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SHORT COMMUNICATION

Hypercapnia and low pH induce neuroepithelial cell proliferation and emersion behaviour in the amphibious fish *Kryptolebias marmoratus*

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ABSTRACT

Aquatic hypercapnia may have helped to drive ancestral vertebrate invasion of land. We tested the hypothesis that amphibious fishes sense and respond to elevated aquatic P_{CO_2} by behavioural avoidance mechanisms, and by morphological changes at the chemoreceptor level. Mangrove rivulus (*Kryptolebias marmoratus*) were exposed to 1 week of normocapnic control water (pH 8), air, hypercapnia (5% CO_2, pH 6.8) or isocapnic acidosis (pH 6.8). We found that the density of CO_2/H^+ chemoreceptive neuroepithelial cells (NECs) was increased in hypercapnia or isocapnic acidosis-exposed fish. Projection area (a measure of cell size) was unchanged. Acute exposure to progressive hypercapnia induced the fish to emerse (leave water) at water pH values ~6.1, whereas addition of HCl to water caused a more variable response with a lower pH threshold (~pH 5.5). These results support our hypothesis and suggest that aquatic hypercapnia provides an adequate stimulus for extant amphibious fishes to temporarily transition from aquatic to terrestrial habitats.

KEY WORDS: P_{CO_2}, Chemoreceptor, Phenotypic plasticity

INTRODUCTION

The evolution of air breathing and the invasion of land by ancestral fishes is thought to have occurred in unfavourable aquatic environments such as stagnant and hypoxic swamps (Graham, 1997). Many extant amphibious fishes live in similar habitats; and poor aquatic conditions, such as hypoxia, are known to cause these fishes to leave water, or emerse (Regan et al., 2011). Hypoxic tropical waters often have high partial pressures of CO_2 (P_{CO_2}; hypercapnia) (Ultsch, 1996). However, the effects of hypercapnia on emersion are unknown. If amphibious fish avoid hypercapnia by emersing, and because emersion behaviour is of survival value, then peripheral CO_2 sensing may be of critical importance to these animals.

Fishes are thought to sense both O_2 and CO_2/H^+ in the environment using externally oriented branchial chemoreceptors named neuroepithelial cells (NECs; Jonz et al., 2004; Qin et al., 2010; Abdallah et al., 2015; Jonz et al., 2015). Branchial NECs display extensive plasticity in response to changes in environmental P_{O_2}. Chronic exposure to hyperoxia can reduce NEC density, while chronic hypoxia can cause both hypertrophy and proliferation in different cell populations. However, there is no evidence of NEC plasticity in response to chronic hypercapnia in the fully aquatic zebrafish *Danio rerio* (reviewed in Jonz et al., 2015). Chemoreceptor plasticity may play a role in adaptation to hypercapnic environments, facilitating CO_2 sensing in amphibious fishes, which have evolved under stagnant, 'swampy' conditions and may therefore frequently encounter acute changes in water chemistry; however, this hypothesis remains untested.

NECs have been directly linked to the hypoxic emersion response in the amphibious mangrove rivulus, *Kryptolebias marmoratus* Poey 1880, and the distribution of NECs containing the neurotransmitter serotonin (5-hydroxytryptamine, 5-HT) has been characterized in the gills of this species (Regan et al., 2011). Moreover, a subpopulation of NECs that respond to both hypoxia and hypercapnia has been reported in zebrafish *in vitro* (Qin et al., 2010; Jonz et al., 2015). Thus, NECs may be critically important for sensing aquatic hypercapnia and controlling the emersion response in amphibious fishes. The primary mechanism through which NECs sense hypercapnia is unclear but may involve intracellular acidification, caused by the conversion of CO_2 to carbonic acid by carbonic anhydrase (Qin et al., 2010), detection of extracellular H^+ as a proxy for CO_2 (Abdallah et al., 2015), or sensing of changes in P_{CO_2} directly.

