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ORIGINAL PAPER

Gulf toadfish (*Opsanus beta*) gill neuroepithelial cells in response to hypoxia exposure

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Abstract

Neuroepithelial cells (NECs) within the fish gill contain the monoamine neurochemical serotonin (5-HT), sense changes in the partial pressure of oxygen (PO_2) in the surrounding water and blood, and initiate the cardiovascular and ventilatory responses to hypoxia. The distribution of neuroepithelial cells (NECs) within the gill is known for some fish species but not for the Gulf toadfish, *Opsanus beta*, a fish that has always been considered hypoxia tolerant. Furthermore, whether NEC size, number, or distribution changes after chronic exposure to hypoxia, has never been tested. We hypothesize that toadfish NECs will respond to hypoxia with an increase in NEC size, number, and a change in distribution. Juvenile toadfish ($N=24$) were exposed to either normoxia (21.4 ± 0.0 kPa), mild hypoxia (10.2 ± 0.3 kPa), or severe hypoxia (3.1 ± 0.2 kPa) for 7 days and NEC size, number, and distribution for each O_2 regime were measured. Under normoxic conditions, juvenile toadfish have similar NEC size, number, and distribution as other fish species with NECs along their filaments but not throughout the lamellae. The distribution of NECs did not change with hypoxia exposure. Mild hypoxia exposure had no effect on NEC size or number, but fish exposed to severe hypoxia had a higher NEC density (# per mm filament) compared to mild hypoxia-exposed fish. Fish exposed to severe hypoxia also had longer gill filament lengths that could not be explained by body weight. These results point to signs of phenotypic plasticity in these juvenile, lab-bred fish with no previous exposure to hypoxia and a strategy to deal with hypoxia exposure that differs in toadfish compared to other fish.

Keywords Serotonin · Filament · Lamellae · NEC size · NEC number · NEC distribution

Introduction

Gulf toadfish, *Opsanus beta*, are benthic marine teleost fish that frequently experience hypoxia in their natural seagrass environment (Frank et al. 2023; Hall et al. 1999; Hopkins et al. 1997; Lapointe and Clark 1992; Serafy et al. 1997) and are able to easily tolerate periods of hypoxia in a laboratory setting (Amador et al. 2018; Amador and McDonald 2022; Frank et al. 2023; McDonald et al. 2007; McDonald et al. 2010; Panlilio et al. 2016; Sebastiani et al. 2022; Walsh et al. 2007). In response to progressive hypoxia, the strategy of larval toadfish is to reduce oxygen consumption upon first detecting low oxygen levels (Frank et al. 2023) – indicating that these fish likely have a robust oxygen sensing mechanism. This survival strategy is reflected in a low regulation index (RI; Mueller and Seymour 2011) of 0.10 ± 0.05 (Frank et al. 2023), suggesting that cellular O_2 supply is not maintained when faced with lowering partial pressures of oxygen (PO_2). In contrast, juvenile toadfish have better regulating abilities than larvae and an RI (0.33; Frank et al. 2023) that is approaching adult Gulf toadfish (0.49 ± 0.05; Amador et al. 2018). That the RI in larvae, juvenile, and adult toadfish is substantially lower than 1.0 suggests that toadfish across all life stages use strategies other than just maintaining cellular oxygen levels to tolerate hypoxia (e.g., Nilsson and Renshaw 2004).

Not yet characterized in toadfish are the gill neuroepithelial cells (NECs) that are involved in O_2 sensing and are important in hypoxia survival (Jonz et al. 2004; reviewed

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by Jonz et al. 2016). Gill NECs, present in all fish species studied to date (e.g., Coolidge et al. 2008; Dunel-Erb et al. 1982; Jonz et al. 2004; Regan et al. 2011; Saltys et al. 2006 Tzaneva and Perry 2010; Zhang et al. 2011), are specialized cells that may be homologues to the glomus cells of the carotid body or the neuroendocrine cells of the lung epithelia (Hockman et al. 2017; Jonz and Nurse 2003; Yokoyama et al. 2015). NECs can be oriented both outwardly towards the surrounding waters and internally towards the circulation of blood in the body to allow fish to sense changes in PO_2 in both the water and blood (Leite et al. 2007; Perry and Reid 2002). NECs contain dense core vesicles and the majority are enriched with 5-HT (Dunel-Erb et al. 1982; Jonz and Nurse 2003; Porteus et al. 2012; Saltys et al. 2006). In response to hypoxia, the NECs depolarize and the dense core vesicles within the NECs appear to degranulate or release their contents (Dunel-Erb et al. 1982; Jonz et al. 2004). These changes in NECs are believed to initiate various behavioral and physiological responses, for example, increases in ventilation that work to optimize gas exchange (Burleson and Milsom 1995; Holeton and Randall 1967a, b; Randall 1982; Randall et al. 1965; Shelton et al. 1986; reviewed by Perry et al. 2023).

