trout also exhibited increased chloride cell surface area (Goss et al., 1994), a result that was inconsistent with the morphological model for acid–base regulation and led to the suggestion that 'chloride cells' represented a mixed population of acid- and base-secreting ionocytes (Goss et al., 1995). The subsequent identification of PNA^+ and PNA^- ionocytes suggests a need to revisit the morphological model for acid–base regulation. Thus, the second objective of the present study was to test the hypothesis that changes in the relative abundance of PNA^+ versus PNA^- ionocytes within the branchial epithelium contribute to acid–base responses to systemic acidosis and alkalosis. An immunohistochemical approach was used to quantify the relative abundance of PNA^- and PNA^+ ionocytes, as well as ionocytes expressing CAc, in rainbow trout subjected to acid–base disturbances. Treatment with cortisol, which increases the number of ionocytes without changing the proportion of PNA^- to PNA^+ cells (Goss et al., 2001) but does not cause an acid–base disturbance, was used to evaluate whether an acid–base disturbance is necessary to elicit changes in the abundance of CAc-expressing ionocytes.

MATERIALS AND METHODS

Experimental animals

Juvenile rainbow trout, *Oncorhynchus mykiss* (Walbaum 1792) (mass 174±5 g, mean±s.e.m., N=38), purchased from Linwood Acres Trout Farm (Campbellcroft, ON, Canada), were transported to the University of Ottawa where they were held in 1275 l fibreglass tanks. Holding tanks were supplied with flowing, aerated, dechloraminated city of Ottawa tap water ('system water'; 0.25 mmol l^{-1} Ca^{2+}, 0.78 mmol l^{-1} Na^+, 0.02 mmol l^{-1} K^+, 0.19 mmol l^{-1} Cl^-; pH 7.6) at 13°C. The photoperiod was maintained at 12 h light:12 h dark and fish were fed commercial trout pellets at a ration of 0.5% body mass per day. Trout were acclimated to these holding conditions for at least 2 weeks prior to experimentation. All experimental protocols were approved by the institutional animal care committee (protocol BL-2118) and were in compliance with the guidelines of the Canadian Council on Animal Care (CCAC) for the use of animals in research and teaching.

Experimental protocols

An immunohistochemical approach was used to quantify the abundance of PNA^- and PNA^+ ionocytes in the gills of rainbow trout exposed to control conditions, acid–base disturbances or exogenous cortisol. Ionocytes were identified on the basis of staining with MitoTracker Red CMXRos (MitoRos; Molecular Probes, ThermoFisher Scientific, Ottawa, ON, Canada), and biotin-conjugated PNA (Sigma-Aldrich, Oakville, ON, Canada) was used to distinguish between PNA^- and PNA^+ ionocytes (Galvez et al., 2002). The PNA^- and PNA^+ ionocytes were further classified as CAc positive or negative using a custom antibody raised against a synthetic peptide corresponding to amino acids 122–141 of trout CAc (Georgalis et al., 2006). The three experimental series that were used are detailed below; in all cases, MitoRos (30 nmol l^{-1}) was added to the water holding the fish for the final 6 h of a treatment period (Tzaneva et al., 2014), after which fish were killed by a cephalic blow and gill tissue was collected, fixed and processed for immunohistochemistry.

In the first experimental series, rainbow trout were exposed to conditions that elicit systemic acidosis. Rainbow trout randomly selected from the holding tank were placed in individual opaque 2.75 l experimental chambers (26.5 cm long×11 cm wide×11.5 cm deep) for a 24 h acclimation period prior to experimentation. Control and treated fish were held in static, aerated water, with water changes every 12 h during the acclimation (24 h) and experimental (72 h) periods, and the experimental chambers were held in a water bath supplied with 13°C water for temperature control. Control fish (N=4) were held for 72 h in aerated system water (pH 7.43±0.04, N=8). To elicit a metabolic acidosis [i.e. a systemic acidosis where the partial pressure of CO_2 (P_{CO_2}) in the blood remains constant], trout were exposed to water of low pH using the approach described by Wood et al. (1999). These fish (N=3) were held for 72 h in aerated system water that was prepared in advance by titration to pH 4.5 with HCl and vigorous aeration to ensure normocapnia (measured pH 4.40±0.03, N=18). Water pH was re-measured immediately prior to each water change and averaged 6.09±0.09 (N=18), reflecting the alkalinizing influence of the fish (Wood et al., 1999). Addition of HCl to lower water pH would have increased water [Cl^-]. However, because water [Cl^-] under control conditions was above the K_m for chloride transport (135 µequiv l^{-1}; Goss and Wood, 1991) and chloride influx decreases during metabolic acidosis (Goss and Wood, 1991), the increase in water [Cl^-] was not expected to impact compensatory responses to metabolic acidosis. To elicit a respiratory acidosis (i.e. a systemic acidosis accompanied by increased blood P_{CO_2}), trout (N=4) were exposed to hyperoxia achieved by gassing the water in the experimental chambers with pure O_2 (instead of air) for 72 h (Wood and Jackson, 1980; Goss and Wood, 1990a; Goss et al., 1994). Exposure to hypercapnia was also used to elicit respiratory acidosis, although with the potential contribution of a moderate reduction in water pH (Perry et al., 1987; Goss and Perry, 1993). In this case, trout (N=4) were held for 72 h in water gassed with 1% CO_2 in air (water pH 6.52±0.03, N=24). The gas mixture was provided by mass flow controllers (SmartTrak 100 series, Sierra Instruments, SRB Controls, Markham, ON, Canada). An additional group of trout (N=4) was exposed to hypercapnia followed by recovery; these fish underwent the same 72 h hypercapnic exposure followed by a 6 h recovery period in water that was vigorously aerated. During recovery from hypercapnia, trout experience a metabolic alkalosis

because blood P_{CO_2} rapidly falls, but the HCO_3^- that accumulated during hypercapnic exposure is cleared from the blood more slowly (Perry et al., 1987; Goss and Perry, 1993). For example, Perry et al. (1987) reported that during recovery from hypercapnia, blood P_{CO_2} had returned to near-normocapnic values within 1 h, whereas blood HCO_3^- levels remained significantly elevated even after 12 h.

