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RESEARCH ARTICLE

Aquatic surface respiration and swimming behaviour in adult and developing zebrafish exposed to hypoxia

Sara J. Abdallah, Benjamin S. Thomas and Michael G. Jonz*

ABSTRACT

Severe hypoxia elicits aquatic surface respiration (ASR) behaviour in many species of fish, where ventilation of the gills at the air–water interface improves O_2 uptake and survival. ASR is an important adaptation that may have given rise to air breathing in vertebrates. The neural substrate of this behaviour, however, is not defined. We characterized ASR in developing and adult zebrafish (*Danio rerio*) to ascertain a potential role for peripheral chemoreceptors in initiation or modulation of this response. Adult zebrafish exposed to acute, progressive hypoxia (P_{O_2} from 158 to 15 mmHg) performed ASR with a threshold of 30 mmHg, and spent more time at the surface as P_{O_2} decreased. Acclimation to hypoxia attenuated ASR responses. In larvae, ASR behaviour was observed between 5 and 21 days postfertilization with a threshold of 16 mmHg. Zebrafish decreased swimming behaviour (i.e. distance, velocity and acceleration) as P_{O_2} was decreased, with a secondary increase in behaviour near or below threshold P_{O_2}. In adults that underwent a 10-day intraperitoneal injection regime of 10 μg g^{-1} serotonin (5-HT) or 20 μg g^{-1} acetylcholine (ACh), an acute bout of hypoxia (15 mmHg) increased the time engaged in ASR by 5.5 and 4.9 times, respectively, compared with controls. Larvae previously immersed in 10 μmol l^{-1} 5-HT or ACh also displayed an increased ASR response. Our results support the notion that ASR is a behavioural response that is reliant upon input from peripheral O_2 chemoreceptors. We discuss implications for the role of chemoreceptors in the evolution of air breathing.

KEY WORDS: Chemoreceptor, Serotonin, Acetylcholine, ASR, Evolution, Feedback

INTRODUCTION

The regulation of O_2 uptake, and its matching with metabolic requirements, are critical factors in the survival of all vertebrates. For most aquatic organisms that are incapable of using air as a respiratory medium, there is a reliance on gills for gas exchange (Randall and Daxboeck, 1984; Evans et al., 2005). In fish, environmental hypoxia produces reflex physiological responses, such as hyperventilation, bradycardia and changes in vascular resistance (Randall and Shelton, 1963; Holeton and Randall, 1967; Perry et al., 2009), as well as behavioural changes to decrease the physiological and biochemical demands imposed by hypoxia (Dalla Via et al., 1998; Domenici et al., 2000; Sloman et al., 2006; Chapman and McKenzie, 2009). One such behaviour is aquatic surface respiration, or ASR (Sloman et al., 2008; Chapman and McKenzie, 2009; Perry et al., 2009; Taylor et al., 2010; Richards, 2011). ASR involves ventilation at the air–water interface, where equilibration with the atmosphere raises the partial pressure of O_2 (P_{O_2}) above that of the rest of the water column (Kramer and Mehegan, 1981). ASR is typically initiated near the P_{O_2} of peak gill ventilation (reviewed by Chapman and McKenzie, 2009; Perry et al., 2009), and the cumulative time spent in ASR increases rapidly as P_{O_2} decreases (Kramer and Mehegan, 1981; Kramer and McClure, 1982). Once at the air–water interface, fish actively skim the surface to ventilate their gills whilst maintaining a posture in which the dorsal aspect of the head lies just at or below the surface, with the body at a slight angle (Kramer and McClure, 1982; Timmerman and Chapman, 2004; Perry et al., 2009).

Performance of ASR under conditions of hypoxia effectively maintains or decreases gill ventilation rate (McNeil and Closs, 2007), increases arterial blood O_2 content (Burggren, 1982), and increases survival (Kramer and Mehegan, 1981; Kramer and McClure, 1982; Perry et al., 2009). The behaviour is believed to have evolved as an adaptation for surviving environmental hypoxia, and is thought to be an evolutionary precursor to aerial respiration in vertebrates (Kramer and McClure, 1982; Gee and Gee, 1995; Shingles et al., 2005). Despite extensive documentation of ASR in the literature, very little attention has been placed on the control of this behaviour. Specifically, it is not adequately understood how ASR is initiated by hypoxia, and the underlying neurochemical pathways associated with this response have not been sufficiently addressed.

In the tambaqui (*Colossoma macropomum*), ASR was abolished by bilateral section of the mandibular branch of the trigeminal nerve (cranial nerve V, which innervates the orobranchial region), whilst section of the glossopharyngeal (IX) or vagus (X) nerves to the gills had no effect (Sundin et al., 2000; Florindo et al., 2006); and in the flathead grey mullet (*Mugil cephalus*), application of sodium cyanide, a potent metabolic inhibitor and chemoreceptor stimulant, induced ASR (Shingles et al., 2005). These studies suggest that, like hyperventilation and bradycardia, ASR may be a reflex response to hypoxia driven by peripheral O_2 chemoreceptors.

O_2-chemosensitive neuroepithelial cells (NECs) of the gill filaments have been characterized in three fish species, including the model vertebrate, the zebrafish *Danio rerio* (Jonz et al., 2004; Burleson et al., 2006; Zachar and Jonz, 2012). NECs receive innervation primarily from cranial nerves IX and X (Dunel-Erb et al., 1982; Nilsson, 1984; Jonz and Nurse, 2003, 2005). Hypoxia stimulates zebrafish NECs via inhibition of membrane-bound K^+ channels and subsequent membrane depolarization, and this presumably leads to the generation of cardioventilatory reflexes (Jonz et al., 2004). Interestingly, adult zebrafish immersed in quinidine, a blocker of O_2-sensitive K^+ channels in NECs (Jonz et al., 2004), induced hyperventilation and ASR (Jonz and Nurse, 2005).

Gill NECs retain serotonin (5-HT) as well as a number of other neurochemicals (reviewed in Jonz, 2014). These cells first appear in the gill filaments of zebrafish larvae between 5 and 7 days postfertilization (d.p.f.) and are preceded by a cutaneous population of NEC-like cells in embryos (Jonz and Nurse, 2005; Coccimiglio

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List of symbols and abbreviations

5-HT	5-hydroxytryptamine (serotonin)
ACh	acetylcholine
ANOVA	analysis of variance
ASR	aquatic surface respiration
dpf	days postfertilization
NEC	neuroepithelial cell
PBS	phosphate-buffered solution
P_{crit}	critical P_{O_2} level
P_{O_2}	partial pressure of oxygen

and Jonz, 2012). In addition, cells expressing an acetylcholine (ACh) transporter were reported in the gills of zebrafish (Shakarchi et al., 2013) and the amphibious fish *Kryptolebias marmoratus* (Regan et al., 2011). Both 5-HT and ACh have been implicated in the chemoreceptor control of ventilatory reflexes in developing and adult fish (Burleson and Milsom, 1995a,b; Shakarchi et al., 2013; Jonz et al., 2015).

Given the advantages of understanding the biological features of O_2 sensing and respiratory development in zebrafish, we designed experiments to characterize ASR in this animal model, and to begin studies aimed at defining the control of this behaviour. We hypothesized that at P_{O_2} levels near maximal gill ventilation, zebrafish would begin to perform ASR; and that this behavioural response would be subject to modulation by factors that affect O_2 chemoreception, such as progressive changes in water P_{O_2}, acclimation to hypoxia, development, and administration of 5-HT or ACh.