Because most NECs are replete with 5-HT, they are typically identified by exploiting their 5-HT immunoreactivity, however, some NECs are not 5-HT immunoreactive (5-HT-IR) and are distinguished by their proximity to 5-HT-IR cells and immunoreactivity to SV-2 (SV-2-IR), a marker of synaptic vesicles (Jonz et al. 2004). NECs can be distributed along the length of the filament, sometimes unevenly, with higher cell numbers typically found at the distal end, and many fish have NECs within the lamellae (Porteus et al. 2012; Saltys et al. 2006; Coolidge et al. 2008; Jonz and Nurse 2003). While no clear pattern has emerged suggesting that the distribution of NECs is related to hypoxia tolerance, the movement of NECs towards the very tips of the lamellae has been shown in hypoxia-tolerant-goldfish (*Carassius auratus*) when exposed to hypoxia demonstrating that NECs can be sensitive to environmental condition (Tzaneva and Perry 2010; Tzaneva et al. 2011). NEC size can be sensitive to hypoxia exposure; an increase in NEC size has been measured in zebrafish (*Danio rerio*) when exposed to up to 60 days of 35 mmHg (4.7 kPa) hypoxia (Jonz et al. 2004; Pan et al. 2021) and in the gill and skin NECs of the mangrove rivulus (*Kryptolebias marmoratus*) in response to a 7-day exposure to 20% air-saturated water (Regan et al. 2011). With respect to NEC number, mild hypoxia (80 mmHg, 10.7 kPa) exposure reduced the natural reduction in cutaneous NECs that is typically measured in developing zebrafish larvae in normoxia and severe hypoxia (30 mmHg, 4.0 kPa) exposure halted the reduction of cutaneous NECs entirely (Coccimiglio and Jonz 2012; Dean et al. 2016). Furthermore, NECs found in the gills of adult zebrafish were found to have an increase in the number of SV2-IR NECs, but not 5-HT-IR NECs, when exposed to 60 days of 35 mmHg (4.7 kPa) hypoxia (Jonz et al. 2004). This is supported by a recent study that demonstrated an increase in gill cells that express vesicular monoamine transporter (*vmat2*), a protein that mediates the storage of 5-HT into synaptic vesicles, when adult zebrafish were exposed for 14 days to severe hypoxia (Pan et al. 2021).

The objective of this study was to determine whether toadfish 5-HT-IR NEC size, number, or distribution are sensitive to hypoxia exposure. We hypothesize that toadfish NECs will respond to hypoxia with an increase in NEC size, number, and a change in distribution. This hypothesis was tested by determining the size, number, and distribution of 5-HT-IR NECs within the toadfish gill during normoxia and after exposure to mild or severe hypoxia for 7 days.

Methods and materials

Experimental animals

This experiment utilized 13- to 16-month-old juvenile Gulf toadfish, *Opsanus beta* (Goode & Beane 1880) that were bred in the lab at the Rosenstiel School of Marine, Atmospheric, and Earth Science in 2020 (Frank et al. 2023). Briefly, adult toadfish were sexed by ultrasound (Cartolano et al. 2019) and male/female pairs were placed into 20-gallon aquaria or large pools with a sandy bottom and artificial seagrass to mimic a natural environment. Both tanks and pools contained a large PVC pipe as a nesting site and were provided with flow-through, filtered seawater from Bear Cut, Biscayne Bay, FL that was aerated so that fish were held under constant normoxic conditions. Once hatched, the juvenile fish were housed in 5-gallon aquaria initially and then moved to larger aquaria as they grew. Fish received frozen artemia five days a week to satiation, and all fish were treated with Rid-Ich Plus (Kordon, Hayward, CA) once every 2.5 weeks to prevent parasite infestations. Approval for experimental protocols using animals was given by the University of Miami Institutional Animal Care and Use Committee (Protocol No. 21–139).

Experimental protocol

Juvenile toadfish (1.63 ± 0.09 g, $N = 24$) were selected semi-randomly using Random.org to choose the tank number for each fish, targeting juveniles that were between approximately 1–2 g, and placing them into one of three groups: control (normoxia-exposed; $PO_2 = 21.4 \pm 0.0$ kPa, 160.4 ± 0.1 Torr), mild hypoxia-exposed

($PO_2 = 10.2 \pm 0.3$ kPa, 76.4 ± 2.1 Torr), and severe hypoxia-exposed ($PO_2 = 3.1 \pm 0.2$ kPa, 22.9 ± 1.8 Torr). The PO_2 of the mild group was above the estimated P_{crit} of adult Gulf toadfish (~6.7 kPa, Amador et al. 2018) and the P_{crit} established for the closely related *Opsanus tau*, of 3.9 ± 0.5 kPa (Ultsch et al. 1981). The PO_2 of the severe group was below the estimated toadfish P_{crit}. Normoxia was achieved by bubbling air from the building's air supply through an air stone into a header tank fed from a seawater reservoir kept at room temperature (24ºC). A second header tank, also fed from the reservoir, was used to create hypoxic conditions by bubbling nitrogen gas (N_2) from a gas cylinder (Airgas) through an air stone and placing a layer of bubble wrap on the water's surface to ensure the least amount of air-water O_2 exchange. To maintain the correct PO_2 for the hypoxia groups, the flow of N_2 from a tank was controlled by a custom relay system. An O_2 probe (Vernier, Beaverton, OR), plugged into a LabQuest Stream (Vernier, Beaverton, OR) interface connected to a laptop running Logger Lite (Vernier, Beaverton, OR), was placed into the hypoxia header tank to take continuous O_2 readings in real time. Logger Lite was set up to send a signal through the interface to a digital control unit (Vernier, Beaverton, OR) to trip a relay when the air saturation dropped to a specified level; 50% air saturation (10.2 ± 0.3 kPa) for mild hypoxia and 12.5% air saturation (3.1 ± 0.2 kPa) for severe hypoxia. The relay caused a solenoid valve (Burkert Fluid Control Systems, Ingelfingen, Germany) attached to the N_2 tank to open or close to adjust the flow of N_2. If the valve was open, N_2 could flow through the air stone and decrease the PO_2 of the water. Once the PO_2 dropped to the desired level, the valve would close and stop the flow of N_2. A maximum of four fish were exposed at a time, each in its own 250 mL experimental chamber, with a layer of bubble wrap on the surface of the water and lid on the chamber, which was fed from the header tank with the appropriate level of O_2 for 7 days. This length of exposure was decided upon based on previous literature on hypoxia-tolerant fish in which changes had been measured within 7–60 d (Jonz et al. 2004; Pan et al. 2021; Regan et al. 2011). We decided on a shorter exposure because chronic hypoxia exposures had never been done on toadfish and juvenile toadfish may be more sensitive to hypoxia exposure than adult toadfish (Amador et al. 2018; Frank et al. 2023). Each 7-d exposure was completed with at least one control fish, and 1–3 fish exposed to either mild or severe hypoxia at the same time. During exposures, juvenile fish were fed frozen artemia to satiation for 4 of the 7 days, and debris was removed from the chamber when necessary. At the end of the 7-d exposure, fish were sacrificed with an overdose of tricaine methanesulfonate (MS 222) (3 $g \cdot L^{-1}$) and weighed.

