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RESEARCH ARTICLE

Neuroepithelial cells and the hypoxia emersion response in the amphibious fish *Kryptolebias marmoratus*

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SUMMARY

Teleost fish have oxygen-sensitive neuroepithelial cells (NECs) in the gills that appear to mediate physiological responses to hypoxia, but little is known about oxygen sensing in amphibious fish. The mangrove rivulus, *Kryptolebias marmoratus*, is an amphibious fish that respires *via* the gills and/or the skin. First, we hypothesized that both the skin and gills are sites of oxygen sensing in *K. marmoratus.* Serotonin-positive NECs were abundant in both gills and skin, as determined by immunohistochemical labelling and fluorescence microscopy. NECs retained synaptic vesicles and were found near nerve fibres labelled with the neuronal marker zn-12. Skin NECs were 42% larger than those of the gill, as estimated by measurement of projection area, and 45% greater in number. Moreover, for both skin and gill NECs, NEC area increased significantly (30–60%) following 7 days of exposure to hypoxia (1.5 mg l^{-1} dissolved oxygen). Another population of cells containing vesicular acetylcholine transporter (VAChT) proteins were also observed in the skin and gills. The second hypothesis we tested was that *K. marmoratus* emerse in order to breathe air cutaneously when challenged with severe aquatic hypoxia, and this response will be modulated by neurochemicals associated chemoreceptor activity. Acute exposure to hypoxia induced fish to emerse at 0.2 mg l^{-1}. When *K. marmoratus* were pre-exposed to serotonin or acetylcholine, they emersed at a significantly higher concentration of oxygen than untreated fish. Pre-exposure to receptor antagonists (ketanserin and hexamethonium) predictably resulted in fish emersing at a lower concentration of oxygen. Taken together, these results suggest that oxygen sensing occurs at the branchial and/or cutaneous surfaces in *K. marmoratus* and that serotonin and acetylcholine mediate, in part, the emersion response.

Key words: neuroepithelial cell, amphibious fish, rivulus.

INTRODUCTION

Hypoxia tolerance in fish is dependent on oxygen chemoreceptors that send appropriate signals to the central nervous system to modulate physiological changes. Oxygen sensing has been linked to a population of chemoreceptive neuroepithelial cells (NECs) located on the distal portion of the primary epithelium in the filaments of each gill arch and, in some species, throughout the respiratory lamellae (Dunel-Erb et al., 1982; Jonz and Nurse, 2003; Saltys et al., 2006). NECs are either oriented externally towards the incident flow of water, where they may respond to aquatic hypoxia, or internally, to detect blood chemistry changes (Perry and Reid, 2002). In zebrafish (*Danio rerio*), gill NECs responded to acute hypoxia by membrane depolarization; these NECs were shown to undergo hypertrophy and develop process extensions in response to chronic hypoxia (Jonz et al., 2004). Many neurochemicals are found within gill NECs, including serotonin (Perry et al., 2009) and acetylcholine (Milsom and Burleson, 2007). Furthermore, changes in discharge patterns recorded from the afferent nerves indicated that chemoreceptors of the gill were responsive to stimulation by hypoxia, cyanide, acetylcholine, serotonin and dopamine (Burleson and Milsom, 1995).

The gills are thought to be the key site of oxygen sensing in fully aquatic fish, but less is known about oxygen sensing in air-breathing fish. Air-breathing organs (ABOs) – such as the buccal pharyngeal cavity, swim bladder, intestine and fortified gills – have evolved in many hypoxia-tolerant fish (e.g. Johansen, 1970; Graham et al., 1978; Geiger et al., 2000; Affonso and Rantin, 2005). Air-breathing bichirs (*Polypterus delhezi* and *P. ornatipinnis*) detect hypoxia with chemoreceptors in the swim bladder epithelium (Zaccone et al., 2008). Denervation studies have provided indirect evidence of chemoreceptor activity within the orobranchial cavity of tambaqui (*Colossoma macropomum*) (Florindo et al., 2006) and along the pseudobranch of bowfin (*Amia calva*) (McKenzie et al., 1991). Therefore, there is some evidence that oxygen-sensitive chemoreceptors are present in non-branchial sites in some species. NEC-like cells that contained serotonin and received nervous innervation were described in the skin of larval zebrafish during stages when primarily cutaneous respiration occurred (Jonz and Nurse, 2006). To our knowledge, similar cells have not been described in the skin of adult amphibious fish, even though cutaneous gas exchange occurs in many amphibious species.

To determine the localization and characteristics of NECs in an air-breathing amphibious fish, we studied the mangrove rivulus, *Kryptolebias marmoratus* (Poey 1880) (formerly *Rivulus*). *Kryptolebias marmoratus* is a cyprinodont fish that lives amongst the mangrove forests of Florida, Central America and South America. They are often found in the ephemeral burrows of the blue land crab (*Cardisoma guanhumi*). Although *K. marmoratus* are relatively tolerant of extreme water conditions, they are often found out of water (emersed) on mangrove roots, in leaf litter or

within the insect-carved crevices of rotting logs (Taylor et al., 2008). When emersed in the laboratory, *K. marmoratus* remained relatively inactive with the operculum closed, and respired cutaneously (Ong et al., 2007). There is also evidence that, when submerged in water (immersed), *K. marmoratus* use both the branchial and cutaneous surface for excretion of nitrogenous wastes (Frick and Wright, 2002), but whether both the gills and the skin play a role in oxygen sensing is unknown. Abel et al. concluded that *K. marmoratus* were insensitive to aquatic hypoxia (Abel et al., 1987), but they tested a level of dissolved oxygen (2 mg l^{-1}) that may have been too high to elicit a response. However, *K. marmoratus* emersed in response to elevated aquatic levels of hydrogen sulphide with an effective concentration (EC_{50}) of 0.12 mg l^{-1} (Abel et al., 1987). It is possible that emersion is a respiratory response when water conditions limit oxygen uptake across the gills.