RESULTS

ASR behaviour in adult zebrafish

Adult zebrafish exposed to water of progressively decreasing P_{O_2} first display an increase in ventilation frequency at ~110 mmHg (Vulesevic and Perry, 2006; Vulesevic et al., 2006). We observed similar hyperventilatory responses to mild hypoxia in the test chamber in the present study (data not shown). However, when zebrafish were exposed to severe levels of hypoxia, aquatic surface respiration (ASR) was observed in addition to hyperventilation. Once at the surface, the zebrafish adopted a position in which the body was at an angle with the air–water interface, with the top of the head in contact with the water surface and the mouth just under the surface layer. In some instances, zebrafish would perform ASR while hyperventilating and remaining stationary at the surface. As P_{O_2} decreased further, fish became more active and would skim the surface layer.

Fig. 1 illustrates the results from tracking experiments in the test chamber at three levels of P_{O_2}, where vertical position is plotted against time. These experiments were performed on 11 adults at each P_{O_2} level. When zebrafish were exposed to normoxic levels of O_2 (P_{O_2}~158 mmHg; Fig. 1A), movements to the surface were uncommon and zebrafish were more likely to explore the lower region of the chamber. By contrast, zebrafish exposed to a P_{O_2} of 23 mmHg or 15 mmHg displayed regular and sustained trips to the surface (i.e. within 10 mm of the air–water interface) associated with ASR behaviour, and swimming activity was reduced elsewhere in the chamber (Fig. 1B,C). Raw data from tracking experiments indicate the preferred regions of the test

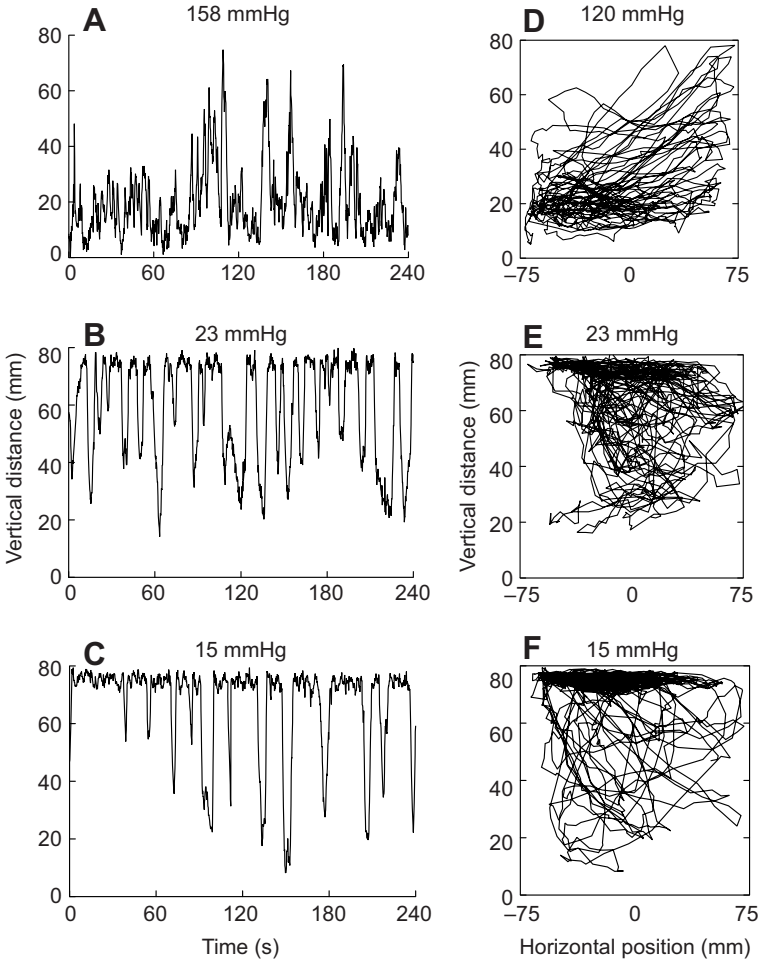

Fig. 1. Adult zebrafish perform aquatic surface respiration and alter their swimming behaviour during hypoxia. Vertical movements (in mm) plotted against time for a single adult zebrafish exposed to (A) normoxic levels of O_2 (P_{O_2}=158 mmHg), (B) 23 mmHg and (C) 15 mmHg. In normoxia, vertical movement was random and ASR was not observed. In two levels of severe hypoxia, ASR behaviour becomes progressively more pronounced as the duration and frequency of trips to the surface increased. (D–F) Representative traces of zebrafish vertical and horizontal movements in the test chamber that correspond to panels A–C. Note that in D, P_{O_2}=120 mmHg.

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chamber for zebrafish during the trials at these levels of P_{O_2} (Fig. 1D–F).

During 5 min trials, in which adult zebrafish were subjected to acute progressive hypoxia (P_{O_2} from 158 to 15 mmHg), zebrafish generally spent progressively more time engaged in ASR as water P_{O_2} was decreased (Fig. 2). From the mean of 11 individuals (Fig. 2C), it was evident that over a range of P_{O_2} values zebrafish displayed no observable ASR activity until the P_{O_2} reached ~50 mmHg, where 2 out of 11 fish participated in ASR and cumulative time began to increase (Fig. 2C, filled circles). A significant increase in cumulative time in ASR above that of normoxic zebrafish was apparent at P_{O_2} values of 30 mmHg and below (P<0.001, repeated-measures ANOVA, Bonferroni *post hoc* test, N=11). We therefore estimate a threshold P_{O_2} for ASR behaviour at 30 mmHg. Additional analysis by two-way ANOVA indicated significant effects of acclimation to hypoxia on the ASR response (Fig. 2C; P<0.001, N=11). Acclimation to hypoxia (P_{O_2}=30–45 mmHg) for 7 days significantly reduced cumulative time in ASR during acute hypoxic exposure, compared with unacclimated zebrafish, at P_{O_2} values of 25 mmHg and below. In addition, the line of best fit for acclimated zebrafish was shifted to the left by ~7 mmHg (at 70.5 s), indicating a potential decrease in threshold P_{O_2} compared with unacclimated controls.

