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CONTROL OF GILL VENTILATION AND AIR-BREATHING IN THE BOWFIN *AMIA CALVA*

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Summary

The purpose of this study was to investigate the roles of branchial and gas bladder reflex pathways in the control of gill ventilation and air-breathing in the bowfin *Amia calva*. We have previously determined that bowfin use two distinct air-breathing mechanisms to ventilate the gas bladder: type I air breaths are characterized by exhalation followed by inhalation, are stimulated by aquatic or aerial hypoxia and appear to regulate O_2 gas exchange; type II air breaths are characterized by inhalation alone and possibly regulate gas bladder volume and buoyancy. In the present study, we test the hypotheses (1) that gill ventilation and type I air breaths are controlled by O_2-sensitive chemoreceptors located in the branchial region, and (2) that type II air breaths are controlled by gas bladder mechanosensitive stretch receptors. Hypothesis 1 was tested by examining the effects of partial or complete branchial denervation of cranial nerves IX and X to the gill arches on gill ventilation frequency (f_G) and the proportion of type I air breaths during normoxia and hypoxia; hypothesis II was tested by gas bladder inflation and deflation. Following complete bilateral branchial denervation, f_G did not differ from that of sham-operated control fish; in addition, f_G was not significantly affected by aquatic hypoxia in sham-operated or denervated fish. In sham-operated fish, aquatic hypoxia significantly increased overall air-breathing frequency (f_{AB}) and the percentage of type I breaths. In fish with complete IX–X branchial denervation, f_{AB} was also significantly increased during aquatic hypoxia, but there were equal percentages of type I and type II air breaths. Branchial denervation did not affect the frequency of type I air breaths during aquatic hypoxia. Gas bladder deflation *via* an indwelling catheter resulted in type II breaths almost exclusively; furthermore, f_{AB} was significantly correlated with the volume removed from the gas bladder, suggesting a volume-regulating function for type II air breaths. These results indicate that chronic (3–4 weeks) branchial denervation does not significantly affect f_G or type I air-breathing responses to aquatic hypoxia. Because type I air-breathing responses to aquatic hypoxia persist after IX–X cranial nerve denervation, O_2-sensitive chemoreceptors that regulate air-breathing may be carried in other afferent pathways, such as the pseudobranch. Gas bladder deflation reflexly stimulates type II breaths, suggesting that gas bladder volume-sensitive stretch receptors control this particular air-breathing mechanism. It is likely that type II air breaths function to regulate buoyancy when gas bladder volume declines during the inter-breath interval.

Key words: hypoxia, air-breathing, gill ventilation, gas bladder, buoyancy, stretch receptor, bowfin, *Amia calva*.

Introduction

The bowfin *Amia calva* is a primitive actinopterygian fish with functional gills for aquatic respiration and a well-vascularized gas bladder for aerial ventilation. In response to aquatic hypoxia, *Amia calva* increase gill ventilation frequency and air-breathing, which serves to maintain oxygen homeostasis (Johansen et al., 1970; Randall et al., 1981; McKenzie et al., 1991; Hedrick and Jones, 1993). During air-breathing in *Amia calva*, gas flow is generated by a buccal force pump which has been described in detail using X-ray cine film and electromyographic analyses (Deyst and Liem, 1985). The latter study experimentally characterized the muscular basis for air-breathing that had been described by direct observations in earlier studies (Wilder, 1877; Johansen et al., 1970); specifically, that *Amia calva* breathe air by a mechanism that involves exhalation followed by an inhalation. Subsequent to those studies, Hedrick and Jones (1993) discovered a previously undescribed air-breathing mechanism in *Amia calva* that involves inhalation alone with no associated expiratory phase. In that study, the two air-breathing mechanisms were designated as type I and type II breaths. Type I breaths are characterized by the previously described exhalation–inhalation sequence, and type II breaths are the newly described

inhalation-only mechanism. Because type I breaths are preferentially stimulated by aquatic or aerial hypoxia (Hedrick and Jones, 1993), we hypothesized that type I breaths are regulated by O_2-sensitive chemoreceptors (see Shelton et al., 1986). In our previous study, *Amia calva* exposed to aerial hyperoxia, regardless of aquatic oxygen levels, used predominantly type II breaths, which led us to hypothesize that type II breaths are regulated by gas bladder volume stretch receptor feedback (Milsom and Jones, 1985), rather than having an oxygen-acquisition function. The proximate function of type II breaths is thought to be adaptive for regulating buoyancy in the aquatic environment (Hedrick and Jones, 1993).