The second experimental series focused on systemic alkalosis, using base infusion to induce a metabolic alkalosis (Goss and Wood, 1990b; Goss et al., 1994; Goss and Perry, 1994). Rainbow trout were anaesthetized by immersion in an aerated solution of benzocaine (0.05 g l^{-1} ethyl *p*-aminobenzoate, Sigma-Aldrich) and placed on a surgery table that allowed continuous irrigation of the gills with the anaesthetic solution. An indwelling cannula (PE-50 polyethylene tubing, Instech Laboratories, Plymouth Meeting, PA, USA) was implanted into the dorsal aorta using the basic method of Soivio et al. (1975). Fish were allowed to recover for 24 h in experimental chambers that were supplied with flowing system water, and cannulae were flushed with heparinized (100 IU ml^{-1} ammonium heparin, Sigma-Aldrich) modified (4.5 mmol l^{-1} $NaHCO_3$) Cortland saline (Wolf, 1963). To achieve metabolic alkalosis, trout (*N*=4) were infused (syringe pump model 780220, KD Scientific, ThermoFisher Scientific) via the dorsal aortic cannula for 72 h with 140 mmol l^{-1} $NaHCO_3$ at a rate of 2 ml kg^{-1} h^{-1}. Infusion of 140 mmol l^{-1} NaCl for 72 h at a rate of 2 ml kg^{-1} h^{-1} served as a control for the effects of cannulation and infusion (*N*=4). In addition, trout that had not been cannulated (*N*=3) served as a true control. In all cases, water flow to the experimental chambers was stopped for the final 6 h of the treatment period for exposure of the fish to MitoRos.

The final experimental series used cortisol injections to increase the number of ionocytes without exposing the fish to an acid–base challenge (Laurent and Perry, 1990; Goss et al., 2001). Rainbow trout were randomly allocated to either a control group (*N*=4) or a cortisol-treated group (*N*=4), with all fish being held in individual experimental chambers supplied with flowing system water. Cortisol-treated fish were lightly anaesthetized (by immersion in an aerated solution of benzocaine, 0.05 g l^{-1} ethyl *p*-aminobenzoate, Sigma-Aldrich) and given an intramuscular injection of cortisol (10 mg kg^{-1} hydrocortisone 21-hemisuccinate, Sigma-Aldrich, in 0.25 ml of modified Cortland saline; Goss et al., 2001) daily for 7 days; control fish were untreated. On the eighth day, water flow to the experimental chambers was stopped for 6 h for exposure of the fish to MitoRos.

Tissue collection, immunocytochemistry and cell quantification

The first and second gill arches were excised from the fish and rinsed in phosphate-buffered saline (PBS) to wash away blood and mucus. Gill tissue was fixed by immersion overnight in five volumes of 4% paraformaldehyde in PBS, followed by two washes in PBS and immersion overnight in 30% sucrose (in PBS). Tissues were kept at 4°C throughout the fixation procedure. Pieces of gill tissue consisting of approximately 10 filaments from the middle of the gill arch were frozen in Shandon Cryomatrix embedding medium (ThermoFisher Scientific), and 12 µm sections were prepared using a cryostat (CM 1850, Leica, Richmond Hill, ON, Canada) at −27°C. Sections were collected onto electrostatically charged slides (Fisherbrand SuperFrost Plus, ThermoFisher Scientific), allowed to dry for approximately 30 min, and stored at −20°C until use.

To identify PNA^- and PNA^+ ionocytes and localize CAc by immunocytochemistry, a hydrophobic barrier was created around each section with a Pap pen (Sigma-Aldrich). Sections were incubated for 2 h at room temperature in a solution of 1% Triton-X in PBS, and then overnight at room temperature in a 1:400 dilution (in PBS) of rabbit anti-trout CAc antibody (Georgalis et al., 2006) containing 20 µg ml^{-1} biotin-conjugated PNA (Sigma-Aldrich). Negative control sections were incubated in PBS lacking the primary antibody and biotin-conjugated PNA. Following washes (3×5 min) in PBS, slides were incubated overnight at room temperature in a 1:500 dilution (in PBS) of Alexa 488-coupled goat anti-rabbit IgG (ThermoFisher Scientific) for the detection of CAc. The incubation solution also contained 10 µl ml^{-1} Alexa 633-coupled streptavidin (ThermoFisher Scientific) to detect PNA binding. The slides were then washed again (3×5 min in PBS) and cover-slipped with Vectashield Antifade mounting medium (Vector Laboratories, Burlington, ON, Canada) containing 4′,6′-diamidino-2-phenylindole (DAPI) for the visualization of nuclei.

Sections were viewed using a conventional epifluorescence microscope (Nikon Eclipse Ni-U upright microscope, Nikon Instruments, Melville, NY, USA) and Andor iXon Ultra EMCDD camera (Andor Technology, Belfast, UK). Images were captured using NIS-Elements AR 4.13.04 imaging software (Nikon Instruments). For each fish, four gill sections were examined (two each from the first and second gill arches). Four photos per gill section were taken from randomly selected areas near the mid-regions of the gill filaments. Typically, the photos covered ∼0.4 mm of filament length with approximately 10 lamellae from one or two different filaments. Digital images were analysed using NIS-Elements AR 4.13.04 to determine the two-dimensional surface area of the lamellae within an image. For each image, the total number of PNA^- ionocytes (MitoRos positive) and PNA^+ ionocytes (MitoRos positive and PNA positive) were counted, as well as the number of each of these ionocytes that expressed CAc, by an observer who was blind to the treatment group to which the images belonged.