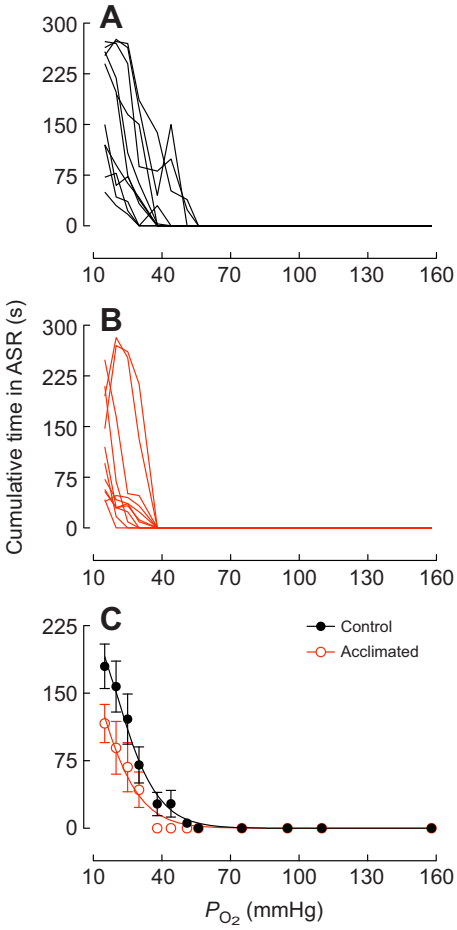

Fig. 2. Aquatic surface respiration behaviour (ASR) is dependent upon water P_{O_2}. (A) Adult zebrafish spent progressively more time engaged in ASR as water P_{O_2} was decreased from 158 to 15 mmHg. For each individual, data points (see C) have been converted to a continuous line for clarity. (B) As above, but zebrafish were previously acclimated to chronic hypoxia. (C) Mean±s.e.m. cumulative time engaged in ASR increases as water P_{O_2} decreases (filled circles). A significant increase in cumulative time in ASR above that of normoxic zebrafish (158 mmHg) was apparent at 30 mmHg and below (P<0.001, repeated-measures ANOVA, Bonferroni *post hoc* test, N=11). A line of best fit was used to analyze data points (r^2=0.99, see Materials and methods). Zebrafish acclimated to chronic hypoxia display a blunted ASR response to acute progressive hypoxia (open circles, r^2=0.97). Acclimation to hypoxia significantly reduces mean±s.e.m. cumulative time in ASR during acute hypoxic exposure, compared with unacclimated zebrafish, at P_{O_2} values of 25 mmHg and below (two-way ANOVA; P<0.001; N=11).

A biphasic change in swimming behaviour was associated with an increased ASR response with decreasing P_{O_2}. Adult zebrafish exposed to progressive, acute hypoxia displayed only a marginal decline in swimming behaviour from 110 to 32 mmHg, but minimum values for total distance travelled (4.8±1.1 m), velocity (16.1±5.6 mm s^{-1}) and acceleration (42.4±13.3 mm s^{-2}) were recorded at a P_{O_2} of 24 mmHg (Fig. 3A,B), just below the ASR threshold of 30 mmHg. When the P_{O_2} was reduced below 24 mmHg, zebrafish then began to display an increase in all three parameters up to 8 mmHg, where maximum distance (10.9±1.1 m), velocity (37.4±3.7 mm s^{-1}) and acceleration (108.1±15.2 mm s^{-2}) were significantly greater compared with minimum values at 24 mmHg (paired t-test; P<0.05; N=6).

Through development of our procedures for intraperitoneal drug injection and behavioural assay, we found that injections of phosphate-buffered solution (PBS, as a sham control) resulted in a decrease in the ability of adults to perform ASR during exposure to acute hypoxia. Adults that received intraperitoneal injections of PBS achieved a lower cumulative time spent in ASR of 10.6±2.8 s (N=38) during a 5 min trial exposed to a P_{O_2} of 15 mmHg, compared with 179.9±24.7 s at the same P_{O_2} in uninjected zebrafish (see Fig. 2C). Despite the attenuating effects of the injection regime on ASR behaviour, it was evident that injection of 10 µg g^{-1} 5-HT or 20 µg g^{-1} ACh significantly increased cumulative time engaged in ASR during hypoxia by 5.5 and 4.9 times, respectively, compared with PBS-injected controls; and by 9.8 and 3.3 times, compared with lower concentrations of the same drug (Fig. 4; Kruskal–Wallis, Dunn's *post hoc* test; P<0.05). Neither 5 µg g^{-1}ACh nor 10 µg g^{-1} 5-HT had a significant effect compared with PBS-injected controls.

ASR behaviour in developing zebrafish

As illustrated in Fig. 5, an increase in the number of frames s^{-1} in which younger larvae were first observed near the surface increased at higher O_2 tensions compared with larvae at 14–21 dpf. Larvae displayed ASR behaviour at 32 mmHg at a mean frequency of 1.9±0.6 frames s^{-1} at 5 dpf and 1.3±0.4 frames s^{-1} at 7 dpf. At 14 dpf, the frequency of ASR events at 32 mmHg had decreased to 0.14±0.1 frames s^{-1}. At all stages of development tested, the frequency of larvae observed at the air–water interface was significantly greater only at 8 and 16 mmHg compared with the number of frames s^{-1} at higher levels of P_{O_2} (ANOVA; Bonferroni *post hoc* test; P<0.05). We therefore estimate an ASR threshold of 16 mmHg for zebrafish larvae at all stages tested, although this may be an underestimate in younger larvae.

Developing zebrafish generally displayed a progressive reduction in swimming behaviour as P_{O_2} was decreased (Fig. 6). Significant reductions in total distance travelled, velocity and acceleration were observed at 5, 18 and 21 dpf; and these first occurred at a P_{O_2} of 16, 32 and 47 mmHg, respectively (P<0.05; ANOVA; Bonferroni *post hoc* test). Though not as prominent as was observed in adults, parameters of swimming behaviour began to increase below the estimated ASR threshold of 16 mmHg at 5, 14 and 18 dpf.

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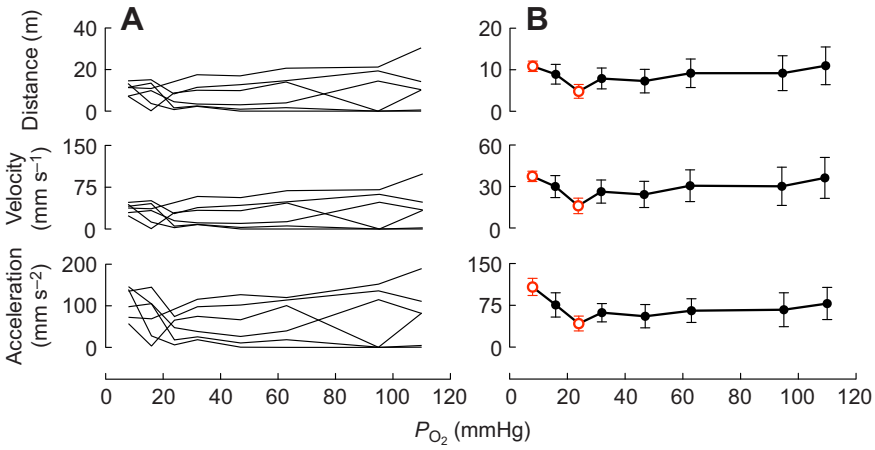

Fig. 3. Swimming behaviour in adult zebrafish is dependent upon water P_{O_2}. (A) Distance travelled in m (top trace), velocity in mm s^{-1} (middle trace) and acceleration in mm s^{-2} (bottom trace) was measured in zebrafish while exposed to progressive acute changes in P_{O_2} from 110 to 8 mmHg. (B) Mean±s.e.m. values for the data shown in A. In each panel, a significant difference between the maximum values at a P_{O_2} of 8 mmHg and minimum values at a P_{O_2} of 24 mmHg (indicated by red data points) was detected using the paired *t*-test (P<0.05; N=6).