Three hypotheses were tested in this study. First, we hypothesized that both the skin and gills are sites of oxygen sensing in *K. marmoratus* because serotonin-positive NECs are present in the skin of cutaneous breathing larval fish (Jonz and Nurse, 2006) and may be retained in adult amphibious species that are skin breathers. If so, then NECs would be present in the skin and gills of *K. marmoratus* and NEC size or number would change in response to chronic hypoxic exposure. Second, we hypothesized that *K. marmoratus* are intolerant of severe hypoxia and emerse in order to breathe air cutaneously. Finally, we hypothesized that the putative hypoxia emersion response would be dependent, in part, on neurochemicals associated with NEC function. The effects of the neurochemicals serotonin and acetylcholine, found previously to stimulate oxygen chemoreceptors in the gills of *Onchorynchus mykiss* (Burleson and Milsom, 1995), were tested. We predicted that these neurotransmitters, and antagonists for their postsynaptic receptors, would alter the hypoxia emersion response by increasing or decreasing the sensitivity of fish to aquatic hypoxia.

MATERIALS AND METHODS

Experimental animals

All fish were reared in the mangrove rivulus breeding colony housed at the Hagen Aqualab at the University of Guelph, Guelph, Ontario, Canada. The fish used in these experiments were 1–2 year old hermaphrodites with a mean mass of 0.18±0.00 g. Fish were kept at 25°C with a 12:12 h light dark cycle in individual plastic cups containing 60 ml half strength seawater (15‰ salt water) made from reverse osmosis water and marine salt (Instant Ocean, Spectrum Brands Inc., Atlanta, GA, USA). The fish were maintained on a diet of *Artemia salina* nauplii three times per week and frozen chopped bloodworms once per month. Chamber water was replaced once per week. Prior to each experiment, the fish were acclimated to air-saturated (90–100%) 15‰ seawater for 2 weeks. During this acclimation period, fish were fed *Artemia salina* nauplii three times per week and water was changed twice per week to reduce waste accumulation.

Experimental protocol

Chronic hypoxia exposure

To determine whether gill and skin NEC morphometrics were altered with longer-term hypoxia exposure, fish were held for 10 days in hypoxia or normoxia (control). Ten fish were placed in perforated containers (120 ml) in a recirculation tank (20 l) where the oxygen level was reduced from 100% (7.3 mg l^{-1}) to 20% air-saturated water (1.5 mg l^{-1}) in decrements of 10–15% over 3 days and exposed to 20% air-saturated water for 7 days for a total exposure time of 10 days. Oxygen levels were regulated by introducing nitrogen gas into a header tank that fed into the recirculation tank. The control group of fish was exposed to 100% air-saturated water (7.3 mg l^{-1}) for 10 days. Fish were prevented from emersing during the experiment by filling each chamber to the lid (120 ml). Fish were fed the day prior to entering the chamber and fasted for the duration of the experiment. At the end of the experiment, fish were killed (400 mg l^{-1} MS-222, Sigma-Aldrich Canada Ltd, Oakville, ON, Canada), and gills and skin were removed and fixed as described below.

Emersion response to hypoxia

Emersion was recorded for individual fish using a web cam (QuickCam Pro™, Logitech, Fremont, CA, USA). Fish were recorded as water oxygen levels were reduced quickly by passing nitrogen gas through chamber water (60 ml), with or without neurotransmitter agonists or antagonists added to the water. The decrease in water oxygen levels followed an exponential curve with a logarithmic slope of –0.312±0.005 mg l^{-1} min^{-1}. Dissolved oxygen (DO) was monitored continuously until the moment of emersion. Emersion was defined as the first instance when the fish left the water, clearing at least more than half their body from the water. Emersed fish adhered to the vertical side of the chamber. In each treatment, fish were pre-exposed for 10 min to agonists or antagonists. Preliminary experiments exposing fish to drugs for 120 min showed similar effects on fish emersion behaviour as a 10 min pre-exposure period.

The drug concentrations selected were based on concentrations used previously in other studies on aquatic organisms and preliminary tests. In preliminary trials, a concentration of 10 μmol l^{-1} serotonin hydrochloride, 100 μmol l^{-1} acetylcholine chloride and 50 μmol l^{-1} ketanserin (+)-tartrate salt (5-HT_{2A} receptor antagonist) had no affect on *K. marmoratus* emersion. However, higher concentrations (100 μmol l^{-1} serotonin and ketanserin and 1 mmol l^{-1} acetylcholine) were effective. Fish were also exposed to 100 μmol l^{-1} hexamethonium chloride (acetylcholine nicotinic receptor antagonist) or 100 μmol l^{-1} atropine (acetylcholine muscarinic receptor antagonist). All pharmaceuticals used, with the exception of ketanserin (+)-tartrate salt, were water-soluble. Dimethyl sulfoxide (DMSO) was used as a vehicle for ketanserin (+)-tartrate salt in a final concentration of DMSO not exceeding 0.47%. A control experiment was performed with 0.47% DMSO added to the water. Treatment concentrations were selected based on concentrations used in previous studies: ketanserin in *K. marmoratus* (Rodela and Wright, 2006), serotonin in *Helisoma trivolvis* (Shartau et al., 2010), atropine in *Pagrus major* (Endo et al., 1992) and acetylcholine and hexamethonium in *Gadus morhua* (Nilsson et al., 1976).

Analysis

Water oxygen measurements

Vernier Clark-type electrodes and Logger Pro™ software (Vernier Software and Technology, Beaverton, OR, USA) were used during the emersion experiments to monitor DO in the water. The electrode was calibrated each day before use with 2 mol l^{-1} sodium sulphite solution and air-saturated water (same temperature and salinity of the experimental water) to set the threshold for fully air-saturated water. DO was calculated (mg l^{-1}) using estimated atmospheric pressure and measured water salinity and temperature as described by the manufacturer. Salinity was measured with a hand-held salinity refractometer (Foster and Smith Aquatics, Rhinelander, WI, USA). For the chronic hypoxia experiment, a field oxygen probe (LDO101 IntelliCAL Standard Dissolved Oxygen Probe, Hach, Loveland, CO,

Table 1. Antibodies used to label neuroepithelial cells and surrounding innervation for immunohistochemistry

Antisera	Dilution	Antigen	Host	Source	Fluorescent marker*
Primary					
Serotonin	1:500	Serotonin	Rabbit	Sigma-Aldrich	FITC
SV2	1:200	SV2	Mouse	DSHB	Alexa Fluor 594
zn-12	1:25	zn-12	Mouse	DSHB	Alexa Fluor 594
VAChT	1:250	VAChT	Rabbit	Sigma-Aldrich	Alexa Fluor 594
Secondary*					
FITC	1:50	Rabbit	Goat	Cedarlane	–
Alexa Fluor 488	1:200	Mouse	Goat	Invitrogen	–
Alexa Fluor 594	1:200	Rabbit	Donkey	Invitrogen	–
Alexa Fluor 594	1:100	Mouse	Goat	Invitrogen	–

*Fluorescent markers were conjugated to secondary antibodies.
DSHB, Developmental Studies Hybridoma Bank, University of Iowa, IA, USA; FITC, fluorescein isothiocyanate; SV2, synaptic vesicle transmembrane protein; VAChT, vesicular acetylcholine transporter; zn-12, zebrafish neuron.