Statistical analyses

Data are reported as mean values±1 s.e.m. One-way ANOVA was used to test for significant treatment effects within the first two experimental series, with *post hoc* multiple comparisons being carried out against the control group in the first experimental series on acidosis and all-pairwise comparisons in the second experimental series using base-infused trout. Student's *t*-tests were used to test for significant effects of cortisol treatment in the third experimental series. Where the underlying assumptions of normality and equal variance were not met, equivalent non-parametric tests were used. Statistical analyses were carried out using Sigmaplot v13 (Systat Software) with a fiducial limit of significance of 0.05.

RESULTS

Rainbow trout gills were examined by immunohistochemistry to identify PNA^+ ionocytes, which were positive for both MitoRos and PNA, and PNA^- ionocytes, which were positive for MitoRos but not PNA (Fig. 1A–C). These cells were then assessed for the presence/absence of CAc (Fig. 1D,E). Negative control sections incubated in solutions from which the primary antibody against CAc and biotin-conjugated PNA were omitted exhibited only the fluorescence associated with MitoRos (Fig. 1F). Quantification of the number of ionocytes of the different types revealed that whereas most PNA^- cells expressed CAc, only a small fraction of PNA^+ cells

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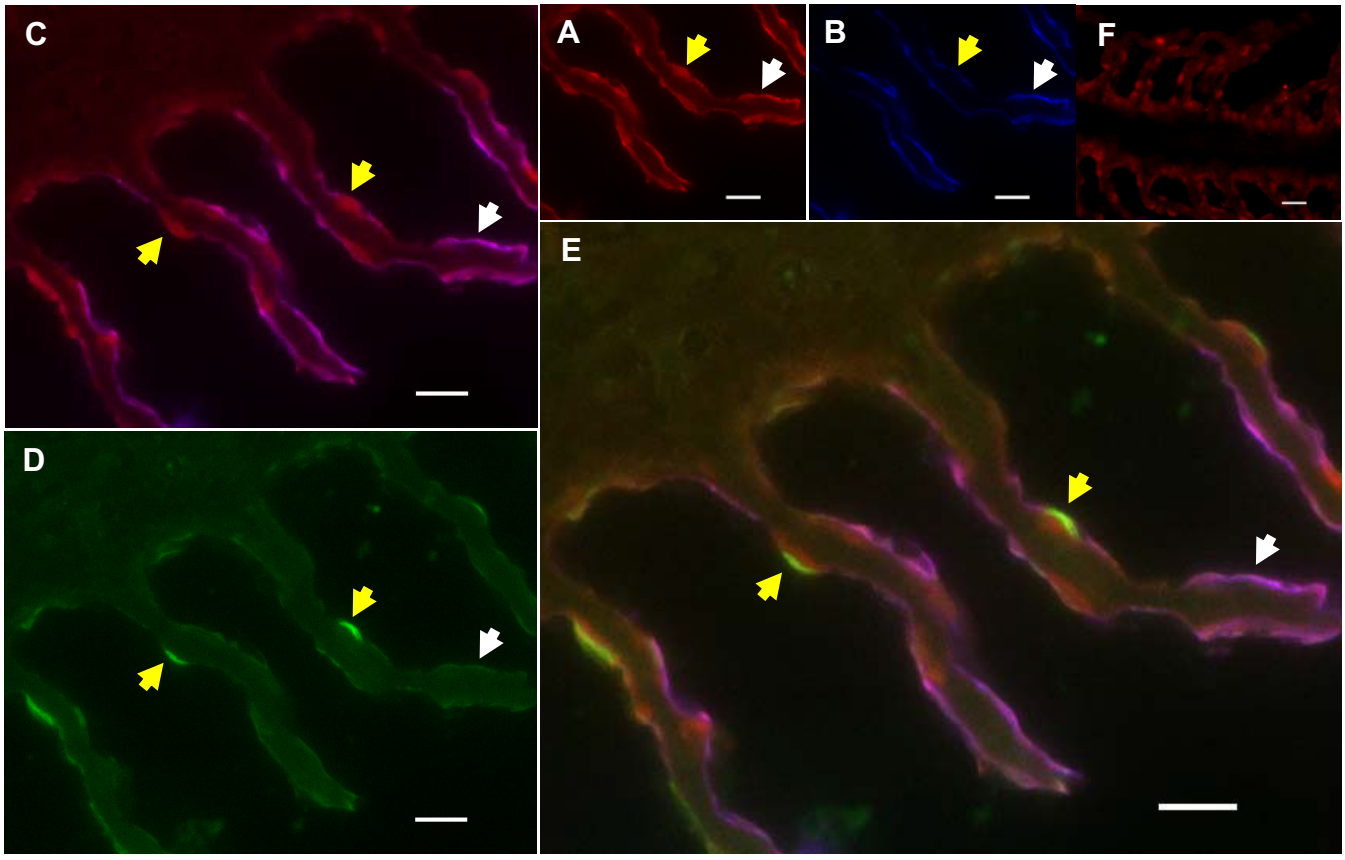