Given the prominent effects of 5-HT and ACh on increasing ASR behaviour in adults upon exposure to hypoxia, the same drugs were tested in behavioural assays of larvae. We recorded frames having a range of only 0–2 ASR events in response to hypoxia (as a control) between 7 and 25 dpf. The number of frames containing these events is plotted in Fig. 7. Note that in groups where few ASR events are present, the data distribution is skewed to the left. ASR events in response to hypoxia in 40 dpf larvae were more frequent. When larvae from 14 to 25 dpf were first immersed in 10 μmol l^{-1} 5-HT or ACh and then exposed to hypoxia, the number of frames having 3 ASR events recorded increased, and the number of ASR events increased to 4 in 40 dpf larvae previously immersed in 5-HT (Fig. 7). Mean ASR events $frame^{-1}$ were significantly greater in hypoxia for 18 dpf (1.29±0.15 $frame^{-1}$), 25 dpf (1.63±0.19 $frame^{-1}$) and 40 dpf (1.9±0.19 $frame^{-1}$) larvae pre-exposed to 5-HT, compared with hypoxia alone (0.63±0.11, 0.37±0.09 and 0.79±0.15 $frame^{-1}$, respectively); whilst pre-exposure to ACh had a significant effect only in 18 dpf larvae (1.24±0.14 $frame^{-1}$) compared with hypoxia alone (rank sum Kruskal–Wallis and Dunn's multiple comparison test; P<0.05).

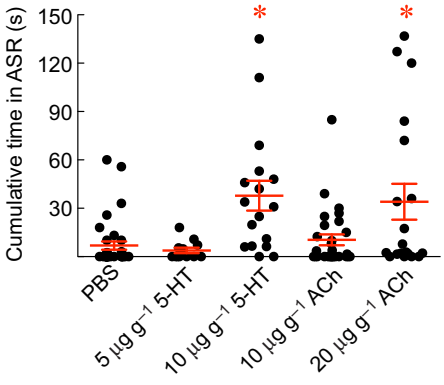

Fig. 4. Injection of exogenous neurotransmitters involved in O_2 chemoreception enhances aquatic surface respiration responses in adult zebrafish. Animals previously received intraperitoneal injection of phosphate-buffered solution (PBS, control), 5 or 10 μg g^{-1} serotonin (5-HT), or 10 or 20 μg g^{-1} acetylcholine (ACh). Illustrated in each group is the mean±s.e.m. cumulative time performing ASR during acute severe hypoxia (P_{O_2}=15 mmHg) in injected animals. Asterisks indicate significant increases in the ASR response following administration of higher 5-HT and ACh concentrations, compared with PBS and lower drug concentrations (Kruskal–Wallis, Dunn's multiple comparison *post hoc* test; P<0.05; N=34, 12, 17, 29 and 19, respectively).

DISCUSSION

The present study has demonstrated that zebrafish exhibit ASR behaviour in response to severe hypoxic stress as early as 5 days postfertilization, and that ASR is increased with decreasing water P_{O_2}. We have further shown that the threshold of the ASR response is dependent upon development and previous exposure to hypoxia,

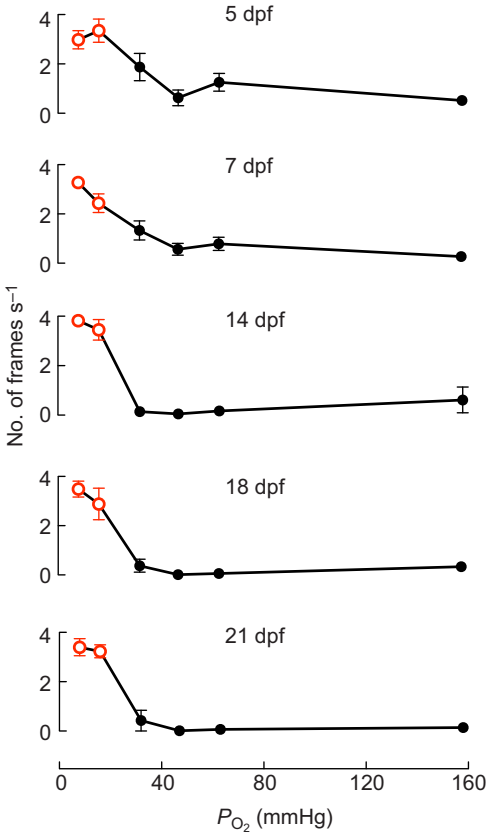

Fig. 5. Development of ASR behaviour in zebrafish larvae. Mean±s.e.m. number of frames s^{-1} in which a zebrafish larva was recorded near the surface of the test chamber as P_{O_2} was reduced from 158 to 8 mmHg. Each larva between 5 and 21 days post-fertilization (dpf) was tested individually at a single P_{O_2}. Data are presented in frames s^{-1}, where 5 frames s^{-1} would indicate a maximal ASR response. In every panel, a significant difference among groups at different levels of P_{O_2} was detected by ANOVA (P<0.05). Red data points indicate a significant difference between means compared with normoxic values at 158 mmHg, as indicated by a Bonferroni *post hoc* test (P<0.05). N=6 for 5, 7, 18 and 21 dpf; N=4 for 14 dpf.

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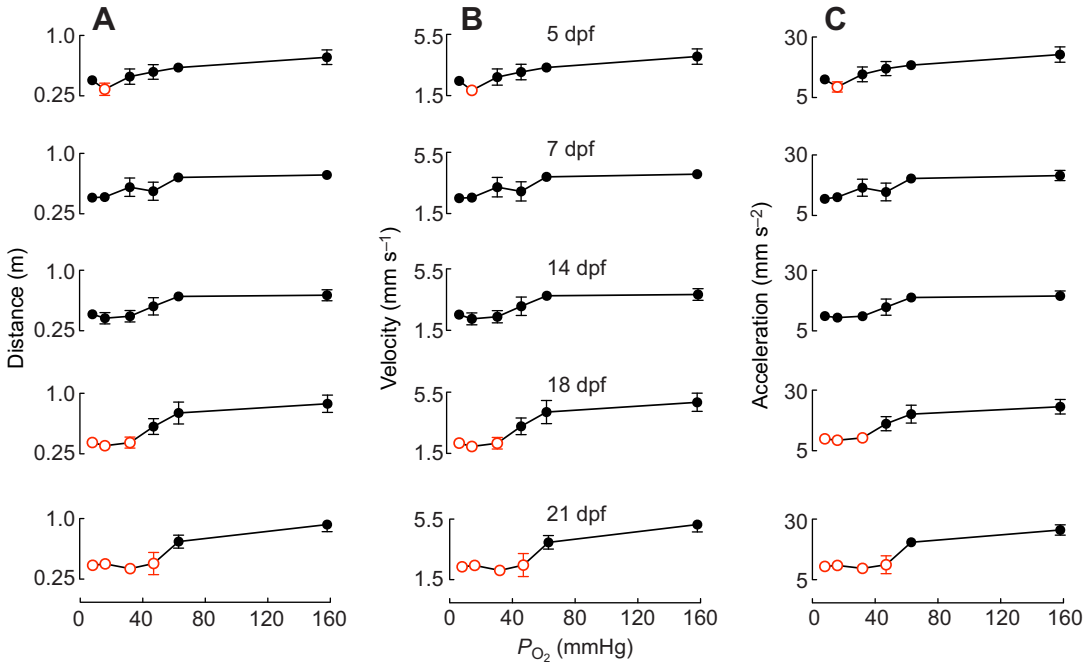

Fig. 6. Swimming behaviour is dependent upon water P_{O_2} and developmental stage. Mean±s.e.m. distance travelled in m (A), velocity in mm s^{-1} (B) and acceleration in mm s^{-2} (C) of zebrafish larvae measured at 5–21 days postfertilization (dpf) while larvae were exposed to acute changes in P_{O_2} from 158 to 8 mmHg. Significant differences among groups at different levels of P_{O_2} were detected by ANOVA (P<0.05). Red data points indicate a significant difference between means compared with normoxic values at 158 mmHg, as indicated by a Bonferroni *post hoc* test (P<0.05). For most data points N=6. Otherwise, N=5 for 158 mmHg at 5 dpf, for 32 mmHg at 7 dpf, for 8, 16 and 32 mmHg at 18 dpf, and for 8, 16, 32 and 158 mmHg at 21 dpf; N=4 for each point at 14 dpf; and N=3 for 63 mmHg at 21 dpf.

and that ASR can be enhanced by exogenous application of neurochemicals known to activate O_2-chemosensory pathways.