USA) was used daily to ensure that experimental levels of oxygen were maintained.

Immunohistochemistry and fluorescence microscopy

The immunohistochemical procedures were previously described by Jonz and Nurse (Jonz and Nurse, 2003). Tissues were immersed in solutions containing working concentrations (Table 1) of the following primary antibodies: anti-serotonin, antibody specific for a synaptic vesicle transmembrane protein (SV2), a zebrafish neuron specific antibody (zn-12) and the vesicular acetylcholine transporter antibody (VAChT). These antibodies have previously been used in other teleost species to label NECs and their innervation (Jonz and Nurse, 2003; Saltys et al., 2006; Coolidge et al., 2008). Working concentrations of secondary antibodies [fluorescein conjugated affinity purified secondary antibody (FITC) and Alexa Fluor 594 and 488 (Table 1)] were applied to the tissue samples once the excess primary antibodies had been removed. Tissues were immediately mounted on standard microscope slides with Fluoromount mounting media (Sigma-Aldrich) and analyzed on an epifluorescence microscope (Nikon Eclipse 90i™, Tokyo, Japan).

NEC morphometrics

Total NECs in the gills and skin were estimated by multiplying the number of gill filaments or the surface area of the skin of each fish by the calculated NEC density. The total number of gill filaments was determined by counting the number of gill filaments per arch and taking the mean value of all gill arches (386±9). Given the body morphology of mangrove rivulus (i.e. relatively slender), the surface area of the skin (A_s) was calculated as a cylinder and a half cone (head shape):

$$A_s = 2\pi r l_b + \pi r\sqrt{(r^2 + l_h^2)}, \quad (1)$$

where r is the body radius (half the diameter of the middle of the fish), l_b is the total length of the fish's body minus the fish's head length and l_h is the length of the fish's head. This is considered to be a conservative estimate because it excludes parts of the fins that were found to contain NECs.

The density of NECs on the skin of mangrove rivulus was measured using a 20× objective lens (field area=0.86 mm^2) and obtained for several fields of view for the entire skin surface from just behind the operculum to the tip of the caudal fin. Values from all fields were divided by the total surface area and expressed as the number of NECs per mm^2. NEC density of the gills was determined by counting the number of NECs per filament on 10 filaments on each of four gill arches on the left side. For this tissue, density is reported as the number of NECs per filament. The auto detect area tool from NIS-Elements AR 3.1™ imaging software (Nikon Instruments Inc., Melville, NY, USA) was used to measure the total projection area of the cells for 30 randomly selected NECs on the skin and for 30 NECs on the gill filaments, thus providing a measure of cell size.

Statistical analysis

To determine significant differences in DO level at the moment of emersion between control and serotonin- or ketanserin-treated fish, an unpaired Student's *t*-test was used. Differences between control and fish treated with acetylcholine, atropine and hexamethonium were determined with a one-way ANOVA and *post hoc* multiple comparisons testing was done using Dunn's method for unequal treatment group sizes. Significant differences were noted for $P \leq 0.05$. Logistic regression was used to calculate the effective concentration at which 50% of mangrove rivulus emersed (EC_{50}) in response to hypoxia and neurochemical agonists or antagonists. The goodness of fit for the logistic curves was described by the probability of the Chi-square test on the log ratio. For probabilities lower than 0.05, it was concluded that significant information was given by the logistic regression. Student's *t*-tests were used to distinguish significant differences ($P \leq 0.05$) of NEC density or area between and within fish exposed to 20 or 100% air-saturated water. All tests were preformed using XLstat™ for Microsoft Excel™ (Addinsoft USA, New York, NY, USA).

RESULTS

Immunohistochemistry

NECs immunopositive for serotonin were found in the gills of *K. marmoratus* (Fig. 1). The majority of these cells were also immunopositive for SV2 and clustered at the tips of the gill filaments of all arches. In addition, we observed SV2-positive NECs that were not immunolabeled by serotonin (Fig. 1). These were located at a more proximal region of the gill filaments than serotonergic NECs. No NECs were found on gill lamellae, nor was there any serotonin labelling on gill lamellae. Cells immunopositive for serotonin that resembled NECs were also found over the entire cutaneous surface and appeared to occupy the epithelial layer (Fig. 2). These cells were also SV2-positive (Fig. 2) and located amongst a network of zn-12-immunoreactive nerve fibres (Fig. 2). In some cases, serotonergic cells of the skin produced membrane processes or specializations (Fig. 2).

Cells labelled by antibodies against VAChT proteins were also found in the gills and skin of *K. marmoratus* (Figs 3, 4). These cells were probably a separate population of cells from the serotonin- and SV2-positive NECs. Both cell types were found in close