We tested the hypothesis that the gill NECs of the mangrove rivulus sense changes in environmental P_{CO_2} and undergo plastic changes with chronic hypercapnia (e.g. proliferation or hypertrophy). Furthermore, we tested the competing hypotheses that NECs respond to (1) CO_2 directly, (2) CO_2 via changes in pH, or (3) a combination of CO_2 and pH. To test the first hypothesis, we measured gill NEC morphology following 1 week of aquatic hypercapnia (P_{CO_2}=5.1 kPa), low pH control (isocapnic acidosis, pH 6.8) and air exposure. We predicted that aquatic hypercapnia (external P_{CO_2}) would induce NEC plasticity if there was no possibility of avoidance, but similar changes would not occur with air exposure, where internal (not external) P_{CO_2} would be elevated. We also predicted that the mangrove rivulus would emerse at a threshold level when exposed to acute aquatic hypercapnia similar to their emersion response to hypoxia.

RESULTS AND DISCUSSION

Our results support the hypothesis that mangrove rivulus are able to sense and respond to changes in environmental CO_2. Here, we provide combined *in vivo* morphological and behavioural evidence that NECs are sensitive to changes in P_{CO_2} as well as external acidosis independent of P_{CO_2} and mediate avoidance behaviour in an amphibious fish.

Gill NECs

We found that the NECs of the gill proliferated in response to both chronic hypercapnia and acidosis. Serotonin-positive cells (NECs) were evident within the primary epithelium of gill filaments of all

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Received 1 April 2015; Accepted 29 July 2015

Journal of Experimental Biology (2015) 218, 2987-2990 doi:10.1242/jeb.123133

fish studied, but NECs were more numerous in fish chronically exposed to aquatic hypercapnia or acid water (Fig. 1A–D). Correspondingly, the absolute number of NECs per filament was significantly higher in fish exposed to high CO_2 or acid water (Fig. 1E, $P<0.05$). This difference remained if the number of cells per filament was standardized to body size (standard length; supplementary material Fig. S1).

While both chronic hypercapnia and acidosis clearly induced NEC proliferation in the mangrove rivulus gill, we found no evidence of NEC hypertrophy in this study as NEC projection area was unaffected by manipulation of CO_2 or pH (Fig. 1F). By contrast, chronic hypoxia exposure resulted in NEC hypertrophy in mangrove rivulus, but not proliferation (Regan et al., 2011). Chronic hypoxia caused both hypertrophy and proliferation of NECs in zebrafish; however, these responses occurred in two separate cellular populations (Jonz et al., 2004). Data from these two studies (Regan et al., 2011; Jonz et al., 2004) suggest that there may be two independent populations of gill NECs in fish that respond to different stimuli with different morphological outcomes. It is possible that some NECs of the mangrove rivulus gill are selectively sensitive to CO_2/H^+. Whether these are the same cells that undergo hypertrophy during hypoxia acclimation, or are from a different subset of the population, is unclear.

Interestingly, air exposure results in an accumulation of tissue CO_2 similar to that in hypercapnia (C.E.R., A.J.T. and P.A.W., manuscript in preparation) but does not induce NEC proliferation (Fig. 1B,E). These data suggest that NECs sense changes in environmental P_{CO_2} rather than arterial P_{CO_2}.

Behavioural response to acute acidosis/hypercapnia

The current view among respiratory physiologists is that the ventilatory responses of fishes to aquatic hypercapnia are mediated by changes in water P_{CO_2} independent of changes in pH (see Milsom et al., 2012, for review). This view is in direct contrast to the *in vitro* finding that changes in external H^+ concentration, but not increased P_{CO_2}, initiated Ca^{2+} signalling in isolated gill NECs of zebrafish, which would presumably lead to neurotransmitter release and activation of reflex pathways (Abdallah et al., 2015). In the present study, both acute acidosis and acute acidic hypercapnia caused mangrove rivulus to emerse (Fig. 2A). However, two distinct