ASR behaviour in zebrafish

Behavioural changes performed by fish during environmental hypoxia play an integral role in survival at times when O_2 is critically limited and changes in gill ventilation can no longer support needs for increased O_2 uptake. Among these behavioural changes are: transitions from water to air breathing, emersion from water and ASR (Graham, 1997; Ong et al., 2007; Chapman and McKenzie, 2009; Perry et al., 2009; Taylor et al., 2010; Richards, 2011; Milsom, 2012). In adult zebrafish, threshold P_{O_2} for the ASR response, i.e. the P_{O_2} at which a significant increase in ASR behaviour first occurred, was 30 mmHg. Zebrafish exposed to hypoxia begin to hyperventilate at a P_{O_2} of approximately 110 mmHg and reach maximal gill ventilation near 40 mmHg (Vulesevic and Perry, 2006; Vulesevic et al., 2006). Therefore, in agreement with previous reports of ASR behaviour in different species (see table 5.5 in Perry et al., 2009), zebrafish engage in ASR at or below the P_{O_2} of maximal gill ventilation. Moreover, Chapman and McKenzie (2009) summarized the ASR thresholds of 63 species and suggested a negative correlation with hypoxia tolerance. McNeil and Closs (2007) determined ASR thresholds of ∼14 mmHg for goldfish (*Carassius auratus*) and common carp (*Cyprinus carpio*) – two cyprinid species related to zebrafish. Both goldfish and carp exhibit high levels of tolerance to hypoxia (Stecyk and Farrell, 2002; Lutz and Nilsson, 2004; Bickler and Buck, 2007), and this may account for the relatively lower ASR thresholds observed in these species compared with zebrafish.

The effects of hypoxia on spontaneous swimming activity have been studied in a number of fish species and appear to be both variable and species dependent (reviewed by Chapman and McKenzie, 2009). Generally, if swimming activity is reduced during hypoxia at levels above the critical P_{O_2} (P_{crit}), this may be regarded as a strategy to conserve energy in an environment of potentially limited O_2 availability. By contrast, the intense agitation of fish in severe hypoxia below P_{crit} has been interpreted as an acute escape or avoidance response (Bejda et al., 1987; Randall et al., 1992; van Raaij et al., 1996). In adult zebrafish, we measured a gradual decline in total distance travelled, velocity and acceleration as water P_{O_2} fell. These parameters reached minimum values at a P_{O_2} at 24 mmHg, and then increased as P_{O_2} was further reduced. A similar biphasic change in activity associated with decreasing P_{O_2} was observed in sole (*Solea solea*; Dalla Via et al., 1998; McKenzie et al., 2008). Reports for P_{crit} in adult zebrafish are ∼20 mmHg (Barrionuevo and Burggren, 1999; Barrionuevo et al., 2010), although a more recent study presented a considerably higher value that varied between males and females (Robertson et al., 2014). We therefore interpret the biphasic trends in swimming behaviour in zebrafish exposed to progressive hypoxia as a strategy to reduce activity and conserve energy at water P_{O_2} levels above P_{crit}; but as P_{O_2} falls below P_{crit}, zebrafish may become agitated and initiate a response in an effort to escape hypoxia. An alternative interpretation of increased swimming activity under these conditions of severe hypoxia may be to increase gas exchange at the surface, as in the case of ram-assisted ASR (Chapman et al., 1994). These data may match our observations that zebrafish first remained stationary when at the air–water interface during the early stages of ASR, but increased swimming activity as P_{O_2} continued to decline. However, when performing ASR at any level of P_{O_2}, zebrafish did not remain at the surface of the chamber but would alternate between states of ASR and brief bouts of rapid swimming behaviour throughout the chamber.

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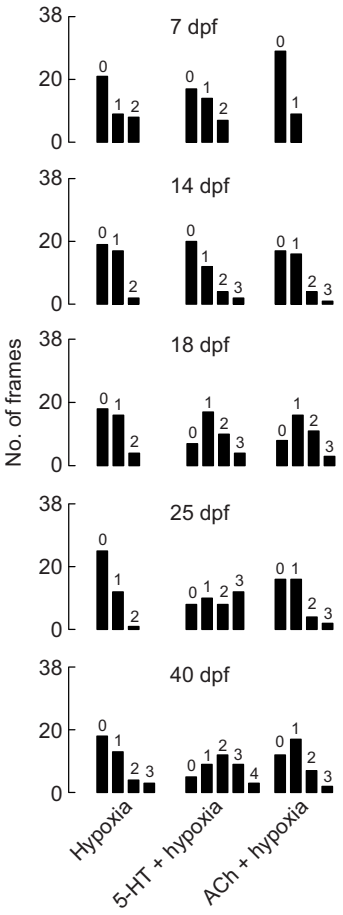

Fig. 7. Pharmacological characterization of ASR behaviour in developing zebrafish. Number of frames in which 0, 1, 2, 3 or 4 (indicated in the figure) ASR events were recorded during behavioural assays, where larvae from 7–40 days post-fertilization (dpf) were exposed to hypoxia in the test chamber. Larvae were first pre-exposed to either 10 μmol l^{-1} serotonin (5-HT) or 10 μmol l^{-1} acetylcholine (ACh) and subsequently exposed to hypoxia (Hox, P_{O_2}=25 mmHg). Controls were not treated with any drugs. For each treatment, data from 20 zebrafish are shown. Where data distributions are skewed to left ASR events were fewer in number; where data are not skewed ASR events were more numerous.

Development of ASR

The development of ASR behaviour in fish is not well defined in the literature. Sloman et al. (2008) reported that juvenile sculpins (*Oligocottus maculosus*) performed ASR, and this occurred at lower O_2 tensions compared with adults; and in juvenile guppies (*Poecilia reticulata*), ASR behaviour increased growth rate during hypoxia (Weber and Kramer, 1983). But the progression in ASR throughout early life in fish has not previously been correlated with events of respiratory development within the same species.

Zebrafish embryos respond to hypoxia by first increasing the frequency of pectoral fin and whole body movements as early as 2 dpf in order to increase flow across respiratory surfaces and improve diffusion; they develop a maximal ventilatory response to hypoxia between 5 and 7 dpf that coincides with innervation of gill NECs (Jonz and Nurse, 2005; Coccimiglio and Jonz, 2012), and this response develops an increasing reliance on central *N*-methyl-D-aspartate (NMDA) receptors at 8 dpf (Turesson et al., 2006). In the present study, developing zebrafish initiated ASR behaviour at lower O_2 tensions than adults. We have estimated an ASR threshold of 16 mmHg in zebrafish larvae, although our observations (Fig. 5) indicate that this may be an underestimate in larvae younger than 14 dpf. P_{crit} for developing zebrafish is ~75 mmHg in embryos and is later reduced to ~70 mmHg at 20 dpf (Barrionuevo and Burggren, 1999; Barrionuevo et al., 2010). Thus, unlike in adults, the ASR threshold in larvae is markedly lower than P_{crit}. This suggests that larvae utilize a different strategy when confronted with severe hypoxia.