Fig. 1. Representative immunohistochemistry images of the rainbow trout (*Oncorhynchus mykiss*) gill. Images show markers used for ionocytes (A; MitoRos, red), peanut lectin agglutinin-positive (PNA^+) cells (B; blue) and trout cytosolic carbonic anhydrase (CAc) (D; green). (C) An overlay of MitoRos and PNA staining, with co-localization of these markers shown in pink. (E) An overlay of MitoRos, PNA and CAc staining. Whereas PNA^+ ionocytes exhibited staining for both MitoRos and PNA (white arrows), PNA^- ionocytes lacked PNA staining (yellow arrows). Most PNA^- ionocytes exhibited immunofluorescence for CAc, but few PNA^+ ionocytes were CAc positive (none in this image). (F) A negative control from which PNA and the primary antibody against CAc were omitted; only fluorescence associated with MitoRos is visible. The scale bars represent 20 µm.

expressed CAc under control conditions (Fig. 2). Of the ionoyctes identified on the basis of MitoRos fluorescence, 70% also bound PNA, but less than 5% of these PNA^+ ionocytes exhibited immunofluorescence for CAc (Fig. 2B,C). By contrast, CAc immunofluorescence was present in over 90% of the MitoRos-positive cells that did not exhibit PNA binding, i.e. PNA^- ionocytes (Fig. 2C).

Exposure of rainbow trout to conditions that elicited metabolic acidosis (low pH), respiratory acidosis (hyperoxia, hypercapnia) or metabolic alkalosis (recovery from hypercapnia) had no significant impact on the abundance of PNA^+ ionocytes (Fig. 3A; ANOVA, $F_{4,14}$=2.878, P=0.062). The abundance of PNA^+ ionocytes that exhibited immunofluorescence for CAc also was unaffected by systemic acidosis, but increased significantly in fish experiencing

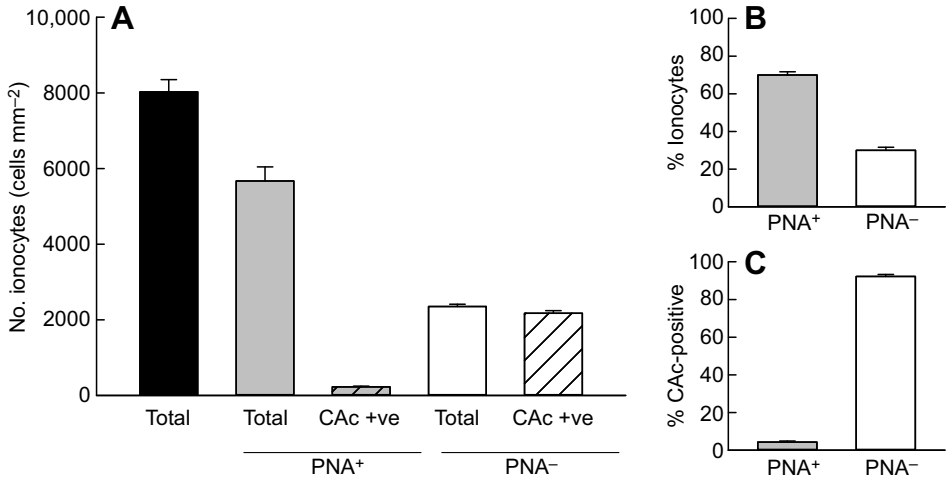

Fig. 2. The number of ionocytes of different types summarized for control rainbow trout used across all three experimental series (*N*=11). (A) Densities of ionocytes, shown both for total number and broken down by ionocyte type (PNA^+ and PNA^-, and those of each type that are CAc positive). (B) Percentage of total ionocytes that are PNA^+ or PNA^-. (C) Percentage of PNA^+ or PNA^- ionocytes that are CAc positive. Values are means+s.e.m.

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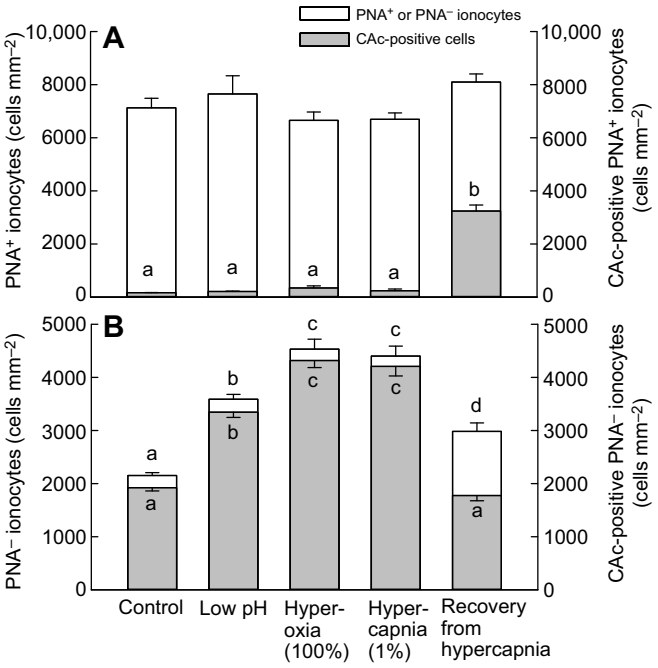

Fig. 3. Quantification of ionocytes in trout exposed to systemic acidosis or metabolic alkalosis. Trout were exposed to water of low pH (*N*=3), hyperoxia (*N*=4) or hypercapnia (1% CO_2; *N*=4) for 72 h to induce a systemic acidosis, to hypercapnia for 72 h followed by 6 h in normoxic conditions to induce a systemic alkalosis (recovery from hypercapnia; *N*=4), or to control conditions (*N*=4). (A) Quantification of PNA^+ ionocytes identified on the basis of double staining for MitoRos and PNA, and PNA^+ ionocytes that exhibited immunofluorescence for CAc. (B) Quantification of ionocytes that were MitoRos positive but lacked PNA staining (PNA^- ionocytes), and PNA^- cells that were CAc positive. Values are means±s.e.m. Treatment groups that share a letter are not significantly different from one another (ANOVA, see Results for details); analysis of PNA^+ ionocyte abundance (A) yielded a *P*-value of 0.062.