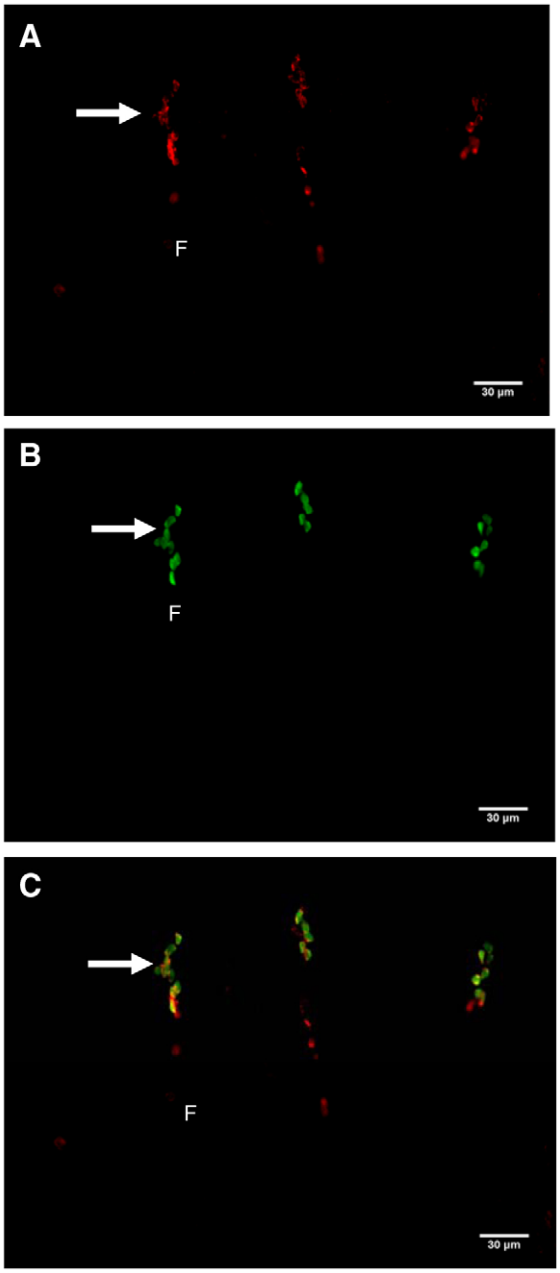

Fig. 1. Epifluorescent images (400×) of neuroepithelial cells (NECs) on the gill filaments of *Kryptolebias marmoratus*. NECs are labelled with antibodies against (A) synaptic vesicles (SV2) (red) and (B) serotonin (green). Arrows mark NECs. F, gill filament. (C) Composite image of A and B.

proximity to each other. In addition, one would expect that acetylcholine would be confined to synaptic vesicles. Although the synaptic vesicle antibody did not colocalize with the VAChT protein antibody, it may be that other markers for synaptic vesicles in these acetylcholine-positive cells would be more effective.

There were no significant differences in the density of gill and skin NECs between fish exposed to 100% (control) or 20% air-saturated (hypoxic) water for 7 days (Fig. 5A,B). However, the projection area of NECs increased significantly in both the gills (+60%, P=0.003) and the skin (+32%, P=0.013) following exposure to hypoxia for 1 week (Fig. 6). In addition, skin NECs were 42% larger in projection area relative to gill NECs (P<0.0001) (Fig. 6). Although the estimated total number of NECs in the skin was greater relative to that in the gills in both control (+45%, P=0.007) and hypoxia-treated (+42%, P=0.014) groups, hypoxia did not appear to have any effect on cell number (Fig. 5C). The distribution of cutaneous NECs is variable; Figs 2 and 4 show images taken from different areas on the same section of skin and demonstrate this.

Emersion response to hypoxia

Fish became increasingly more agitated with an acute decrease in water oxygen levels from 7.3 to 0 mg l^{-1} over a 15 min period. They appeared to increase swimming activity and spent more time at the air–water interface prior to emersion. *Kryptolebias marmoratus* showed a consistent emersion response (21 out of 21 fish) when DO levels were close to zero. The EC_{50} for DO was 0.23 mg l^{-1} (Fig. 7).

The threshold concentration of DO that provoked emersion in *K. marmoratus* was influenced by whole body pre-exposure to neurochemicals. When fish were pre-exposed to 100 μmol l^{-1} serotonin, the threshold low oxygen level that induced emersion increased by 72% (P=0.007; Fig. 8). When a separate group of fish were pre-exposed to 100 μmol l^{-1} ketanserin (serotonin receptor antagonist) dissolved in 0.47% DMSO, emersion occurred at a significantly lower concentration of oxygen (–51%, P=0.015) compared with controls exposed only to 0.47% DMSO (Fig. 8).

Acetylcholine also altered the emersion response to hypoxia. Following a pre-exposure to 1000 μmol l^{-1} acetylcholine, *K. marmoratus* emersed from the water at a 92% higher DO concentration (P<0.01; Fig. 9). Atropine (100 μmol l^{-1}), a known inhibitor of the muscarinic acetylcholine receptors, induced the fish to emerse at a significantly higher concentration of DO (82%, P<0.01; Fig. 9). In contrast, the nicotinic acetylcholine antagonist, 100 μmol l^{-1} hexamethonium, resulted in a suppressed emersion response (–63%, P<0.05; Fig. 9). In a final experiment, fish were pre-exposed simultaneously to 100 μmol l^{-1} serotonin or 1 mmol l^{-1} acetylcholine and the emersion response to hypoxia was recorded to determine whether the effects of these neurochemicals were additive. DO concentration at emersion when fish were pre-exposed to both neurochemicals was not significantly different from fish exposed to either serotonin or acetylcholine alone (Fig. 10).

DISCUSSION

NEC morphology and chronic hypoxia

We hypothesized that both branchial and cutaneous tissues are sites of oxygen sensing in *K. marmoratus*. Indeed, NECs were identified in the gills that were of similar morphology and distribution to gill NECs of most other fish examined (Dunel-Erb et al., 1982; Bailly et al., 1992; Jonz and Nurse, 2003; Saltys et al., 2006; Coolidge et al., 2008). In addition, NECs of *K. marmoratus* gills contained serotonin and cytoplamsic vesicles, as do those of other species. A similar population of cells, having both serotonin and secretory vesicles, was found in the skin of *K. marmoratus*. These cells appeared to be similar in general morphology to the serotonergic cells previously reported in the skin of zebrafish larvae (Jonz and Nurse, 2006), and we may consider these serotonergic cells of the skin to be neuroepithelial or paraneurons, as they exist in the epithelial layer and have the capacity to store and release neurotransmitters (Zaccone et al., 1994). The projection area of skin NECs was substantially larger than those of the gill, which may reflect the high degree of membrane specializations that were observed in these cells.