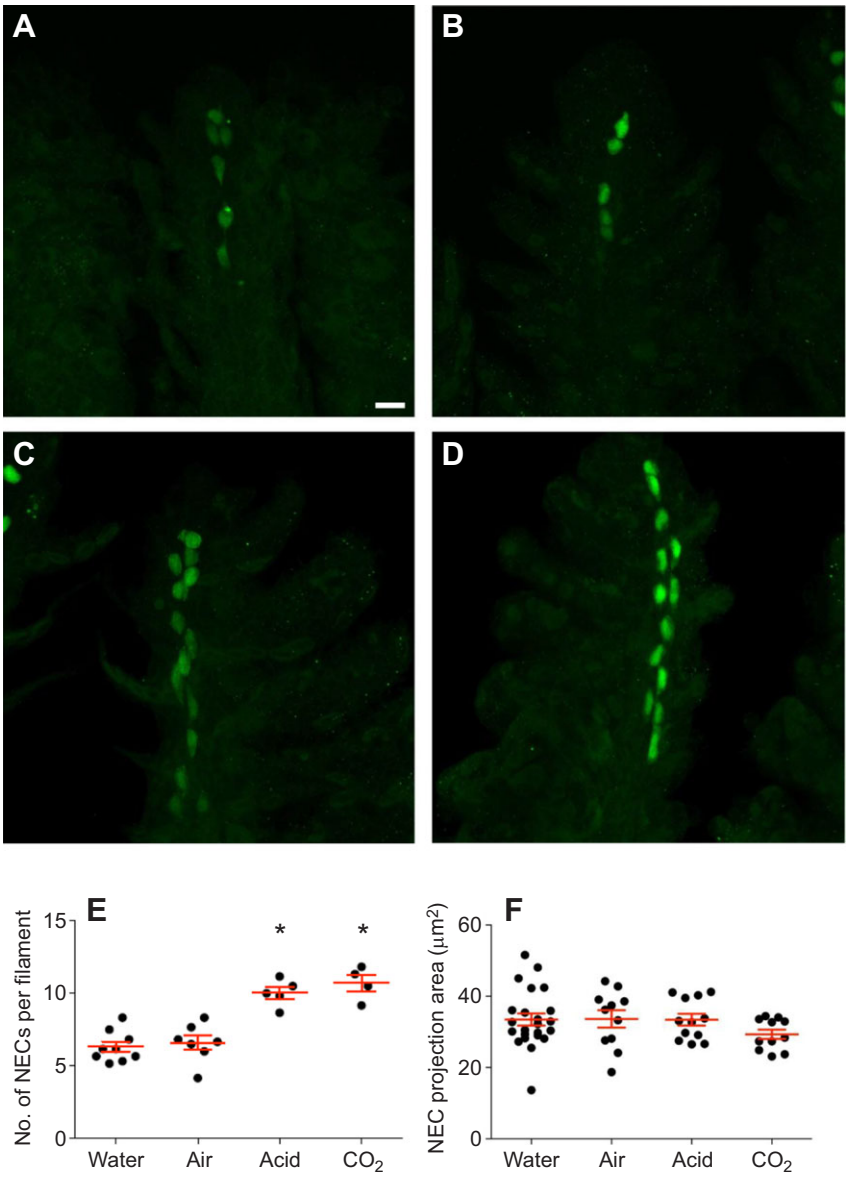

Fig. 1. Immunohistochemical labelling of neuroepithelial cells (NECs) of the primary filament epithelium in mangrove rivulus (*Kryptolebias marmoratus*). Fish were exposed to 7 days of (A) brackish water (control, pH 8), (B) air, (C) isocapnic acidosis (pH 6.8) or (D) aquatic hypercapnia (5% CO_2, pH 6.8). NECs (green) were identified using antibodies against serotonin. Scale bar in A is 10 µm and corresponds to all panels. (E) A greater number of serotonergic NECs per filament were recorded following exposure to acid or hypercapnia, compared with water or air. (F) Projection area (a measure of cell size) did not change. *Significant difference from brackish water controls ($P<0.05$). Data in E and F are presented as means±s.e. (N=6–10).

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behavioural patterns were observed. Acute acidic hypercapnia caused fish to emerse in a consistent manner at a mean pH of 6.10±0.01 (effective dose at which 50% of fish emerse, ED_{50}: pH 6.18; Fig 2A). A similar tight emersion threshold is seen in response to decreasing O_2 (Regan et al., 2011) and corresponds with the P_{O_2} at which mangrove rivulus can no longer O_2 regulate (Turko et al., 2012). Acute acidosis, without an accompanying rise in CO_2, caused a much less consistent emersion threshold (ED_{50}: pH 5.85; Fig. 2A). Emersion occurred over a much broader pH range (3.4–7.3) than emersion in fish exposed to hypercapnia. This is particularly interesting given that all the fish in this study were isogenic, i.e. from the same clonal lineage. Therefore, underlying genetic factors do not influence this behavioural variation.