metabolic alkalosis (Fig. 3A; ANOVA on log-transformed data, $F_{4,14}$=43.071, P<0.001). As a result, the percentage of PNA^+ ionocytes that were CAc positive increased from 2.4±0.1% (*N*=4) and 3.6±0.8% (*N*=4) in control and hypercapnic fish, respectively, to 40.3±3.5% (*N*=4) in fish recovering from hypercapnia. Unlike the situation with PNA^+ ionocytes, the abundance of PNA^- ionoyctes increased significantly and in a treatment-specific fashion in response to both acidosis and alkalosis, with the magnitude of the increase being largest in trout exposed to hypercapnia or hyperoxia and smallest in trout recovering from hypercapnia (Fig. 3B; ANOVA, $F_{4,14}$=45.152, P<0.001). Under control conditions, 23.3±1.3% (*N*=4) of ionocytes were PNA^- and this proportion increased to 40.5±0.3% (*N*=4) in hyperoxic fish and 39.6±0.6% (*N*=4) in hypercapnic trout, falling again to 26.9±1.6% (*N*=4) in trout recovering from hypercapnia. The abundance of CAc-positive cells increased essentially in proportion with the increase in PNA^- ionocytes in all treatments except trout recovering from hypercapnia, where PNA^- ionocyte abundance did not differ significantly from the control value (Fig. 3B; ANOVA, $F_{4,14}$=100.987, P<0.001). Thus, the percentage of PNA^- ionocytes that was CAc positive fell from 89.4±2.1% (*N*=4) in control animals and 95.6±1.1% (*N*=4) in hypercapnic trout to 60.5±6.2% (*N*=4) in trout recovering from hypercapnia.

Given the marked effects of recovery from hypercapnia on CAc expression in PNA^+ and PNA^- ionocytes, base infusion was examined as an alternative approach to inducing metabolic alkalosis. Saline-infused (sham) trout served as a control for the effects of infusion. Relative to control and saline-infused trout, base-infused fish exhibited a significant increase in the abundance of PNA^+ cells (Fig. 4A; ANOVA, $F_{2,8}$=20.193, P<0.001), from 66.0±0.9% (*N*=3) of ionocytes being PNA^+ under control conditions to 78.7±0.4% (*N*=4) in base-infused fish. The abundance of CAc-positive PNA^+ cells also increased significantly in base-infused fish, and tended to increase in sham trout (ANOVA on reciprocal-transformed data, $F_{2,8}$=10.759, P=0.005). As a result, the percentage of PNA^+ ionocytes that were CAc positive increased from 4.7±0.6% (*N*=3) in control fish to 10.1±2.2% (*N*=4) in saline-infused fish and 13.8±1.0% (*N*=4) in base-infused trout. Significant reductions in the abundance of PNA^- ionocytes (Fig. 4B; ANOVA, $F_{2,8}$=24.423, P<0.001) and CAc-positive PNA^- ionocytes (ANOVA, $F_{2,8}$=57.586, P<0.001) were detected in both saline- and base-infused trout. The percentage of ionocytes that were PNA^- decreased from 34.0±0.9% (*N*=3) under control conditions to 28.4±0.8% (*N*=4) and 21.3±0.4% (*N*=4) in sham- and base-infused trout, respectively. However, the magnitude of the fall in CAc-positive PNA^- ionocyte abundance was significantly greater in base-infused fish, with the percentage of PNA^- cells that were CAc positive decreasing from 94.3±0.7% (*N*=3) in control fish to 87.9±2.5% (*N*=4) in saline-infused fish and 72.6±3.9% (*N*=4) in base-infused fish.

In a final series of experiments, cortisol treatment was used to increase the number of ionocytes (Goss et al., 2001) in the absence of an acid–base challenge. As in previous work (Goss et al., 2001), cortisol treatment increased the abundance of PNA^- and PNA^+ ionocytes in a similar fashion (Fig. 5; rank sum test, T=10.000,

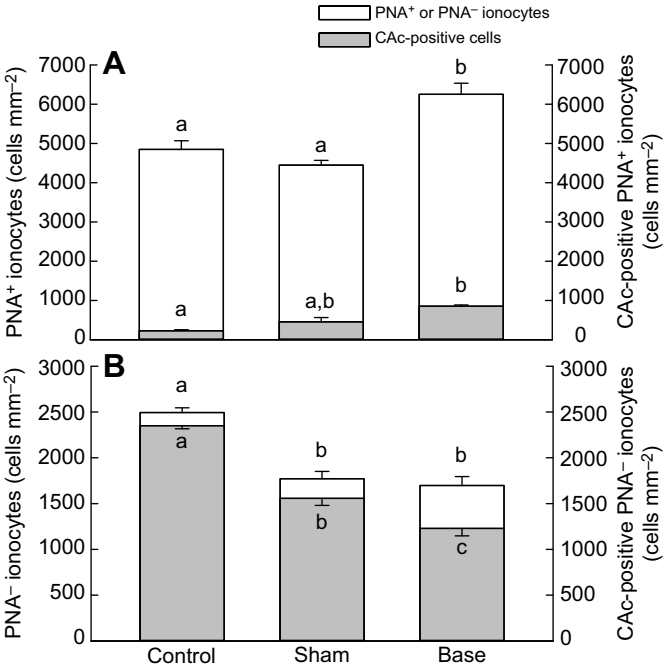

Fig. 4. Quantification of ionocytes in trout exposed to metabolic alkalosis by base infusion. Trout were infused with 140 mmol l^{-1} $NaHCO_3$ (base, *N*=4) for 72 h to induce a systemic alkalosis, infused with 140 mmol l^{-1} NaCl as a control for infusion (sham, *N*=4) or held untreated under control conditions (*N*=3). (A) Quantification of PNA^+ ionocytes identified on the basis of double staining for MitoRos and PNA, and PNA^+ ionocytes that exhibited immunofluorescence for CAc. (B) Quantification of ionocytes that were MitoRos positive but lacked PNA staining (PNA^- ionocytes), and PNA^- cells that were CAc positive. Values are means±s.e.m. Treatment groups that share a letter are not significantly different from one another (ANOVA, see Results for details).