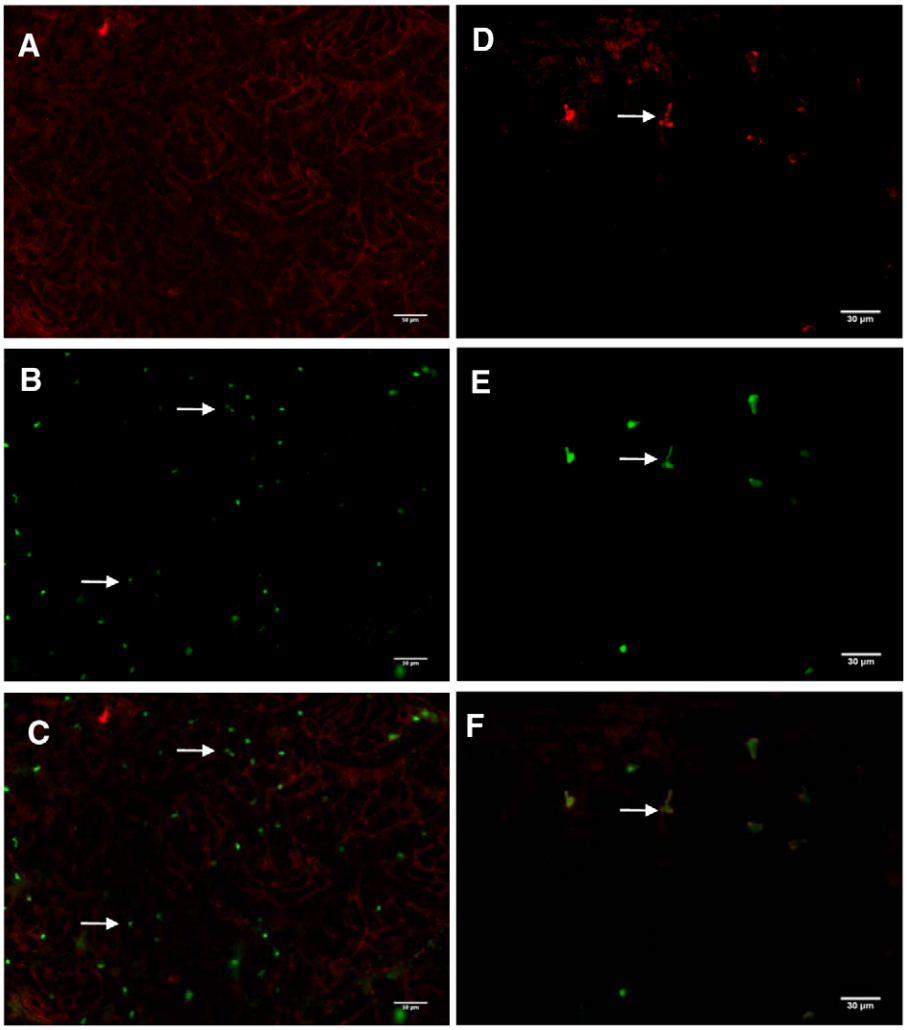

Fig. 2. Epifluorescent images of NECs on the skin of *K. marmoratus*. NECs are labelled with antibodies against (A) neurons (zn-12) (red), (B,E) serotonin (green) and (D) synaptic vesicles (SV2) (red). (C) Composite image of A and B. (F) Composite image of D and E. Arrows mark selected NECs.

Cellular hypertrophy, but not hyperplasia, of both gill and skin NECs occurred in response to chronic hypoxia in the present study. Hypertrophy of chemoreceptors in the fish gill, as well as in other tissues of higher vertebrates, has been documented. The serotonin-immunopositive NECs in the gill filaments of zebrafish increased in size, but not number, following hypoxic exposure (Jonz et al., 2004). In mammals, type I cells of the rat carotid body increase in size and are more numerous after chronic exposure to hypoxia, and this occurs along with neurochemical and physiological changes in the carotid body that are important during acclimatization (Wang and Bisgard, 2002; Powell, 2007). Our findings of NEC hypertrophy in the gills and skin of *K. marmoratus* may guide future investigations in this species to characterize potential functional changes in oxygen sensing at these sites following chronic hypoxia that may underlie acclimitization.

Acetylcholine has been shown to stimulate chemoreceptors in trout gills (Burleson and Milsom, 1995) and is a major excitatory neurotransmitter in the mammalian carotid body (Nurse, 2010). However, NECs of the gills and skin in *K. marmoratus* were not labeled with the VAChT antibody. This may suggest that if there is a cholinergic mechanism involved in O_2 sensing in the mangrove rivulus, then serotonergic NECs may not be invloved. However, non-serotonergic NECs as described in this study and others (Jonz and Nurse, 2003; Saltys et al., 2006) should not be ruled out because a neurotransmitter for these cells has not yet been identified.

Emersion response to hypoxia

Mangrove rivulus were previously reported to be insensitive to hypoxia (Abel et al., 1987) and field studies indicate that they often inhabit stagnant hypoxic crab burrows (see Introduction). However, our results indicate that, in the laboratory environment, the mangrove rivulus consistently emersed in response to aquatic oxygen depletion, and there was very little variation between individual fish with respect to DO at emersion. Therefore, there appears to be a critical oxygen threshold, at approximately 0.2 mg l^{-1}, at which the mangrove rivulus must switch from branchial to cutaneous respiration upon emersion. *Kryptolebias marmoratus* may first invoke a ventilatory response at moderate levels of hypoxia, described in many fish species (Perry et al., 2009), but at severe levels of hypoxia, alternative strategies, such as air breathing, appear to be required for survival.

It is not clear whether the hypoxic emersion response can be characterized as a reflex response to low oxygen or a behavioural preference to be in air during extreme bouts of hypoxia. Reflexes are involuntary, such as the bradycardia and hyperventilation experienced by most fish following stimulation of their chemoreceptors by hypoxia (Burleson and Milsom, 2003; Perry et al., 2009). The fact that the critical oxygen level for emersion was extremely consistent in *K. marmoratus* suggests that emersion is partially controlled by an oxygen-sensing mechanism and could be considered a reflex. Similar gill chemoreceptors that modulate