While isocapnic acidosis did eventually result in emersion in all fish, the average pH of emersion (5.5±0.077) was significantly lower than that with acute hypercapnia (Fig. 2B). There are two possible explanations for these findings. The first is that mangrove rivulus sense both CO_2 and H^+ separately and that aquatic hypercapnia, with its associated change in water pH, stimulates both of these pathways. These two stimuli together may result in a more consistent and robust response to hypercapnia rather than H^+ alone. The second possibility is that NECs respond to intracellular acidification, and this is induced by either intracellular hydration of CO_2 or from addition and subsequent diffusion of extracellular H^+. Interpretation of our results in this manner may suggest that the rate of emersion responses in acidic hypercapnia versus low pH is related to the rate of change of intracellular pH in NECs. In intracellular H^+ imaging experiments in zebrafish NECs, extracellular addition of HCl resulted in a slow, modest change in intracellular pH, which did not stabilize within the 3 min measurement time, while addition of CO_2 produced a rapid (<2 min), intracellular acidification response, which was 4.5-fold greater (Abdallah et al., 2015). Therefore, the emersion threshold may be dictated by a threshold intracellular pH, which would be reached faster and at a higher extracellular pH in acidic hypercapnia than with HCl alone.

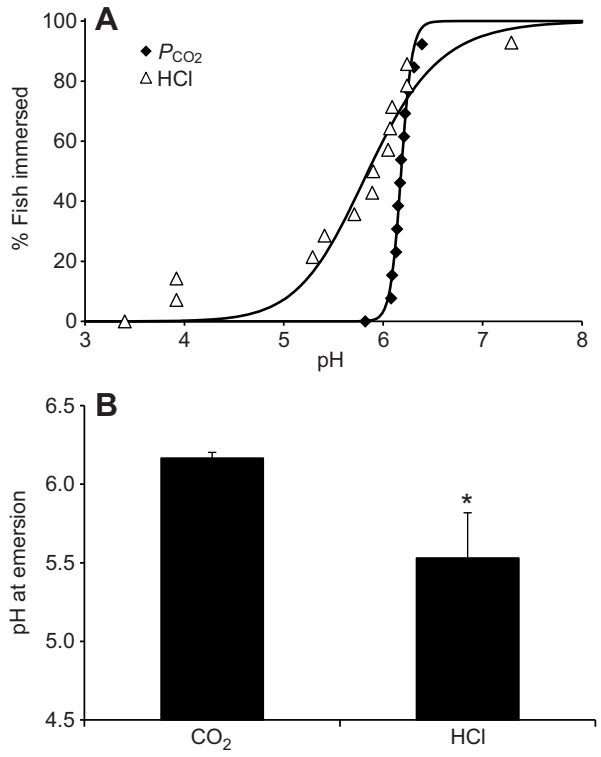

Fig. 2. Emersion response of mangrove rivulus to acute hypercapnia and isocapnic acidosis. (A) Proportion of fish immersed in water at low pH, achieved by increasing water P_{CO_2} or adding HCl. The line of best fit for both the P_{CO_2} response experiment and the HCl response experiment follows a sigmoidal curve. (B) Mean (+s.e.m., N=14) pH at emersion of mangrove rivulus exposed to increased P_{CO_2} or HCl. *Significant difference from P_{CO_2} value (P<0.05).

The hypothesis that NECs respond to overall intracellular pH is supported by the observation that glomus cells of the mammalian carotid body, thought to be homologues of NECs, require intracellular acidification in order to sense CO_2 (Putnam et al., 2004). However, the sensing and signalling mechanisms governing NECs are still unclear. While NECs *in vitro* require extracellular acidification to induce a rise in intracellular Ca^{2+} (Abdallah et al., 2015), CO_2 can cause membrane depolarization of zebrafish NECs at a constant external pH (Qin et al., 2010). This is at least in part regulated by carbonic anhydrase-mediated dehydration of CO_2, and subsequent intracellular acidification (Qin et al., 2010). Inhibition of carbonic anhydrase still eventually results in intracellular acidification and membrane depolarization, albeit at a lower magnitude (Qin et al., 2010). These data support our findings that H^+ alone either takes longer or requires a lower pH threshold to elicit a behavioural response and provide a possible explanation for the large variability in the response.