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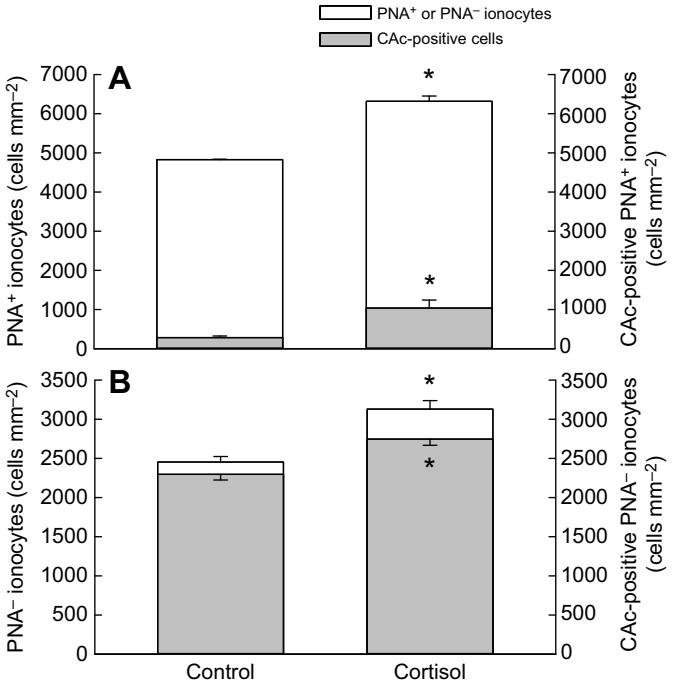

Fig. 5. Quantification of ionocytes in trout treated with cortisol. Trout were treated with cortisol (N=4) or held under control conditions (N=4) for 7 days. (A) Quantification of PNA$^+$ ionocytes identified on the basis of double staining for MitoRos and PNA, and PNA$^+$ ionocytes that exhibited immunofluorescence for CAc. (B) Quantification of ionocytes that were MitoRos positive but lacked PNA staining (PNA$^-$ ionocytes), and PNA$^-$ ionocytes that were CAc positive. Values are means±s.e.m. An asterisk indicates a significant difference between control and cortisol-treated fish (Student's t-test, see Results for details).

P=0.029 for Fig. 5A; Student's t-test, t_6=−5.254, P=0.002 for Fig. 5B), such that the proportion of each ionocyte type remained constant; 66.3±0.7% (N=4) and 66.9±0.4% (N=4) of ionocytes were PNA$^+$, and 33.7±0.7% (N=4) and 33.1±0.4% (N=4) were PNA$^-$ in control and cortisol-treated fish, respectively. For both ionocyte types, the abundance of cells that exhibited immunofluorescence for CAc increased significantly with cortisol treatment (Student's t-tests, t_6=−3.654 and −4.184, P=0.011 and 0.006 for Fig. 5A and B, respectively). However, whereas the percentage of PNA$^+$ cells that were CAc positive tended to increase with cortisol treatment, from 5.9±0.9% (N=4) in control fish to 16.7±3.6% (N=4) in cortisol-treated fish, the percentage of PNA$^-$ cells that were CAc positive tended to fall, from 93.6±0.6% (N=4) in control fish to 87.9±2.6% (N=4) in cortisol-treated fish.

DISCUSSION

In contrast to the expectation that both ionocyte types of the trout gill epithelium would express CAc (Gilmour and Perry, 2009), less than 5% of the cells identified as PNA$^+$ ionocytes on the basis of double staining for MitoRos and PNA exhibited immunofluorescence for CAc under control conditions. However, the abundance of CAc-positive PNA$^+$ ionocytes increased significantly in trout that experienced metabolic alkalosis, either during recovery from hypercapnic acidosis, driven by an increase in the percentage of PNA$^+$ ionocytes that were CAc positive (to ∼40%), or as a result of base infusion, where both the abundance of PNA$^+$ ionocytes (from 66% to 79% of ionocytes) and the percentage of these cells that were CAc positive (to ∼14%) increased. Similarly, cortisol-treated trout exhibited increases in both the abundance of PNA$^+$ ionocytes and the percentage of PNA$^+$ cells that were CAc positive (to ∼17%). About 90% of PNA$^-$ ionocytes (MitoRos-positive cells that lacked staining for PNA) exhibited immunofluorescence for CAc under control conditions, but this fell (to 61–73%) in trout that experienced metabolic alkalosis. In contrast, in trout that experienced metabolic acidosis, the abundance of CAc-positive PNA$^-$ ionocytes increased significantly (to ∼95%), tracking the abundance of PNA$^-$ cells. Collectively, these responses shed new light on the morphological model for acid–base regulation (e.g. Goss et al., 1995, 1998).