Gill whole mounts

Gill arches were dissected from fish to create whole mounts as described by Jonz and Nurse (2003). All six gill arches (three from each side) were used. Each arch was placed into individual 1.5 mL bullet tubes containing 1 mL 4% paraformaldehyde (PFA) in phosphate buffer solution (PBS) and incubated for 48 h at 4°C. Tubes were laid on their sides to allow for the gills to fix relatively flat. After the first incubation period, the PFA was removed, and gill arches were rinsed in 20% sucrose in PBS for 5 min. The sucrose was replaced with 1 mL of fresh 20% sucrose and left to soak for 24 h at 4°C. Once the soaking period was over, gill arches were moved from the bullet tubes into a 96-well plate and overlaid will 200 µl of PBS-TX (pH 7.6 PBS containing 1% fetal calf serum (FCS) and 0.5% Triton X-100) for 5 min. The no primary antibody control arches were overlaid with PBS-TX only and all other arches were overlaid with a 50-fold dilution of a polyclonal antibody against 5-HT [made in rabbit (Sigma-Aldrich, St. Louis, MO, catalogue # S5545)] in PBS-TX. Once gill arches were overlaid with their respective solutions, the 96-well plate was sealed with parafilm and left to incubate for 48 h at 4°C. After the final incubation period, the primary antibody or control solution was removed and gills were washed with PBS-TX three times, for 5 min each. All gill arches were then incubated in a secondary antibody solution (50-fold dilution of anti-rabbit Alexa Fluor™ 488 [made in goat (Life Technologies Corporation, Eugene, OR)] for 2 h at room temperature in the dark. The secondary antibody was removed and washed a final time in PBS-TX for 5 min. Gill arches were placed on a glass microscope slide and mounted with Vectashield (Vector Laboratories Inc., Burlingame, CA) and a cover slip for examination using fluorescence microscopy (Olympus BX61, Olympus Life Science, Tokyo, Japan).

Neuroepithelial cell size and number analysis

Images were taken of each gill arch under the FITC filter using a camera (Retiga Exi Fast 1394, Teledyne Photometrics, Tucson, AZ) that was attached to the microscope and controlled by a personal computer running the program Qcapture Plus, using a 10x objective. Each gill arch was examined to determine the most representative filament of that arch, yielding six representative filaments per fish for each O_2 regime. Any filaments that were damaged in any way (e.g., torn, ripped, or cut), or obscured by other filaments were not used. The representative filament was captured using the camera, sometimes in multiple images if the entire filament did not fit within the aperture of the camera lens. Images were then analyzed using FIJI Imaging Software. The filament, or section of the filament, of interest in

each image was measured using an image of a micrometer to set the image scale and the segmented line tool to accurately follow the shape and curve of the filament. If a filament was captured in its entirety in one image, a single line from base to tip was used to determine total length. Filaments that were captured in two or more images were first aligned at the point of overlap and then marked using the pencil tool. Total length was determined by summing up the measurements from each image, either from base to line, line to line, or line to tip. The total length of the filament was then divided by three to separate the filament into distal, middle, and proximal sections. The 5-HT-IR NECs present in the filament were counted and their surface area was measured using the freehand selection tool, and the values were recorded and put into matrices to be used in Rstudio. After all the data were collected, the straightest filaments with the most well-defined cells were imported into Procreate, a digital illustration app for the iPad. Here, whole filament images were created from filament images taken in parts, done by overlapping the images at their connecting points.

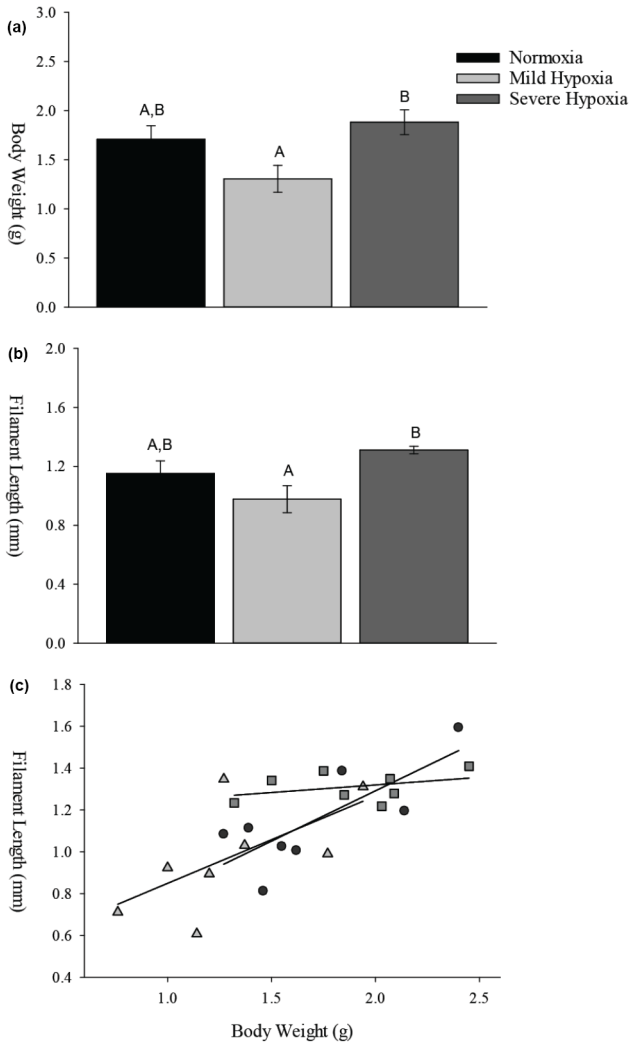

Fig. 1 Significant differences were measured in (**a**) body weight and (**b**) filament length between fish exposed to mild hypoxia and those exposed to severe hypoxia. Values are means ± SEM ($n = 8$); different letters represent differences between the oxygen regimes, $p < 0.05$. (**c**) A significant relationship between body weight and filament length is measured in fish exposed to normoxia (black circles; $y = 0.480x + 0.331$, $r^2 = 0.602$) and a similar relationship is measured in fish exposed to mild hypoxia (light grey triangles; $y = 0.417x + 0.433$, $r^2 = 0.392$). No relationship between body weight and filament length is measured in fish exposed to severe hypoxia (dark grey squares; $y = 0.072x + 1.175$, $r^2 = 0.140$)