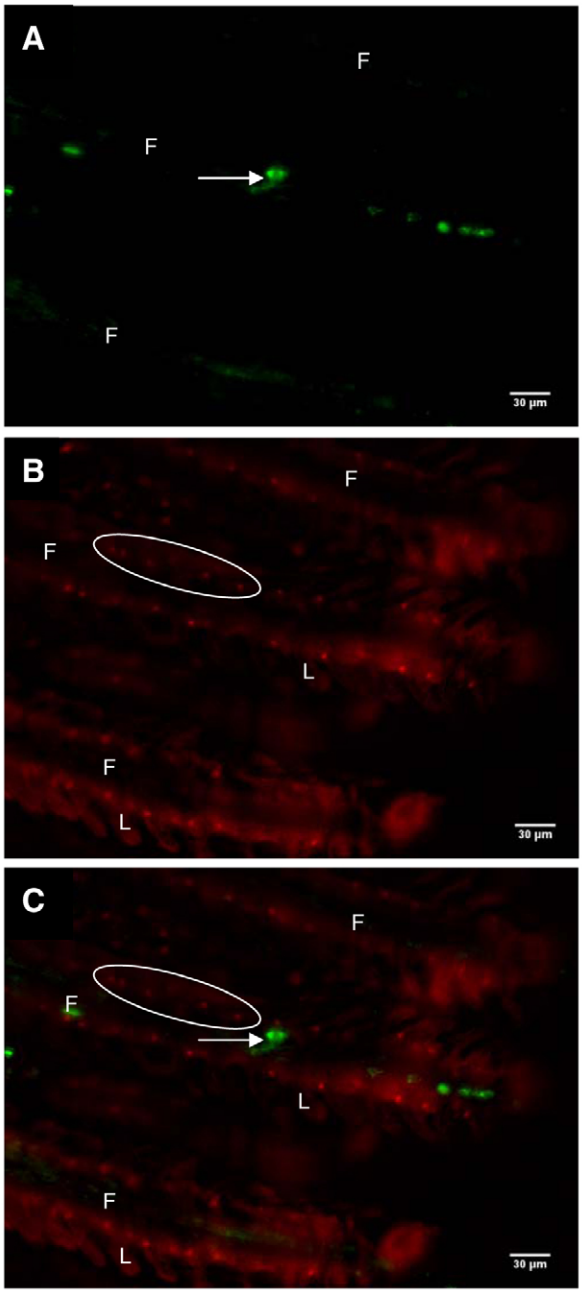

Fig. 3. Epifluorescent images (200×) of NECs and acetylcholine cells on the gill filaments of *K. marmoratus*. (A) NECs are labelled with antibodies against SV2 (green) and (B) acetylcholine cells are labelled with antibodies against VAChT (red). (C) Composite image of A and B. Arrows mark selected NECs; five selected acetylcholine cells are encircled. F, gill filament; L, lamellae.

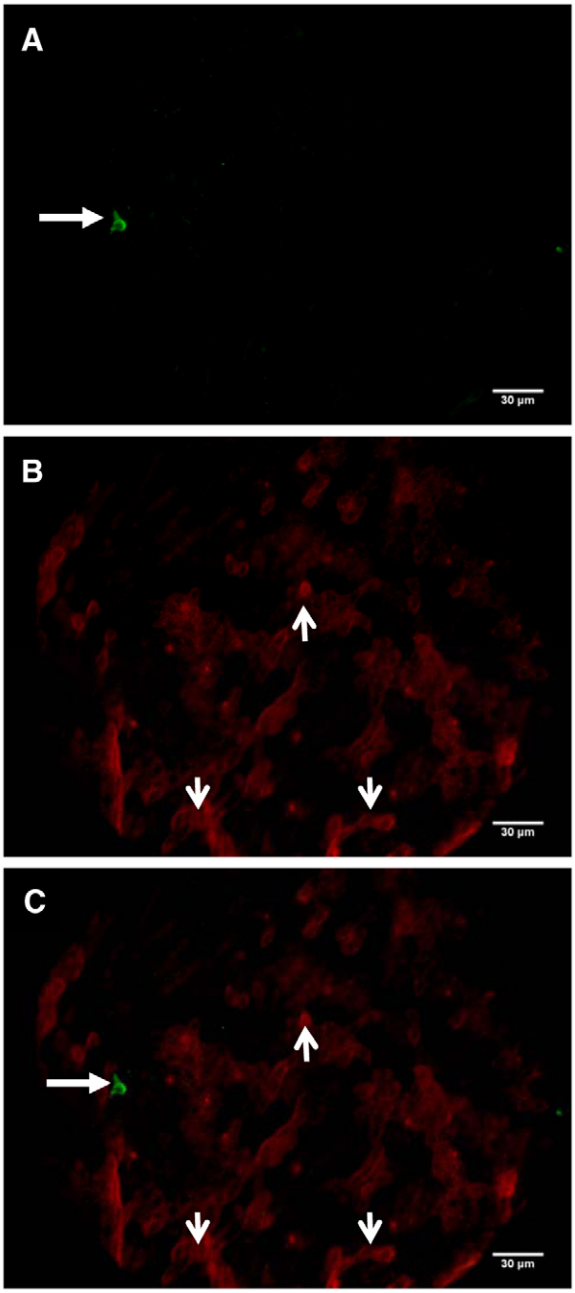

Fig. 4. Epifluorescent images (400×) of NECs and acetylcholine cells on the skin of *K. marmoratus*. (A) NECs are labelled with antibodies against SV2 (green) and (B) acetylcholine cells are labelled with antibodies against VAChT (red). Horizontal arrows mark an NEC; vertical arrows mark selected single or clustered acetylcholine cells. (C) Composite image of A and B.

hypoxia responses in aquatic fish have been shown to mediate surfacing and air gulping in some air-breathing fish (Smatresk, 1986; Shingles et al., 2005; Lopes et al., 2010). However, we have observed that *K. marmoratus* may emerse for a variety of reasons and do so even under unstressed normoxic conditions. In flathead gray mullet (*Mugil cephalus*), it was found that the reflex to hyperventilate and undergo aquatic surface respiration following stimulation with NaCN (chemically induced hypoxia) was altered when the fish were exposed to a model avian predator (Shingles et al., 2005). Likewise, it is possible that emersion in *K. marmoratus*, aside from the possibility of being a reflex to low oxygen, may be partially modulated by higher brain centers.

Role of neurochemicals in hypoxia emersion response

To understand more about the hypoxia emersion response in *K. marmoratus*, we bathed fish in neurochemicals known to be associated with oxygen sensing and NECs in vertebrates. We recognize that exposure to a bath solution of neurochemicals will