Methods for distinguishing the ionocyte subtypes of the trout gill epithelium using PNA were developed by Goss and colleagues (Goss et al., 2001; Galvez et al., 2002). Using dispersed gill cells suspended in solution with MitoTracker green-FM as an ionocyte marker, this group reported that 35–40% of ionocytes were PNA positive (Goss et al., 2001; Hawkings et al., 2004). By contrast, ∼70% of ionocytes were PNA positive in the present study, where cell counts were carried out on gill tissue sections using MitoRos to identify ionocytes. Ivanis et al. (2008) also identified PNA-positive cells in trout gill tissue sections, using NKA as the ionocyte marker. Although these researchers did not quantify cell numbers, scrutiny of their images suggests that PNA$^+$ and PNA$^-$ ionocytes were approximately equal in abundance (e.g. fig. 3A in Ivanis et al., 2008). At least three factors may have contributed to these differences across studies. First, the likelihood of detecting particular ionocyte subtypes probably differs between gill cell suspensions, where all gill arches are used and cells are released from the tissue by enzymatic digestion, and gill sections, where specific areas of the gills are scrutinized and cells are organized into tissues with the lamellar and filament epithelia receiving greatest attention. Second, different ionocyte markers may identify somewhat different populations of ionocytes. In zebrafish (*Danio rerio*), for example, MitoTracker green-FM stains NKA-enriched NaR cells more strongly than H$^+$-ATPase-rich HR cells (Lin et al., 2006), whereas MitoRos staining is weak in NaR cells and strong in HR cells and the Na$^+$/Cl$^-$-cotransporter-expressing NCC cells (Kwong and Perry, 2016). Galvez et al. (2002) reported a small but significant difference in the relative expression of MitoTracker green-FM fluorescence between PNA$^-$ and PNA$^+$ fractions of trout gill cell suspensions, suggesting that this ionocyte marker may not be equally effective for the two ionocyte types. A similar argument may apply to the use of NKA as an ionocyte marker. Both PNA$^-$ and PNA$^+$ ionocytes are enriched in NKA relative to pavement cells, but NKA abundance and activity are higher in PNA$^+$ cells, suggesting that this ionocyte subtype may be detected more effectively by NKA immunostaining (Galvez et al., 2002; Hawkings et al., 2004). Finally, differences among trout populations in the ionocyte composition of the gill epithelium may have contributed to differences among studies in the relative proportions of PNA$^-$ and PNA$^+$ ionocytes. Branchial morphology is widely recognized as being responsive to factors such as water ion concentrations and pH (Perry and Laurent, 1993; Laurent and Perry, 1995; Evans et al., 2005).

Identification of PNA$^-$ and PNA$^+$ ionocytes in the trout gill epithelium enabled the localization of CAc to specific ionocyte types. In agreement with functional data linking CA to acid secretion and Na$^+$ uptake (Kerstetter et al., 1970; Payan et al., 1975; Georgalis et al., 2006; Goss et al., 2011), both of which are attributed to PNA$^-$ ionocytes (Reid et al., 2003; Goss et al., 2011), the present study provided immunohistochemical confirmation of CAc expression in PNA$^-$ ionocytes. These data add to the emerging picture of the PNA$^-$ cell as an ionocyte that possesses the

infrastructure for proton secretion linked to Na^+ uptake, including CAc (present study; Goss et al., 2011) and the V-type H^+-ATPase (Galvez et al., 2002), but interestingly, not the Na^+/H^+ exchangers NHE2 or NHE3, which are expressed in PNA^+ ionocytes (Ivanis et al., 2008). This last finding is particularly unexpected because the ionocyte of the zebrafish gill with a function comparable to the PNA^- ionocyte, the HR cell that secretes protons and takes up Na^+, expresses NHE3 (Guh et al., 2015). Similarly, the ionocyte complements of the freshwater tilapia (*Oreochromis mossambicus*) and medaka (*Oryzias latipes*) include an ionocyte that expresses apical NHE3 and is involved mainly in Na^+ uptake and acid secretion (Hiroi et al., 2008; Inokuchi et al., 2009; Hsu et al., 2014). These differences speak to the diversity of ionocytes among teleosts, but also raise the question of how Na^+ uptake is accomplished in the PNA^- ionocyte of the trout gill. The recent identification of an acid-sensing ion channel (ASIC) in ionocytes (the study did not distinguish between ionocyte types) of the trout gill and its implication in Na^+ uptake suggest that a coupled proton pump–ASIC mechanism localized to the PNA^- ionocyte could account for the Na^+ uptake and acid secretion attributed to this cell type (Dymowska et al., 2014).