Statistics

Data are shown as means ± the standard error of the mean (SEM). Data were first checked for outliers using both the Dixon and Grubbs outlier tests and any present were removed. Data were tested for normality using the Shapiro-Wilk test for normality and variances were compared using the F-test. Data that were not normally distributed were log-transformed. Differences in body weight and filament length were determined using a one-way ANOVA with O_2 regime as the main factor followed by a Holm-Sidak post-hoc test. The strength of the correlation between filament length and body weight were tested using a Peason Product Moment Correlation and differences between the slopes and y-intercept (y_0) of the lines were determined using a Student's t-test. Differences in filament length between arches were determined using a one-way ANOVA with arch # as the main factor followed by a Holm-Sidak post-hoc test. Differences in cell size and cell number were determined using a two-way ANOVA with O_2 regime and arch # as the main factors. Differences in cell distribution were determined using a three-way ANOVA with O_2 regime, arch #, and position (proximal 33%, middle 33%, or distal 33%) as the main factors followed by a Hold-Sidak post-hoc test.

Results

The average weight of toadfish exposed to severe hypoxia was 44% higher than fish exposed to mild hypoxia ($p = 0.019$), with no difference measured between normoxia-exposed fish and either hypoxia exposed group (Fig. 1a). Similarly, the average filament length of toadfish exposed to severe hypoxia was 34% longer than fish exposed to mild hypoxia ($p = 0.014$), with no difference measured between normoxia-exposed fish and either hypoxia exposed group (Fig. 1b). The difference in filament length between severe hypoxia-exposed and mild hypoxia-exposed fish persisted when looking at the individual arches (Table 1). Furthermore, arch 3 filaments were ~ 19% shorter than the filaments on arch 1 or 2 for fish of all O_2 regimes (Table 1). There was a significant relationship between filament length and body

Table 1 Filament lengths of gill arches from fish in normoxia, mild hypoxia, and severe hypoxia

	Arch 1 filament length (mm)	Arch 2 filament length (mm)	Arch 3 filament length (mm)
Normoxia	1.12 ± 0.11^{a}	1.26 ± 0.13^{a}	$1.08 \pm 0.12^{a*}$
Mild hypoxia	1.07 ± 0.14^{ab}	1.08 ± 0.12^{ab}	$0.78 \pm 0.06^{ab*}$
Severe hypoxia	1.52 ± 0.07^{b}	1.34 ± 0.03^{b}	$1.08 \pm 0.09^{b*}$

Values are represented as mean ± SEM.

Different letters denote a difference between O_2 regimes and an asterisk denotes a difference between Arch 1 and 2 (two way ANOVA, O_2 regime $p < 0.001$, arch $p < 0.01$, interaction $p = 0.362$; $n = 8$).

Fig. 2 Representative images of gill filaments containing 5-HT-IR NECs from fish held under (**a**) normoxia, (**b**) mild hypoxia, or (**c**) severe hypoxia. Scale bar is 0.1 mm

weight in normoxia-exposed fish ($r^2 = 0.602$, $p = 0.024$) but that relationship broke down in fish exposed to mild hypoxia ($r^2 = 0.392$, $p = 0.097$). The slopes and y_0 of the normoxia-exposed and mild hypoxia-exposed fish lines were not significantly different (Fig. 1c). In contrast, there was no relationship between body weight and filament length in fish exposed to severe hypoxia ($r^2 = 0.136$, $p = 0.369$). The slope of the severe hypoxia line was significantly different from that of normoxia-exposed fish ($p = 0.036$) but not fish exposed to mild hypoxia (Fig. 1c). The y_0 of the severe hypoxia line was significantly different than both the normoxia-exposed and mild hypoxia-exposed fish ($p < 0.05$; Fig. 1c).

Representative gill images from fish from each exposure are shown in Fig. 2, with bright white cells evident as 5-HT-IR NECs. Within each O_2 regime, there were no significant differences between the first, second, and third gill arches in NEC size; furthermore, there was no impact of hypoxia exposure on NEC size (Fig. 3). There was also no significant correlation between cell size and body weight for fish within any of the O_2 regimes (data not shown). There were no significant differences in NEC number between the first, second, and third gill arches within each O_2 regime (Fig. 4a). However, gills exposed to mild hypoxia had 33% fewer NECs per filament (34.9 ± 2.3, $n = 8$) than both fish exposed to normoxia (51.8 ± 4.1, $n = 8$; $p < 0.005$; Fig. 4a) and fish exposed to severe hypoxia (62.8 ± 3.1, $n = 8$; $p < 0.0005$; Fig. 4a), and fish exposed to severe hypoxia had 21% more NECs per filament than fish exposed to normoxia ($p < 0.05$; Fig. 4a). The difference in cell number persisted between the mild hypoxia exposure and the severe hypoxia exposure when cell numbers were normalized to either body weight (data not shown) or the number of cells per mm of filament (Fig. 4b). Fish exposed to mild hypoxia had 24.1% fewer cells per mm of filament (37.7 ± 2.3 cells/mm of filament) than fish exposed to severe hypoxia (49.6 ± 3.1 cells/mm of filament) ($p < 0.005$; Fig. 4b). However, when filament length was taken into account, there was no longer a difference in NEC count between normoxia and mild or severe hypoxia. For fish exposed to mild and severe hypoxia, 53% and 32% more NECs, respectively, were found on filaments from the third gill arch in opposed to the first gill arch, compared to no difference between gill arches in normoxia (Fig. 4b). The NECs were distributed along the gill filament, with the majority of the cells ($63.0 \pm 1.3\%$, $n = 8$) located in the distal end of the filament, $28.1 \pm 1.3\%$, $n = 8$ of the cells located in the middle and little to no cells ($8.9 \pm 0.2\%$, $n = 8$) located in the proximal end (Figs. 2 and 5; $p < 0.001$). There were no significant effects of O_2 regime on NEC distribution nor were there any impacts of arch # (Figs. 2 and 5). There were no NECs present within the gill lamellae in any group (Figs. 2 and 5).