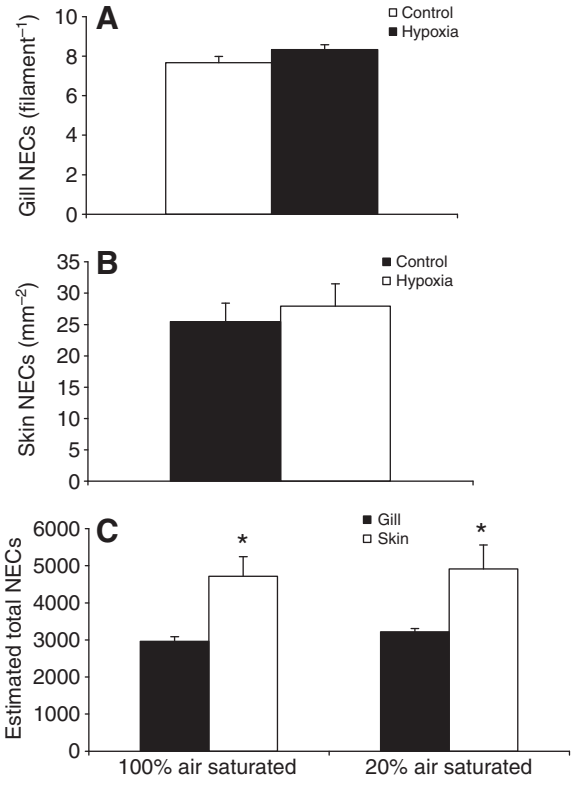

Fig. 5. NEC density in the (A) gills (no. filament^{-1}) and (B) skin (no. mm^{-2}), and (C) estimated total NECs of *K. marmoratus* exposed to 100% (control, *N*=10) and 20% (hypoxia, *N*=10) air-saturated water for 7 days. Data are means ± s.e.m. Asterisks indicate significant differences (P<0.05) between gills and skin.

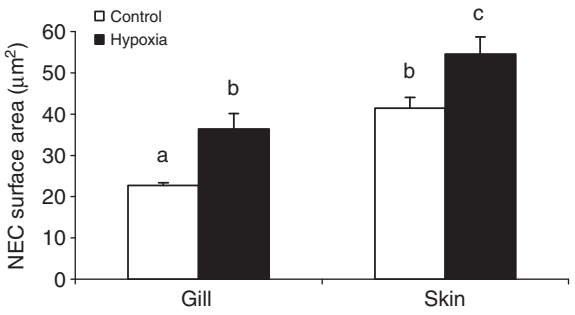

Fig. 6. Surface area of NECs of *K. marmoratus* exposed to 100% (control, *N*=10) and 20% (hypoxia, *N*=10) air-saturated water for 7 days. Data are means ± s.e.m. Letters indicate significant differences (P<0.05) between groups.

have stimulated both NEC populations and may have impacted other cells or tissues. Serotonin and acetylcholine are neurochemicals associated with multiple physiological systems in fish (Johansen and Reite, 1967; Sundin and Nilsson, 1992; Janvier, 1997). For these reasons, we chose a relatively short pre-exposure time of 10 min to reduce the time for drug absorption and to target the epithelial cells of the skin and gills. Despite the non-specific drug delivery, our data show a highly consistent and predicted response between agonists and antagonists. Finally, regardless of whether the observed changes were due to direct NEC interaction, the hypoxia emersion response was modulated in the presence of serotonin or acetylcholine and their respective receptor antagonists, implicating a role for these neurochemicals in oxygen sensing in *K. marmoratus*.

Serotonin exposure resulted in fish emersing at a higher oxygen concentration, thereby increasing their sensitivity to hypoxia, whereas ketanserin, an inhibitor of serotonin receptors (5-HT$_{2A}$), had the opposite effect. There is extensive evidence that serotonin is involved in oxygen sensing in fish and mammals. For example, chronic hypoxia exposure in this study and that of Jonz et al. (Jonz et al., 2004) resulted in an increase in the area of serotonin-immunopositive NECs. Burleson and Milsom also found that serotonin caused a weak burst of chemoreceptor activity when chemoreceptor afferent discharge in the glossopharyngeal nerve (cranial nerve IX) was monitored (Burleson and Milsom, 1995). In mammals, O_2-sensitive neuroepithelial bodies (i.e. clusters of NECs) release serotonin in response to hypoxia (Fu et al., 2002).

Because non-serotonergic NECs have been identified in the gill filaments of fish (Jonz et al., 2004; Saltys et al., 2006), it was useful in the present study to examine the effects of another potent neurochemical, acetylcholine, that has been implicated in oxygen sensing (Burleson and Milsom, 1995; Nurse, 2010). When mangrove rivulus were exposed to acetylcholine, they emersed at ~90% higher water oxygen concentrations relative to control fish, thereby increasing their sensitivity to hypoxia. The nicotinic receptor antagonist, hexamethonium, led to emersion at a 62% lower water oxygen level compared with control fish, thus blunting the emersion response to hypoxia. These results are consistent with findings by Burleson and Milsom, who identified acetylcholine and nicotine as potent neurochemical stimulants of chemoreceptors in rainbow trout (Burleson and Milsom, 1995). In contrast, when we blocked muscarinic receptors with atropine, the emersion response to hypoxia was augmented, such that fish were more sensitive to hypoxia. Therefore, it appears that nicotinic and muscarinic acetylcholine receptors may have very different roles in oxygen sensing in *K. marmoratus*. It is possible in *K. marmoratus* that muscarinic receptors have an inhibitory effect on the release of acetylcholine during hypoxia. Blocking muscarinic receptors may therefore have resulted in more acetylcholine released and an emersion response at a higher concentration of oxygen. This possible scenario is supported by studies in mammals. Carey et al.

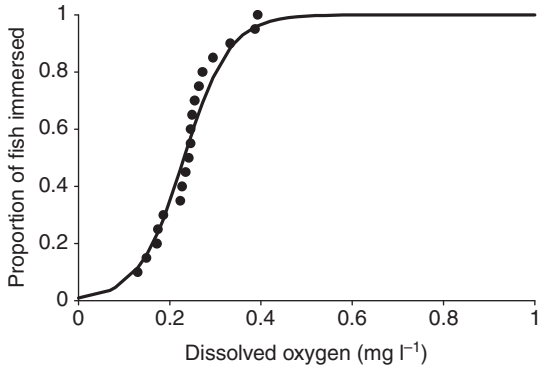

Fig. 7. Proportion of *K. marmoratus* immersed in water at low levels of dissolved oxygen (DO; mg l^{-1}). At lower DO levels (<0.4 mg l^{-1}), fish emersed and adhered to the side of the experimental chamber (*N*=21, EC_{50}=0.23 mg l^{-1} DO). Line of best fit follows logistic regression:

$$y = \frac{1}{1+e^{-(-4.6+20.1x)}} \quad (P<0.05)\ .$$

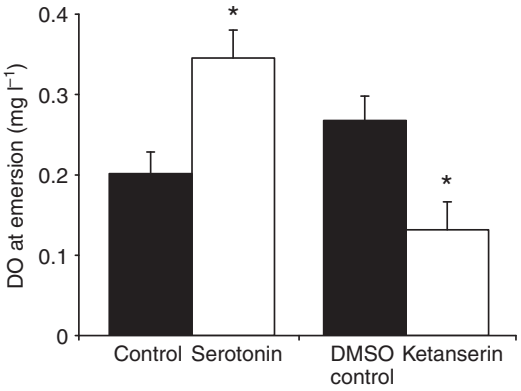

Fig. 8. DO level in the chamber when fish emersed from control water (15‰ salt water) and after pre-exposure to 100 μmol l^{-1} serotonin for 10 min (*N*=11 and 15, respectively) and 0.47% DMSO in 15‰ salt water (DMSO control) and 100 μmol l^{-1} ketanserin (*N*=6 and 8, respectively). Data are means ± s.e.m. Asterisks indicate a significant (*P*<0.05) change in DO level at emersion from controls.