Although earlier studies reported the presence of CAc in trout gill chloride cells (Rahim et al., 1988; Georgalis et al., 2006), which are now recognized as PNA^+ ionocytes (Galvez et al., 2002), the quantitative approach adopted in the present study revealed that relatively few PNA^+ cells exhibited immunofluorescence for CAc under control conditions. However, the abundance of CAc-positive PNA^+ cells increased significantly in response to systemic alkalosis or cortisol treatment, owing to increases in the abundance of PNA^+ cells and/or the proportion of PNA^+ cells that were CAc positive. These responses suggest that increases in CAc expression within existing PNA^+ ionocytes (i.e. to the threshold of detection by immunohistochemistry) as well as the differentiation/proliferation of new cells may contribute to the increased abundance of CAc-positive PNA^+ ionocytes in alkalotic trout. A significant increase in CAc transcript abundance was detected in the gills of base-infused trout, supporting a role for transcriptional regulation of CAc (Gilmour et al., 2011). Regardless of how it is achieved, the increased abundance of CAc-positive PNA^+ ionocytes in trout experiencing systemic alkalosis supports the hypothesis that this ionocyte functions in base secretion (Galvez et al., 2002). Models typically link base secretion to Cl^- uptake through an apical Cl^-/HCO_3^- exchanger that is postulated to operate in a metabolon with cytosolic CA, to provide protons from the catalysed hydration of CO_2, and with basolateral H^+-ATPase (Tresguerres et al., 2006). Both H^+-ATPase (Galvez et al., 2002) and CAc (present study) are expressed by at least a subset of PNA^+ ionocytes. The key element of this model, the apical Cl^-/HCO_3^- exchanger, remains uncertain but recently a member of the solute carrier 26 family of anion exchangers, SLC26a6, was cloned from trout (Boyle et al., 2015) and found to be expressed in a subset of ionocytes (Leguen et al., 2015; this study did not distinguish between ionocyte types). However, the relatively low number of PNA^+ ionocytes that exhibited CAc immunofluorescence under control conditions (i.e. in the absence of an acid–base disturbance) might suggest that mechanisms other than anion exchange are responsible for Cl^- uptake. In zebrafish and freshwater tilapia and medaka, the NCC cell has been implicated in Cl^- uptake (Hiroi et al., 2008; Hsu et al., 2014; Guh et al., 2015). This ionocyte expresses apical NCC2, a Na^+/Cl^--cotransporter, and at least in zebrafish, basolateral ClC-2c, a Cl^- channel (Wang et al., 2015). A subset of trout gill ionocytes also expresses ClC2 (Leguen et al., 2015). Collectively, these data raise the possibility that there is more than one type of PNA^+ ionocyte in the trout gill epithelium. It is tempting to speculate that these include a relatively rare, base-secreting ionocyte that expresses CAc and SLC26a6 and increases in abundance in response to systemic alkalosis, and a trout equivalent of the NCC cell that functions in Cl^- uptake using ClC2 and presumably also expresses NHE2 and/or NHE3. Both NHE2 and NHE3 have been localized to PNA^+ ionocytes (Ivanis et al., 2008; Hiroi and McCormick, 2012), yet it is difficult to reconcile their presence with a primary function of base secretion for this cell type. The existence of PNA^+ ionocyte subtypes would overcome this dilemma.

Both PNA^- and PNA^+ ionocytes responded to acid–base challenges in directions that were consistent with an acid-secreting role for PNA^- cells and a base-secreting role for PNA^+ cells (or at least a subset of PNA^+ cells). Regardless of whether trout were exposed to a metabolic (low-pH water) or respiratory (hyperoxia, hypercapnia) acidosis, the abundance of PNA^- ionocytes increased significantly in the absence of changes in the abundance of PNA^+ ionocytes, a response that would be expected to increase the capacity for acid excretion. Metabolic alkalosis, by contrast, generally reduced the abundance of PNA^- ionocytes (relative to hypercapnic trout in fish recovering from hypercapnia) while increasing the abundance of CAc-positive PNA^+ ionocytes, responses that would be expected to increase the capacity for base excretion. Differences between the responses to recovery from hypercapnia and base infusion may have reflected the different time scales of these treatments: 6 h of recovery from hypercapnia versus 72 h of base infusion. That is, trout recovering from hypercapnia exhibited substantial changes in the proportion of PNA^+ and PNA^- ionocytes that were CAc positive, whereas changes in PNA^+ and PNA^- cell abundance were more marked in base-infused trout, perhaps suggesting rapid regulation of CAc expression as an acute response to acid–base disturbance, followed by somewhat slower changes in ionocyte numbers. In an analogous situation, changes in cell morphology were apparent before changes in cell proliferation in trout exposed to ion-poor water (Perry and Laurent, 1989; Laurent et al., 1994). Collectively, the responses documented in the present study are consistent with the morphological model for acid–base regulation, but whereas the morphological model focused primarily on the covering (during acidosis) or uncovering (during alkalosis) of base-secreting 'chloride cells' by pavement cells so as to reduce or enhance HCO_3^- loss, respectively (Goss et al., 1995, 1998), the data of the present study provide a new perspective. Specifically, these data emphasize the dynamic regulation of both an acid-secreting ionocyte and a base-secreting ionocyte in the compensation of acid–base disturbances, with changes in cell abundance as well as the expression of specific proteins (e.g. CAc) within specific ionocyte types both contributing to compensatory responses.

Ionocyte abundance was also altered in response to NaCl infusion or cortisol treatment. Decreased abundance of PNA^- ionocytes in NaCl-infused fish may account for the reduction in Na^+ influx reported previously (Goss and Perry, 1994), and presumably reflects a decreased need for Na^+ uptake, although stress associated with cannulation cannot be ruled out. As in a previous study (Goss et al., 2001), cortisol treatment increased the overall number of ionocytes without affecting the proportion of PNA^+ to PNA^- types, and, in agreement with this finding, previous work revealed increased Na^+ and Cl^- influx rates in cortisol-treated rainbow trout (Laurent and Perry, 1990). However, cortisol treatment in the present study had differential effects on CAc expression within the ionocyte types, tending to increase the proportion of CAc-positive PNA^+ cells while decreasing the proportion of CAc-positive PNA^- cells. The net

effect of these changes is predicted to be an enhanced capacity for base excretion. Consistent with this prediction, trout treated with a combination of cortisol and ovine growth hormone to increase ionocyte abundance exhibited higher rates of base excretion in response to $NaHCO_3$ infusion, resulting in smaller increases in plasma pH and HCO_3^- concentration (Perry and Goss, 1994). How cortisol treatment leads to differential effects on CAc expression within PNA^+ versus PNA^- cells remains to be determined. Indeed, our knowledge of the sensory mechanisms and signal transduction pathways responsible for detecting acid–base disturbances and initiating appropriate changes in ionocyte abundance within the gill epithelium as well as the expression of specific proteins within ionocyte types remains incomplete.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: K.M.G.; Formal analysis: M.B., K.M.G.; Investigation: M.B.; Writing - original draft: K.M.G.; Writing - review & editing: M.B., K.M.G.

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