Discussion

Unexpectedly, the average body weights of severe hypoxia-exposed fish were significantly higher than that of mild hypoxia-exposed fish at the end of the 7-d exposure period. When setting up the experiment, an attempt was made using visual approximation to ensure an equal distribution of body weights in each of our O_2 regimes; however, the juvenile fish were intentionally not weighed prior to hypoxia exposure in an effort to reduce unnecessary handling. Thus, it is most likely that the difference in body weight existed from the very beginning of the experiment. While less likely, it cannot be ruled out that severe hypoxia-exposed fish weighed

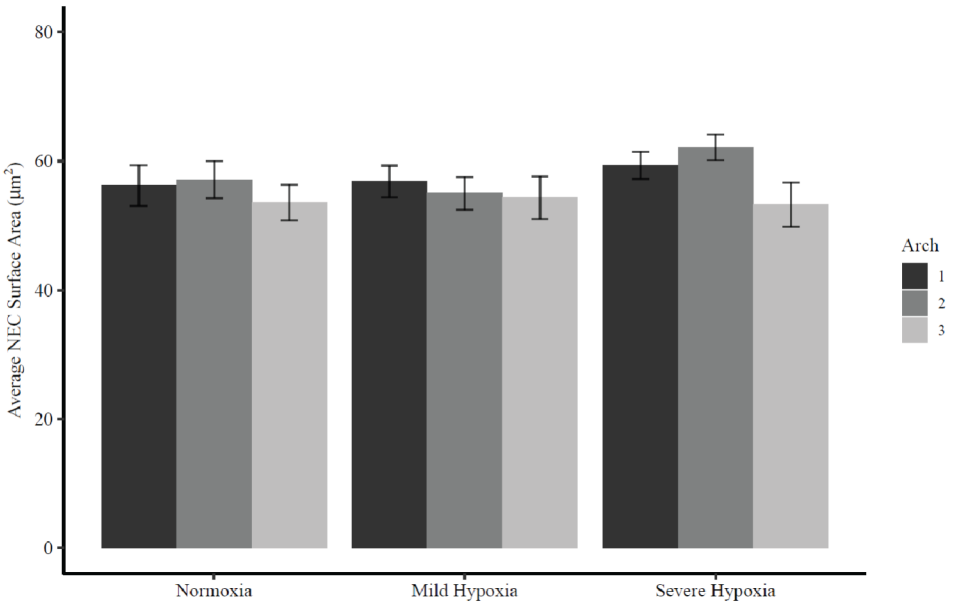

Fig. 3 The average size of the NECs in each gill arch from fish exposed to either normoxia, mild, or severe hypoxia does not change. Values are means ± SEM ($n = 8$)

more as a consequence of the 7-d hypoxia exposure. Gulf toadfish are bottom-dwelling fish that move < 1% of the time when in the lab (Cartolano et al. 2019), have a low routine metabolic rate, and a large aerobic scope (Amador et al. 2018; Frank et al. 2023; Sebastiani et al. 2022). As severe hypoxia-exposed fish were exposed to O_2 levels below their approximate P_{crit}, they had an even lower routine metabolic rate than their mild hypoxia-exposed counterparts. All fish were fed to satiation daily, and so, in theory, a higher proportion of food energy could have been available to go towards growth if food consumption was maintained, and digestive and absorptive processes continued to work sufficiently. Hypoxia primarily results in a reduction in the appetite of fish and can also have negative impacts on assimilation efficiency, resulting in a reduction in growth (reviewed by Wang et al. 2009). However, some fish have been shown to acclimatize over more chronic hypoxia exposures resulting in a recovery in growth rates (Petersen and Pihl 1995; Pichavant et al. 2000). Furthermore, the severe hypoxia-exposed fish of the present study were exposed to O_2 levels that were well within what wild toadfish experience daily in a seagrass environment (Frank et al. 2023; McDonald et al. 2007), and so the reduction in O_2 may not have been a significant limiting stress, even after a 7-d exposure.

Not surprisingly, there was a clear correlation between body weight and filament length in fish held under normoxic conditions, as reported in other studies (e.g., Borowiec et al. 2015; Rees and Matute 2018). However, there was no such relationship in fish exposed to severe hypoxia, indicating that the longer filaments measured in severely hypoxic fish was independent of body size and, thus, most likely a result of hypoxia exposure. Indeed, amongst those fish exposed to severe hypoxia, the significantly higher y_0 compared to both normoxia- and mild hypoxia-exposed fish indicates that smaller fish were the most responsive when it came to lengthening their filaments in response to severe hypoxia. Longer filament lengths in response to hypoxia has been shown in some fish (Mucha et al. 2023) but not in others (Boroweic et al. 2015; Huang and Lin 2016). Aside from the longer filament lengths, there was no other clear evidence of gill remodeling in response to hypoxia exposure (e.g., increased lamellar length, reduction in the size of the interlamellar cell mass) in either mild- or severe hypoxia-exposed fish. However, whole mounted gills rather than sectioned makes interpreting these types of changes more difficult.