Larvae reduced swimming behaviour with hypoxia, as did adults, but statistically significant reductions in total distance travelled, velocity and acceleration were first observed at a P_{O_2} of 32 and 47 mmHg for 18 and 21 dpf larvae, respectively. This indicates that larvae reduce swimming behaviour at higher O_2 tensions compared with adults, and thus may rely primarily upon conserving energy and upon gas exchange via simple diffusion during severe hypoxia. During early developmental stages, the gills may not be fully developed and so zebrafish larvae use both cutaneous and gill respiration to satisfy metabolic needs for O_2 (Rombough, 2002). In addition, a model has predicted that zebrafish can survive by simple diffusion in air-saturated water without the need of a circulatory system until 42 dpf (Rombough and Drader, 2009). ASR may then become important as a survival strategy in larvae at O_2 tensions closer to anoxia, where reduced activity no longer provides any benefit and an escape response is elicited. Indeed, we found that at O_2 tensions below the ASR threshold, at some developmental stages, parameters of swimming behaviour began to increase. Consistent with these results, silverside larvae (*Menidia beryllina*) between approximately 6 and 9 dpf increased swimming activity and moved in an upward direction to avoid hypoxia (Weltzien et al., 1999).

We show that zebrafish displayed ASR as early as 5 dpf. Zebrafish larvae from the time of hatching at 2 dpf have an average density above that of the surrounding water, and so must actively swim to the surface using rapid tail movements and by adhering intermittently to vertical surfaces, in order to take in a small bubble of air and inflate the swim bladder by the end of 4 dpf (Lindsey et al., 2010). This 'swim-up' behaviour is one of the first coordinated behaviours in developing larvae, and must occur in order for larvae to achieve buoyancy control (Spence et al., 2008). We suggest that development of ASR in zebrafish at about 5 dpf is limited primarily by coordination of swim-up behaviour and swim bladder inflation. Moreover, swim-up behaviour may even be a rudimentary form of ASR, occurring over a longer period of time as a result of the inability of newly hatched larvae to rise rapidly to the surface.

ASR as a chemoreflex

An element of some controversy in defining ASR behaviour in fish is that of its control – whether ASR is a reflex initiated by peripheral sensory receptors, or if it is a more complex behaviour. Although ASR may be initiated or dependent upon sensory receptors, it appears at least to have a strong behavioural component that can be modulated by higher brain centres. Shingles et al. (2005) found that even the perceived threat of aerial predation delayed the onset of ASR in the flathead grey mullet (*Mugil cephalus*) and reduced the proportion of fish performing the behaviour. Yet another possibility is that severe hypoxia may be perceived by fish as a noxious or painful stimulus. Some authors have identified an aversive behaviour that may lead fish away from regions of severe hypoxia and toward the surface (Petersen and Petersen, 1990; Weltzien et al., 1999; Poulsen et al., 2011; Cook et al., 2013). In the present study, ASR behaviour in zebrafish was punctuated with brief trips to the bottom of the chamber before returning rapidly to the surface. This

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may have been a searching behaviour directed by a repeated aversive response away from hypoxia. Nociceptors have been identified on the head of rainbow trout (*Oncorhynchus mykiss*) and mediate responses to noxious stimuli, including high CO_2 and acidity (Sneddon, 2003; Mettam et al., 2012).

Evidence in favour of ASR as a reflex response to hypoxia comes primarily from four studies. Application of sodium cyanide, a potent metabolic inhibitor and chemoreceptor stimulant, induced ASR in the mullet (Shingles et al., 2005); and in adult zebrafish immersed in quinidine, a drug that targets O_2-sensitive K^+ channels in gill NECs (Jonz et al., 2004), hyperventilation and ASR were induced (Jonz and Nurse, 2005). In the tambaqui (*Colossoma macropomum*), ASR was abolished by section of the mandibular branch of the trigeminal nerve (cranial nerve V), which innervates the orobranchial region (Sundin et al., 2000; Florindo et al., 2006). The authors therefore suggested the orobranchial cavity as a site for O_2 chemoreceptors that produced the response. However, the trigeminal nerve is also an important source of innervation to cutaneous receptors of the somatic sensory system (Nilsson, 1984), and fibres of the mandibular branch innervate nociceptors of the head and lower jaw (Sneddon, 2003; Mettam et al., 2012).

The present study has demonstrated that administration of 5-HT and ACh increased ASR responses to severe hypoxia in zebrafish adults and larvae. Both 5-HT and ACh have been implicated in chemoreceptor control of ventilatory reflexes in developing and adult fish (Burleson and Milsom, 1995a,b; Shakarchi et al., 2013; Jonz et al., 2015), as they have in mammals (Shirahata et al., 2007; Nurse, 2010). In addition, O_2-chemoreceptive gill NECs in zebrafish retain 5-HT (Jonz and Nurse, 2003), and cells expressing an ACh transporter were reported in the gills of zebrafish (Shakarchi et al., 2013) and the amphibious fish, *Kryptolebias marmoratus* (Regan et al., 2011). In zebrafish larvae, 5-HT_2, 5-HT_3, and nicotinic receptors of the gills appear to play a role in initiation of the hyperventilatory response to hypoxia (Shakarchi et al., 2013; Jonz et al., 2015) and may be involved in initiating the ASR response. Sensory Merkel-like cells containing 5-HT were described in the oropharyngeal epithelium of zebrafish (Zachar and Jonz, 2012), but these cells did not display any morphological changes following long-term exposure to hypoxia (M.G.J., unpublished observations), as did gill NECs (Jonz et al., 2004), suggesting that the former are not O_2 sensitive. Together, these data may suggest that ASR is susceptible to control via O_2 chemoreceptors of the gills. In tambaqui, however, evidence suggests the contrary. Sectioning of the glossopharyngeal and vagus nerves, which supply sensory innervation to gill NECs, did not abolish ASR (Sundin et al., 2000; Florindo et al., 2006); although Florindo et al. (2006) did observe an attenuation of ASR response frequency that was not significantly different from controls.

From the present experiments, adults acclimated to chronic hypoxia for 7 days displayed a lower ASR threshold and spent less time at the surface during severe hypoxia. Other studies have likewise reported that ASR thresholds can be depressed by prior acclimation to hypoxia (Kramer and Mehegan, 1981; Timmerman and Chapman, 2004; Chapman and McKenzie, 2009). Previous studies in zebrafish have demonstrated that acclimation to hypoxia increases the size of O_2-sensitive NECs, and increases the number of nonserotonergic NECs, in the gills (Jonz et al., 2004). Whilst changes in chemoreceptor morphology and proliferation may contribute to the enhanced ventilatory responses observed in fish following acclimation to hypoxia for 1 to 3 weeks (Kerstens et al., 1979; Burleson et al., 2002), as it does in ventilatory acclimatization to hypoxia in mammals (Bisgard, 2000; Powell et al., 1998; Porteus et al., 2011; Kumar and Prabhakar, 2012), it remains to be determined if the blunted ASR response is due directly to these chemoreceptor changes, or due to a putative increased hyperventilatory response resulting from the acclimation period that may have mitigated the need for ASR and effectively reduced the ASR threshold. Confirmation of this will require studies directed at determining the ventilatory response to acute hypoxia in zebrafish after a similar period of acclimation, especially since time-dependent changes in the ventilatory response to hypoxia appear to occur in fish (Porteus et al., 2011). One report has indicated that acclimation of zebrafish to hypoxia for 28 days reduced basal ventilation without a significant effect on the acute response (Vulesevic et al., 2006).