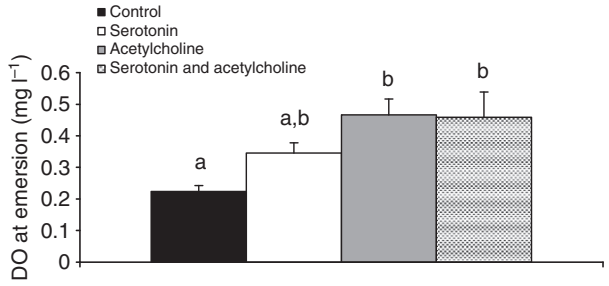

Fig. 10. DO level in the chamber when fish emersed from control water (15‰ salt water) and after pre-exposure to 1 μmol l^{-1} serotonin, 1 mmol l^{-1} acetylcholine and both serotonin and acetylcholine for 10 min (control *N*=21, serotonin *N*=10, acetylcholine *N*=9, serotonin + acetylcholine *N*=7). Data are means ± s.e.m. Letters indicate significant (*P*<0.05) differences in DO at emersion between treatments.

found that acetylcholine release increased in the central nervous system of rats when muscarinic receptors were blocked with an antagonist (SCH 57790) (Carey et al., 2001). Atropine also increased acetylcholine release in mice atria by preventing feedback inhibition through muscarnic receptors (Dawson et al., 1996). In rabbit carotid bodies, hypoxia caused an initial release of acetylcholine followed by a sustained inhibition by muscarinic receptors (Kim et al., 2004). Taken together, there is evidence that atropine may stimulate oxygen sensing during hypoxic exposure, but further work is required to validate this.

Finally, when fish were pre-exposed simultaneously to the neurotransmitters serotonin and acetylcholine, the emersion response was not significantly different from trials where the fish were exposed to either drug alone. Therefore, it is likely that there were no additive effects of these two drugs on emersion. It is also possible that the response was saturated and further stimulation was unable to evoke a stronger response. In light of our immunohistochemical studies, which indicate that serotonin and acetylcholine are not co-localized in gill and skin cells of *K. marmoratus*, the underlying neural pathways of the hypoxia emersion response may be relatively complex. Given this, it may be possible that the serotonin- and acetylcholine-containing cells are part of the same pathway; for instance, the NECs and acetylcholine-containing cells may modulate one another as part of the same pathway. This is an interesting avenue for further experimentation.

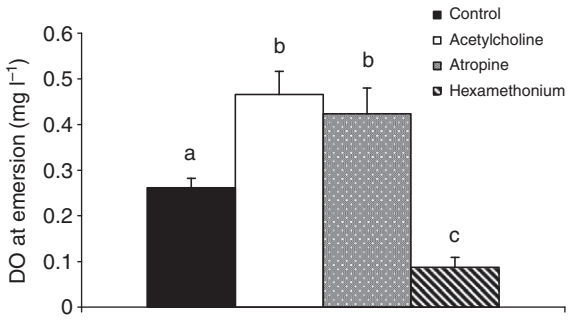

Fig. 9. DO level in the chamber when fish emersed after pre-exposure to a control (15‰ salt water), 1 mmol l^{-1} acetylcholine, 100 μmol l^{-1} atropine or 100 μmol l^{-1} hexamethonium (*N*=13, 9, 7 and 9, respectively). Data are means ± s.e.m. Letters indicate significant (*P*<0.05) differences in DO at emersion between treatments.

Conclusions and perspectives

In conclusion, the mangrove rivulus is an amphibious fish that has distinct populations of NECs in the gills and the skin. This is the first report of cutaneous NECs in an adult fish. In response to chronic hypoxia, there was an increase in the size of gill and skin NECs, suggesting that both NEC populations are sensitive to changes in DO and may play a role in oxygen sensing in an aquatic environment. The hypoxia emersion response was predictably modified by pre-exposure to serotonin, acetylcholine and their respective receptor antagonists, suggesting that emersion in *K. marmoratus* is regulated, in part, by neurotransmitters associated with NECs. A challenge of future studies will be to define the specific role for skin NECs in this species. Similar NECs of the skin in larval zebrafish have been observed (Jonz and Nurse, 2006), but in the air-breathing rivulus, which retains cutaneous gas exchange and NECs as adults, these cells may continue to play an important physiological or adaptive role.

In their natural habitat, *K. marmoratus* are often found in stagnant pools of water with high concentrations of hydrogen sulphide (0.7–5 mg l^{-1}) and ammonia (1 mmol l^{-1}), as well as low oxygen (0.2–2 mg l^{-1}) (Abel et al., 1987; Frick and Wright, 2002). NECs in the skin and gills may be also involved in sensing noxious compounds, such as ammonia and hydrogen sulfide in the aquatic habitat. Evidence in rainbow trout indicates that ventilatory modulation is affected by environmental hydrogen sulfide (Olson et al., 2008) and ammonia (Zhang and Wood, 2009), potentially through stimulation of gill NECs. Although skin NECs may play a crucial role in environmental sensing in aquatic habitats, they would completely dominate when fish emerse onto land. This is because in air, *K. marmoratus* develop a cell mass between gill lamellae that decreases the functional surface area of the gill and fish cease opercular movements (Ong et al., 2007). Therefore, it is likely that skin NECs would be more exposed and sensitive to changes in atmospheric gas composition (e.g. high H_2S) relative to the gill NECs. *Kryptolebias marmoratus* are known to emerse and seek insect-carved tunnels in rotting logs in mangrove forests (Taylor et al., 2008), and in this unusual terrestrial environment,

skin NECs may be important sensors if aerial conditions deteriorate.

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