Under control conditions, Gulf toadfish NECs in the present study, ranged from 41.0 to 71.2 µm^2, and were similar in size to NECs of other teleost species. The NECs of hypoxia-tolerant fish have reported sizes ranging from 22 µm^2 to 104.5 µm^2, with smaller NECs found on the lamellae compared to those along the filament in fish species that have both. For example, the NECs on the lamellae of adult zebrafish (*Danio rerio;* 2.5-3 cm in length) are 28.3 µm^2 and along the filament are 66.1 µm^2 and for adult goldfish (*Carassius auratus;* 4–5 cm in length), the NECs on the lamellae are 41.2 µm^2 and 104.5 µm^2 on the filament (Saltys et al. 200). Adult mangrove rivulus (*Kryptolebias marmoratus*) differ from the others, with NECs located along the most distal tip of the lamellae and filament that are similar in size (~ 22 µm^2; Porteus et al. 2012; Regan et al. 2011). Interestingly, the NECs of hypoxia-sensitive juvenile rainbow trout (*Oncorhynchus mykiss;* 3–5 cm in length) are substantially larger, an average of 148.4 µm^2, than those

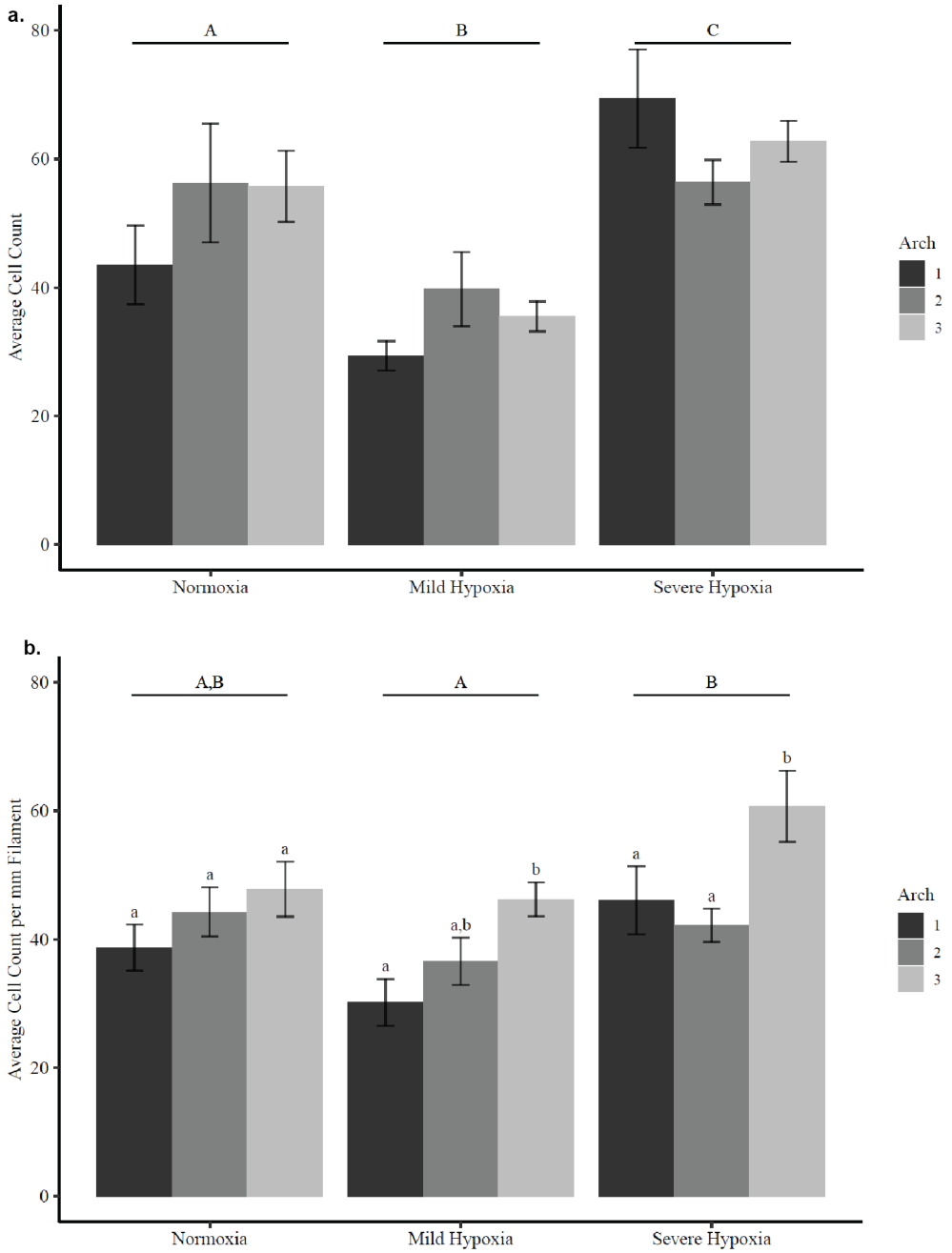

Fig. 4 (**a**) The average number of NECs in each gill arch from fish exposed to either normoxia, mild, or severe hypoxia. (**b**) The average number of NECs per mm of filament for fish exposed to either normoxia, mild, or severe hypoxia. Values are means ± SEM ($n = 8$). Capital letters represent differences between the oxygen regimes, while lowercase letters signify differences within oxygen regimes

measured in the hypoxia-tolerant fish, and are only found along the filament (Saltys et al. 2006). Juvenile toadfish NECs are similar in size to the average size of NECs measured in adult hypoxia-tolerant fish species and smaller than those found in hypoxia-sensitive rainbow trout of a similar life stage, suggesting that sexual maturity does not play a significant role in dictating NEC size. While the length of the toadfish used in the present study was not noted, toadfish used in the study were approximately 2–3 cm, making them comparable in length to the other species used.