As noted above, ASR behaviour was first observed in zebrafish larvae at 5 dpf. Unlike in adults, at this early developmental stage O_2-sensitive gill chemoreceptors are not yet fully innervated (Jonz and Nurse, 2005) and so are not likely to participate in reflex responses. However, cutaneous NECs that receive innervation, retain 5-HT and exhibit O_2-sensitive properties appear in zebrafish as early as 1 dpf (Coccimiglio and Jonz, 2012). Although these cells may be candidate chemoreceptors for initiation of ASR in early larvae, we were not able to accordingly modify ASR responses in larvae by immersion in 5-HT or ACh before 18 dpf. The reason for this is unclear, since serotonergic receptors participate in hypoxic hyperventilation as early as 7–10 dpf, and cholinergic receptors as early as 12 dpf (Shakarchi et al., 2013). Marginal increases in the frequency of ASR events observed with 5-HT at 7 and 14 dpf suggest that a higher sample size in our experiments might have revealed a more prominent change in behaviour. In addition, the loss of response to ACh in 25 and 40 dpf larvae may be due to the increased size in larvae during these later juvenile stages associated with decreased drug diffusion to tissues during immersion experiments. Indeed, only intraperitoneal injection of 5-HT or ACh enhanced ASR responses in adults. Immersion of adults in these drugs had no effect on ASR.

Evolutionary implications

It is recognized that the transition from water to air breathing in vertebrates was an important adaptive change that led away from the diffuse distribution of peripheral O_2 chemoreceptors in the gills of anamniotes and towards the evolution of the carotid body, the primary peripheral chemoreceptive organ that controls cardiorespiratory reflexes in mammals (Milsom and Burleson, 2007). In addition, it has been proposed by some authors that ASR behaviour in fish may have been an adaptive response that facilitated the evolution of air breathing in vertebrates and the transition to terrestrial life (Kramer and McClure, 1982; Gee and Gee, 1995; Shingles et al., 2005). Considering these two hypotheses, and if ASR is dependent upon input from peripheral chemoreceptors as suggested in this and previous studies (Sundin et al., 2000; Florindo et al., 2006; Jonz and Nurse, 2005; Shingles et al., 2005), it would follow that air breathing arose as a result of evolutionary change enhanced by positive feedback. Positive feedback can be generally defined as the evolution of phenotypes in response to selection that serve to further reinforce or strengthen selection for more extreme phenotypes (Robertson, 1991; Crespi, 2004). In this scenario, movement to the surface resulting from chemoreceptor input would have, in turn, modified chemoreceptor distribution so as to enhance or favour further transitions toward aerial respiration. Observations of chemoreceptor distribution in some aquatic vertebrates may support the positive-feedback hypothesis. In amphibians, NECs of the gills are present in aquatic larval and neotenic forms (Jonz and Nurse, 2006; Saltys et al., 2006), but a carotid labyrinth develops

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after metamorphosis in adults as the primary O_2-chemoreceptive organ, when the lungs become functional (Kusakabe, 2002). Although variable between species, in some air-breathing fish specific chemoreceptor loci may stimulate air breathing while others do not (Milsom, 2012). In addition, air-breathing organs in fish are endowed with chemoreceptor-like cells (Zaccone et al., 1997, 2006), although there is currently no physiological evidence that these cells participate in O_2 sensing. Direct confirmation of a link between chemoreceptors and ASR behaviour will strengthen the positive-feedback hypothesis. It will help us define and understand the diversity of O_2-sensing mechanisms and the evolution of air breathing in vertebrates.

MATERIALS AND METHODS

Animals

Wild-type zebrafish, *Danio rerio* (Hamilton 1822), were obtained from a local commercial supplier (Mirdo Importations, Montreal, Canada) and held in a closed recirculated facility. Animals were maintained at 28.5°C on a 14 h:10 h light:dark cycle (Westerfield, 2000). All animal handling and care was carried out in accordance with institutional guidelines, which adhere to those of the Canadian Council on Animal Care (CCAC). Embryos were bred in accordance with methods described in Westerfield (2000). Embryos were stored in an embryo medium and incubated at 28.5°C. Embryo medium consisted of: 5 mmol l^{-1} NaCl, 0.17 mmol l^{-1} KCl, 0.33 mmol l^{-1} $CaCl_2$, 0.33 mmol l^{-1} $MgSO_4$ at pH 7.8. After hatching, larvae were transferred to 1 litre aquaria filled with dechlorinated water at 28.5°C. The water was replaced every other day and treated with 0.01% Methylene Blue to reduce fungal or bacterial growth.

Behavioural assays for adult zebrafish

The ASR response, and the relationship between water P_{O_2} and ASR behaviour, was first established in zebrafish by exposing adults to acute, progressive hypoxia. We identified ASR behaviour based on previous descriptions in other species (Kramer and Mehegan, 1981; Kramer and McClure, 1982). Zebrafish were engaged in ASR behaviour when the top of the head was near the water surface, with the mouth just below the air–water interface, and the body oriented at a slight upward angle. For each level of P_{O_2}, 4 groups of 2–4 adult zebrafish (N=11) were placed in a 1 litre test chamber, with dimensions 80 mm high by 150 mm wide, marked with gradations on the outside that measured distance (in mm) from the bottom to the air–water interface, or surface. 100% N_2 was delivered to the chamber via polyethylene tubing and a porous stone until the desired water P_{O_2} was achieved. Water P_{O_2} ranged from normoxic values of ∼158 mmHg to as low as 8 mmHg and were verified before and after each trial with an O_2 meter (Model 550A, YSI, Yellow Springs, OH, USA). Once water P_{O_2} had stabilized, zebrafish were introduced into the test chamber and given 2 min to recover from handling. At normoxic levels of P_{O_2}, this was sufficient time for all fish to adopt normal swimming and breathing patterns. A 5 min trial was then performed, after which zebrafish were transferred to a recovery tank for an additional 5 min for recovery. For trials characterizing behaviour over a range of P_{O_2} levels, a group of zebrafish was reintroduced to the test chamber after recovery and an additional trial was performed at a lower P_{O_2}. In this manner, zebrafish were exposed to successive bouts of progressively more severe acute hypoxia.

Adult zebrafish were video-recorded using a digital camera (SD780 IS, Canon, Tokyo, Japan), and their swimming behaviour was analyzed using Videopoint video-tracking software (Lenox Softworks, Lenox, MA, USA) at a sampling rate of 1 frame every 200 ms (5 Hz). The distance between each position was calibrated to the gradations marked directly on the outside of the test chamber to obtain the vertical distance (in mm) from the chamber bottom versus time, and vertical distance versus horizontal position (Fig. 1). Video recordings were analyzed *post hoc* with a desktop computer and high-resolution LCD display to determine the effects on ASR behaviour. Cumulative time engaged in ASR was obtained by reviewing videos and recording the total time (in s) each adult spent near the air–water interface (Figs 2 and 4). Additional experiments were performed to study parameters of swimming behaviour. In these experiments, and in order to facilitate video tracking, zebrafish were placed individually into the test chamber. During 5 min trials adults (N=6) were tracked, as above, and the following behavioural parameters were extracted using MatLab software (MathWorks, MA, USA): total distance travelled (in m), velocity (in mm s^{-1}) and acceleration (in mm s^{-2}, e.g. Fig. 3). Acceleration is an indicator of the rate of change in velocity and was measured because our early observations indicated that zebrafish continuously modify their velocity while swimming.