Conclusions

The mangrove rivulus exhibits remarkable phenotypic plasticity in the face of both external CO_2 and H^+ challenges. The NECs of the gills sense these conditions and probably mediate emersion during acute changes. Chronic exposure to elevated CO_2 and isocapnic acidosis that was less severe than the emersion threshold levels caused proliferation of NECs in mangrove rivulus. It remains to be seen whether these cellular changes will allow *K. marmoratus* to better sense acute changes in environmental conditions that approach their tolerance limit. The ability to sense CO_2/H^+ allows the mangrove rivulus to avoid adverse aquatic conditions and may play a significant role in the behavioural ecology of these and other amphibious fishes. It is also possible that these same mechanisms may have driven the emersion response in ancestral vertebrates in an effort to avoid hypercapnic/acidic conditions.

MATERIALS AND METHODS

Experimental animals

All experimental animals were hermaphrodites of the self-fertilizing (Slc 8E strain) *K. marmoratus* housed at the Hagen Aqualab, University of Guelph. Fish were individually reared to at least 6 months of age in 120 ml plastic containers (FisherBrand Collection Containers, Fisher Scientific) under constant conditions (25°C, 15‰, pH 8.3, 12 h light:12 h dark cycle) (Regan et al., 2011). Synthetic brackish water was made by dissolving Instant Ocean sea salt (Spectrum Brands, Blacksburg, VA, USA) in reverse osmosis water (total alkalinity=1.1 mequiv kg^{-1}; Atkinson and Bingman, 1998). All experimental procedures were approved by the University of Guelph's animal care committee.

Experimental procedures

Fish were acclimated for 7 days (25°C) to one of four experimental treatments: water (control, pH 8.1), air, aquatic hypercapnia (P_{CO_2}=5.1 kPa, pH 6.8) or an isocapnic acidosis (pH 6.8). The brackish water control fish were maintained under normal rearing conditions (15‰, pH 8.3) at ambient CO_2 (0.4 kPa). Air exposure was achieved by placing fish on moist filter paper as previously described (Turko et al., 2012). Aquatic hypercapnic conditions were achieved by placing fish in a temperature-controlled CO_2 incubator (Sanyo CO-17A, Sanyo Electric Co., Japan). Aquatic hypercapnia decreased water pH from pH 8.1 to 6.8. To isolate the effects of CO_2 versus

pH, a final group of fish were acclimated to isocapnic (ambient atmospheric CO_2) acidosis by adding dilute HCl daily to well-aerated brackish water for a final pH of 6.8. All fish were fasted for the duration of the experimental acclimation period. Following 7 days, treatment fish were killed and gill baskets were dissected and fixed at 4°C by immersion with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.8) containing (in mol l^{-1}): NaCl, 137; Na_2HPO_4, 15.2; KCl, 2.7; and KH_2PO_4, 1.5.

NEC immunofluorescence and analysis

Procedures for immunohistochemical labelling of serotonergic gill NECs were modified from those previously described (Regan et al., 2011). Fixed gill baskets were washed in PBS and permeabilized for 48 h at 4°C. Permeabilizing solution (PBS-TX) contained 2% Triton X-100 in PBS (pH 7.8). Gill arches were then separated and incubated in polyclonal antibodies raised against 5-HT (diluted 1:250 in PBS-TX) for 24–48 h at 4°C. This antibody (cat. no. S5545, Sigma-Aldrich, Oakville, ON, Canada) was produced in rabbit and has previously been used to identify gill NECs in a number of fish species, including zebrafish and the mangrove rivulus (Jonz and Nurse, 2003; Regan et al., 2011). Tissue was rinsed in PBS-TX and treated with secondary antibodies (diluted 1:50), produced in goat and conjugated with fluorescein isothiocyanate (FITC, cat. no. 111-095-003, Cedar Lane, Burlington, ON, Canada), at 23°C for 1 h in darkness. Arches were rinsed in PBS and mounted on glass microscope slides in Vectashield (Vector Laboratories, Burlingame, CA, USA) to prevent photobleaching. Images were collected using an epifluorescence microscope (Axiovert, Zeiss, Jena, Germany) and Northern Eclipse software (Empix Imaging, Mississauga, ON, Canada).