Based on the literature, we had hypothesized that there would be an increase in NEC size in toadfish when exposed to hypoxia. However, there was no significant change in toadfish NEC size exposed to either

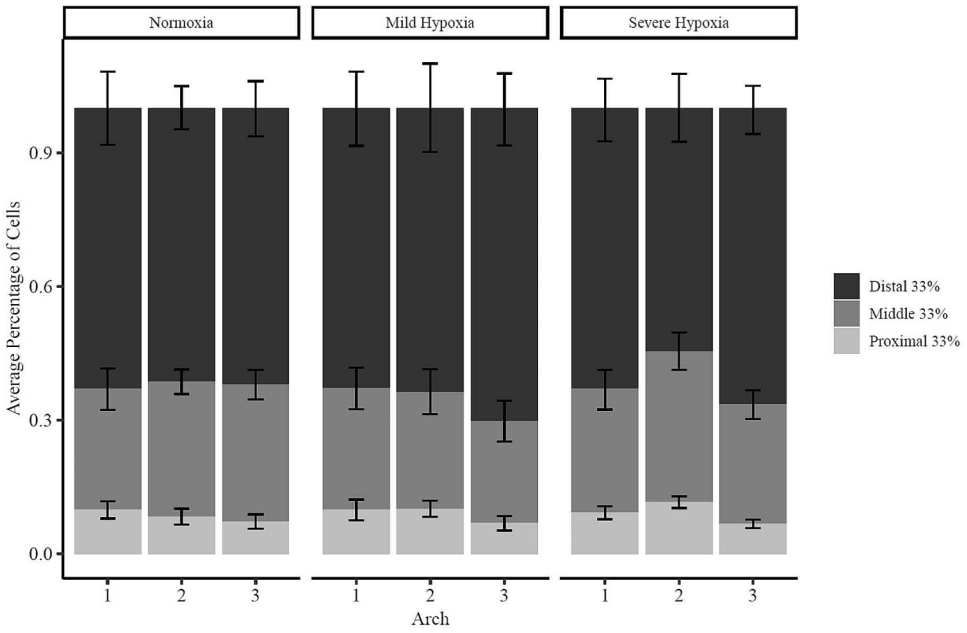

Fig. 5 The distribution of NECs throughout the filament of the gill of fish exposed to either normoxia, mild, or severe hypoxia. The filament is separated into thirds, with the distal 33% being farthest away from the arch and proximal 33% being the closest. Values are means ± SEM ($n = 8$)

chronic mild ($PO_2 = 10.2 \pm 0.3$ kPa) or severe hypoxia ($PO_2 = 3.1 \pm 0.2$ kPa) for 7 days. This was in contrast to zebrafish that also respond to hypoxia ($PO_2 = 35$ Torr, 4.7 kPa) with an increase in NEC size but only after a given time frame – after hypoxia exposure for 60 days zebrafish demonstrate an increase in cell size (Jonz et al. 2004) but show no change in the size of NECs when exposed for only 28 days (Vulesevic et al. 2006). So, the difference could be due to the shorter exposure period of the present study (7-d) or perhaps the difference between toadfish and zebrafish could represent different strategies to deal with hypoxia. One could argue that since the Gulf toadfish is a scaleless fish species, they may be able to respire across their skin in combination with their gills, similar to other species of scaleless fish (Nilsson et al. 2007; Urbina and Glover 2012), and so simply looking at the gill might not give us the full picture. However, unlike toadfish, the NECs in both the gill and skin of mangrove rivulus also increase in size by 30–60% in response to a 7-d exposure to 20% air-saturated water ($PO_2 \sim 4.3$ kPa; Regan et al. 2011). It is possible that toadfish may just respond to hypoxia differently than other fish due to their lifestyle as a lie-and-wait benthic species that stay on the ocean floor, hiding in shelters, waiting for food to come to them (Cordeiro et al. 2020; Hoffman and Robertson 1983).

A comparison of NEC number between species is a bit more complicated as NEC number in the literature is presented in different ways. Due to size differences in fish species, or even in the fish of the present experiment, the more accurate method amongst the two used in the present study is to compare the number of NECs per mm of filament, i.e., the density of NECs, rather than just the total number. When looking at NEC density, juvenile toadfish have 84% more NECs per mm of filament (46.1 ± 3.4 cells/mm of filament) than measured in adult goldfish and 32% more than measured in adult trout (Coolidge et al. 2008). Thus, even though trout NECs are larger than those of toadfish, as described above, toadfish have a greater number of NECs than trout. However, similar to goldfish and trout, no difference in NEC number was measured between the filaments of different arches in toadfish, at least under normoxic conditions (Coolidge et al. 2008). In general, there does not appear to be a clear positive relationship between the hypoxia tolerance of a fish and the number of NECs measured within its gill, as the hypoxia-tolerant goldfish had fewer NECs compared to hypoxia-sensitive trout (Coolidge et al. 2008) while the hypoxia-tolerant toadfish examined in the present study had more NECs compared to trout. The difference in NEC density between fish species could be related to the age of the fish as the fish used in other studies were mature adults in contrast to the juvenile toadfish examined here. Very few studies have looked at NEC density in fish through different developmental stages. Reductions in the number of NECs present on the skin of early-life stage zebrafish as they develop into adults has been documented (Coccimiglio and Jonz 2012). And while the innervation and morphology of NECs present on the gill filament of zebrafish larvae is similar to that in adults, gill NEC number between larvae and adults has not been directly investigated (Jonz and Nurse 2003, 2005).