Behavioural assays for zebrafish larvae

The behaviour of larvae at different developmental stages and P_{O_2} was observed. A small test chamber was constructed in order to assay one larva at a time at pre-determined levels of hypoxia. The chamber (30×25×5 mm) was placed inside a transparent 100 ml water bath. The chamber was designed to produce a vertical water column of sufficient height and width for observation, while at the same time minimizing thickness to prevent zebrafish from moving out of focus during video recording. N_2 was bubbled into the bath until the desired P_{O_2} in the chamber was achieved. Water flow was continuous between the chamber and the bath, and thus allowed for accurate control of chamber P_{O_2} while minimizing turbulence. The P_{O_2} of the chamber was measured directly before and after each trial using an O_2 meter, as above.

A single larva was introduced into the test chamber using a pipette and given 2 min to recover from handling stress. Throughout the subsequent 3 min trial, movement of the larva was video recorded (Sony, Handycam, Model HDR-XR350V, Tokyo, Japan). These assays were performed at P_{O_2} levels of 8, 16, 32, 47, 63 and 158 mmHg. Larvae of ages 5, 7, 14, 18, and 21 days postfertilization (dpf) were tested. Younger larvae lack the ability to make rapid vertical changes in the water column (Lindsey et al., 2010) and so were not tested for ASR. Each larva was tested individually at a single P_{O_2} value. The videos were reviewed, and the position of larvae was noted in 200 ms intervals (i.e. for a 3 min trial, 900 frames in total). ASR was defined as the presence of a larva within 3 mm of the air–water interface in a given frame. The total number of frames in which the larva was performing ASR was noted, and the data are expressed as the number of frames s^{-1} for each developmental stage across a range of water P_{O_2} (Fig. 5). These data were further analyzed as a measure of swimming behaviour using tracking software (Videopoint, Lenox, MA, USA). The x and y coordinates obtained from Videopoint were exported into MatLab (MathWorks, MA, USA) in order to obtain the total distance travelled (m), velocity (mm s^{-1}) and acceleration (mm s^{-2}) of each individual larva (Fig. 6).

Acclimation of adults to hypoxia

Adult zebrafish were placed in a 2.5 litre aquarium and acclimated for 7 days within a P_{O_2} range of 30 to 45 mmHg. Water P_{O_2} was lowered to the desired levels by introducing a mixture of O_2 and N_2 (Pegas 4000 MF gas mixer, Columbus Instruments, Columbus, OH, USA) delivered through a porous stone. Control zebrafish were simultaneously maintained in an identical aquarium at a normoxic level (∼158 mmHg) for the same period of time. Both aquaria were maintained at 28.5°C. Water P_{O_2} was measured as above. Both aquaria were covered with a lid, and a 50% water change with dechlorinated water was performed each day to minimize potential changes in water quality. Acclimated and control adults were tested for acute responses to severe hypoxia as above.

Neurochemical exposures

In order to assess the potential role of O_2-chemoreceptive neuroepithelial cells (NECs) of the gill in initiation of the ASR response, zebrafish were treated with serotonin (5-hydroxytryptamine or 5-HT, cat. no. H9523) and acetylcholine (ACh, cat. no. A6625), both of which stimulate gill O_2 chemoreceptors (Burleson and Milsom, 1995a) and induce hyperventilation in fish, including zebrafish (Burleson and Milsom, 1995b; Shakarchi et al., 2013). Both chemicals were obtained from Sigma-Aldrich (Oakville, ON, Canada).

Adult zebrafish ranging from a body weight of 0.7 to 1.0 g were lightly anaesthetized with 0.25 mg ml^{-1} MS 222 (Syndel Laboratories, Vancouver, BC, Canada) dissolved in dechlorinated system water. Fish were injected intraperitoneally (i.p.) with 5-HT (5 or 10 µg g^{-1} body weight; N=12 or 17,

respectively), ACh (10 or 20 µg g^{-1}; N=29 or 19, respectively) in phosphate-buffered solution (PBS), or PBS as a sham control (N=34). Injections were administered at the ventral aspect below the swim bladder. PBS contained the following: 137 mmol l^{-1} NaCl, 15.2 mmol l^{-1} Na_2HPO_4, 2.7 mmol l^{-1} KCl, 1.5 mmol l^{-1} KH_2PO_4 at pH 7.8 (Jonz et al., 2004). Preliminary experiments indicated that these drug concentrations were optimal in producing a measurable response with minimal mortality. A 10 µl syringe with a 33 gauge needle (Hamilton Company, Reno, NV, USA) was used to deliver a 5 µl g^{-1} body weight volume of these drugs dissolved in PBS. Each adult zebrafish was given 4 injections over a 10 day period and returned to aquaria specific to each treatment group. Preliminary experiments indicated that single injections of these drugs had no observable effect on behaviour. At 48 h after the final injection, all fish were tested for their response to acute hypoxia, as described above.

Developing zebrafish (7–40 dpf) were pre-exposed to 10 µmol l^{-1} 5-HT or 10 µmol l^{-1} ACh dissolved in 10 ml dechlorinated system water in a 20 ml vial for 20 min. Controls were treated in the same way but were not exposed to any drug. Following pre-exposures, 10 larvae from each developmental stage were placed in the test chamber and given 2 min to recover. The effects of hypoxia (P_{O_2}=25 mmHg) on ASR behaviour were recorded for each group and developmental stage by collecting an image (frame) every 10 s for 3 min (for a total of 38 frames). The experiment was repeated with an additional 10 larvae and data were pooled. The number of frames having 0, 1, 2, 3 or 4 ASR events was quantified (Fig. 7). An ASR event was defined as the presence of a larva within 3 mm of the air–water interface in the test chamber during exposure to hypoxia. The total number of events and mean±s.e.m. events per frame were recorded.

Statistics

For data sets containing normal data, the paired t-test was used to compare means, or an analysis of variance (ANOVA) was performed to test for differences among multiple groups. In the latter case, where a statistical difference was detected, a *post hoc* Bonferroni test was used to indicate which groups were significantly different. For data that was not normally distributed, the rank sum Kruskal–Wallis and Dunn's multiple comparison test were used to test for differences between groups. For the data presented in Fig. 2C, cumulative time in ASR versus P_{O_2} was analyzed using a mixed model approach in which the repeated measures ANOVA test was used combined with blocking for groups using SAS (Statistical Analysis System Institute Inc., Cary, NC, USA). All other statistical analyses were performed using Prism v5.0 (GraphPad Software Inc., La Jolla, CA, USA). For cumulative time in ASR versus P_{O_2} (Fig. 2C), lines of best fit were produced following the equation: $y=100/(1+10^{\log EC50-x})$. Note that in some figures, bars indicating s.e.m. were smaller than data points and are not visible.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

All authors participated in the conceptual design of the study and performed analyses. S.J.A. and B.S.T. executed the experiments and collected data. All authors took part in the writing and revision of the manuscript.

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