The number of NECs in the gills of fish from each group was determined as follows. Images were taken of 6 filaments randomly selected from the first gill arches of the left and right side of each fish. Images were confined to the distal filament tips, where NECs are located. The total number of NECs from these images was divided by 6 to provide the mean number of NECs per filament. Statistical analysis was performed on these absolute values of NEC density, as well as on NEC density standardized to body size (number of NECs per filament divided by standard length, the length from the tip of the snout to the base of the caudal fin). The relative differences between treatment groups were the same whether or not the data were corrected for standard length; therefore, only the absolute values are reported here. See supplementary material Fig. S1 for length-corrected NEC density values.

Gill NECs are asymmetrical but give a two-dimensional projection that resembles an ellipse. We therefore estimated NEC size by calculating the elliptic projection area following πab, where a and b equal the semi-major and semi-minor axes, respectively. A previous study used projection area to estimate the size of gill NECs in zebrafish (Jonz et al., 2004).

Emersion threshold

The emersion response of mangrove rivulus was measured as before (Regan et al., 2011). Experiments were conducted on individual fish held in rearing containers (60 ml, 15‰ water). Fish were first habituated overnight to the presence of a pH microelectrode (MI-710, Microelectrodes Inc., Bedford, NH, USA) and a slow stream of bubbled air introduced to the bottom of the cup through PE20 tubing. Fish were observed as water pH was quickly reduced by bubbling CO_2 or adding 0.1 mol l^{-1} HCl into a stream of bubbled air. Water pH was monitored continuously and emersion was defined as the first instance upon which more than half of the fish's body length was above the water. To test the emersion response to increasing CO_2, the flow of bubbled gas into the cup was switched from air to a mix of 45% CO_2/55% air produced by a gas mixing pump (Wösthoff, Dortmund, Germany). This gas mix was chosen based on preliminary experiments because it stimulated emersion after 15.6±0.3 min and allowed us to make comparisons with previously published work (Regan et al., 2011), though it is probably not representative of natural rates of change. To measure the emersion response to isocapnic acidosis, a slow flow (0.12 ml min^{-1}) of 0.1 mol l^{-1} HCl in 15‰ water was delivered directly into the stream of bubbled air using an adjacent piece of PE tubing connected to a peristaltic pump. This concentration/flow rate was chosen to reduce pH at the same rate (0.13 pH units min^{-1}) as we achieved by bubbling CO_2 gas.

Data analysis

One-way ANOVA followed by Bonferroni *post hoc* tests were used to compare NEC density and projection area between groups. A Student's *t*-test was used to compare mean emersion threshold between acute hypercapnia- and acute isocapnic acidosis-exposed fish. Sigmoidal regression was used to calculate the effective dose (effective pH) at which 50% of fish had emersed (ED_{50}) in response to acidic hypercapnia or acute acidosis (SigmaPlot 11, Systat Software, San Jose, CA, USA).

Acknowledgements

The authors wish to thank Mike Davies, Theresa Williams, Matt Cornish and numerous undergraduate volunteers for animal care.

Competing interests

The authors declare no competing or financial interests.

Author contributions

C.E.R., A.J.T., M.G.J. and P.A.W. conceived and designed the project. C.E.R., A.J.T. and M.G.J. executed the experiments and analyzed the data. C.E.R. wrote the draft manuscript. C.E.R., A.J.T., M.G.J. and P.A.W. revised the manuscript.

Funding

Funding was provided by the Natural Sciences and Engineering Research Council of Canada Discovery grants program to P.A.W. (120513) and M.G.J. (342303), and Natural Sciences and Engineering Research Council of Canada graduate scholarships to A.J.T. and C.E.R.

Supplementary material

Supplementary material available online at
http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.123133/-/DC1

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