Based on findings in other fish, we hypothesized that hypoxia exposure would increase the number or density of 5-HT-IR NECs. The density of toadfish 5-HT-IR NECs did change when exposed to hypoxia, with toadfish exposed to

severe hypoxia ($PO_2 = 3.1 \pm 0.2$ kPa) having a higher density of NECs compared to toadfish exposed to mild hypoxia ($PO_2 = 10.2 \pm 0.3$ kPa). These changes indicate that, when in hypoxic environments, there is a cellular-level adjustment that could potentially impact the sensitivity or capacity to sense O_2. For example, in reduced environmental O_2 levels, only very small changes in PO_2 are possible, so an increase in NEC proliferation may serve to amplify the signal that then leads to a physiological or behavioral response. This signal amplification may be particularly important at O_2 levels below P_{crit}, as a lack of a physiological or behavioral response at that point may be lethal. The change in NEC density may also directly correspond to changes in other aspects of physiology or behavior, although this has never been tested in toadfish. Along these lines, it is not clear why the fish would produce less NECs in mild hypoxia. Keeping in mind that the NEC density in mild hypoxia-exposed fish was not significantly different than normoxia-exposed fish, it might simply be because the intensity and duration of hypoxia exposure under these conditions was not great enough to induce an increase in NEC density. If toadfish were exposed to mild conditions for longer than 7 days, a response more similar to fish exposed to severe hypoxia might have been evident.

Alternatively, the lower NEC density measured in mild hypoxia-exposed fish could be an artifact of increased gill 5-HT degradation in these fish that, without the use of SV-2, would have rendered the NECs invisible. Sebastiani et al. (2022) has shown that within 24 h of being exposed to mild hypoxia (10.7 kPa) similar the levels of the present study, adult toadfish experience an immediate increase in gill 5-HT uptake from the circulation that is closely followed by an increase in whole gill 5-HT degradation, measured as an increase in monoamine oxidase (MAO) activity, and a subsequent decrease in plasma 5-HT concentrations (Sebastiani et al. 2022). The removal of 5-HT from the circulation is hypothesized to be responsible for the decrease in systemic blood pressure that is measured in toadfish in response to hypoxia exposure, as 5-HT typically acts as a vasoconstrictor (Fritsche et al. 1992; Kermorgant et al. 2014; McDonald et al. 2010; Sebastiani et al. 2022; Sundin et al. 1998). It is possible, had we used SV-2 in our study, as in Jonz et al. (2004), we might have measured an upregulation in SV-2-IR cells, indicating that the absolute number of NECs stayed constant (or might have even increased). Jonz et al. (2004) measured no change in the number of 5-HT-IR NECs when zebrafish were exposed to severe hypoxia ($PO_2 = 4.7$ kPa) for 60 days but did measure an increase in NECs that were SV-2 immunoreactive (SV-2-IR) and 5-HT negative. They suggested that these SV-2-IR cells may be an immature form of NECs that could eventually lead to an increase in 5-HT-IR NECs (Jonz and Nurse 2003; Jonz et al. 2004). It should be said that juvenile toadfish used in the present study had never been exposed to hypoxia, and so it is possible that they might have reacted to hypoxia in a way that is consistent with their naivety to hypoxic conditions and not how wild toadfish, that experience both daily and seasonal cycles of hypoxia (Hall et al. 1999; Hopkins et al. 1997; Lapointe and Clark 1992; McDonald et al. 2010; Serafy et al. 1997), would respond. Wild toadfish would typically be exposed to their first hypoxic event at the very beginning of their life cycle, unlike lab-reared toadfish that have a constant supply of O_2. Therefore, it is possible that the juveniles used in the present study could be exhibiting signs of phenotypic plasticity, with respect to NEC density, that is related to their first exposure to hypoxia.

Goldfish and zebrafish have NECs spread evenly throughout both the filaments and lamellae of the gill (Coolidge et al. 2008; Jonz and Nurse 2003; Jonz et al. 2004; Porteus et al. 2012), while salmon and trout have NECs along the filament with none present in the lamellae (Coolidge et al. 2008; Nilsson and Östlund-Nilsson 2008; Porteus et al. 2012). This study found that toadfish were similar to salmon and trout, with NECs only being present in the filaments and not the lamellae and similar to Japanese medaka and mangrove rivulus with respect to greater density in the tips (Porteus et al. 2012; Regan et al. 2011). It is possible that if toadfish have NECs present in their skin like other scaleless fish (Regan et al. 2011), the presence of NECs in the lamellae may not be necessary in the way they are in the goldfish and zebrafish. NECs have been shown to be dynamic in some fish with respect to movement during hypoxia – for example, in goldfish exposed to warm and/or hypoxic water the interlamellar mass recedes and the NECs appear all along the lamellae instead of just the lamellar tips when in cold and/or normoxic water (Tzaneva and Perry 2010; Tzaneva et al. 2011). In the present study, the distribution of NECs did not change when toadfish were exposed to either mild or severe hypoxia, suggesting that the location of NECs in the gill is not dependent on the PO_2 in the blood or surrounding waters of toadfish.

In conclusion, the present study provides some insight into how juvenile Gulf toadfish NEC size, number, and distribution during normoxia and when exposed to mild and severe hypoxia compared to other hypoxia-tolerant and hypoxia-intolerant fish species that have been studied. Our findings demonstrate that toadfish exposed to severe hypoxia experience changes in gill filament length that cannot be explained by body weight. Furthermore, toadfish have similar sized 5-HT-IR NECs to other fish that are not responsive to hypoxia exposure, but more 5-HT-IR NECs/mm filament or density compared to other fish that are responsive to hypoxia exposure. With respect to distribution, toadfish NECs are found along the filament, mostly

towards the distal end and this distribution is not sensitive to hypoxia exposure. Toadfish breeding season usually occurs from March to May, meaning that juveniles live through major hypoxia events during the summer (Briceño et al. 2011; Hall et al. 1999; Hopkins et al. 1997; Lapointe and Clark 1992; McDonald et al. 2010; Serafy et al. 1997). Thus, understanding O_2 sensing in juvenile toadfish may be particularly important. This work also highlights the need for future work on the skin of the scaleless toadfish that may play an important role in O_2 sensing as well as respiration, which could contribute to its hypoxia tolerance.

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