The locations of the putative O_2-sensitive chemoreceptors and the afferent pathways that mediate branchial and air-breathing reflexes in *Amia calva* and other air-breathing fishes are uncertain. Evidence from air-breathing and non-air-breathing fish (i.e. those that use exclusively branchial ventilation) suggests that hypoxic ventilatory reflexes are regulated by O_2-sensitive chemoreceptors in the branchial region that monitor aquatic and/or intravascular P_{O_2} (Shelton et al., 1986; Smatresk et al., 1986; Burleson et al., 1992). Because the afferent neural pathways from the presumed branchial locations for these chemoreceptors are carried in the glossopharyngeal (cranial nerve IX) and vagus (cranial nerve X) nerves, studies have attempted to abolish gill ventilatory responses to hypoxia by selective branchial denervation (Saunders and Sutterlin, 1971; Burleson and Smatresk, 1990); however, there is little information on the role of potential chemoreceptor pathways controlling air-breathing reflexes in fish (Smatresk et al., 1986; McKenzie et al., 1991).

The purpose of the present study was to test in *Amia calva* the hypotheses (1) that increased gill ventilation and the frequency of type I air breaths during aquatic hypoxia are regulated by O_2-sensitive chemoreceptors located in the branchial region with afferent pathways carried in cranial nerves IX and X; and (2) that type II air breaths are stimulated by reductions in gas bladder volume and are regulated by volume-sensitive gas bladder stretch receptors.

Materials and methods

Animals

Twenty-two bowfin (*Amia calva* L.) were used in this study. Fish were netted in Lake Ontario, Canada, by commercial fishermen and air-freighted to the University of British Columbia, where they were kept indoors in large circular fibreglass tanks supplied with continuously running dechlorinated tap water that ranged in temperature from 6 to 13 °C during the course of this study.

Branchial denervation experiments

Seventeen fish (mass 433±77 g, mean ± S.D.) were used for denervation experiments. The experiments were carried out from June to November. Surgical procedures were performed at the temperature at which the fish were normally maintained (10–13 °C), but fish were acclimated to room temperature (20–22 °C) for the experimental procedures. Fish were anaesthetized in 0.01 % tricaine methanesulphonate (MS-222; Sydell Laboratories, Vancouver, British Columbia, Canada) dissolved in dechlorinated water (pH 7.4–7.8). When gill ventilation ceased, the fish was transferred to an operating table and artificially ventilated with a lower concentration of MS-222 (0.005 %) in oxygenated (100 % O_2) water. The branchial branches of cranial nerves IX (glossopharyngeal) and X (vagus) were exposed by lifting the operculum and cutting the thin membrane overlying the gill arches. Branches of nerve X innervate all four gill arches, whereas nerve IX innervates the first gill arch and pseudobranch (see Nilsson, 1984). Branches of cranial nerves IX and X to the four gill arches and pseudobranch were identified, separated from the surrounding fascia and cut with scissors. Bleeding was minimal and was controlled with cotton applicators. The procedure was performed bilaterally, and the membrane was closed with 3–4 sutures (3-0 silk). The entire procedure required approximately 45–60 min to complete. The fish recovered in oxygenated, dechlorinated water until spontaneous gill ventilatory movements returned. Sham-operated control fish were treated in the same manner with anaesthetization and bilateral nerve exposure, but the nerves were not cut. After the procedure, the fish were returned to a holding tank maintained at the pre-surgical temperature (10–13 °C) and allowed 3–4 weeks to recover.

Protocol

After the minimum 3 week recovery period, fish were brought into the laboratory in large (40 l) plastic containers at their normal holding temperature (10–13 °C), and the water was allowed to warm slowly to room temperature (20–22 °C) overnight while being continuously aerated. This acclimation procedure was complete by the following day (12–16 h), and the animals were held at room temperature for a total of 24–48 h before being transferred to an aquarium for measurements. After acclimation, a fish was placed into an aquarium for 12–24 h before any recordings were made. Air-breathing behaviour was recorded non-invasively by allowing the fish to breathe from a funnel located at the air–water interface; air flowed continuously through the funnel at 200 ml min^{-1}. Air-breathing was restricted to the funnel by blocking the aquarium surface with an acrylic cover.

Air-breathing behaviour was recorded with a camera and video cassette recorder (JVC Canada, Scarsborough, Ontario, Canada) at 30 frames s^{-1} for each fish in 8 h sessions over a 3 day period with the following sequence: day 1, aquatic normoxia (P_{O_2}>18.6 kPa); day 2, aquatic hypoxia (P_{O_2}=6.52±0.13 kPa, mean ± S.E.M.); day 3, aquatic normoxia. After these sessions had been completed, the fish was killed by overanaesthetization with MS-222, and a *post-mortem* dissection was carried out to determine the extent of branchial denervation. Branchial nerves were identified and compared with a published description (Allis, 1897). Fish were categorised, on the basis of the dissection findings, as sham-operated control fish (SH; N=4), partially denervated (PD; N=5), in which nerve regrowth was indicated or not all the

branchial branches had been cut, and total branchial-denervated fish (TD; N=8).

Air-breathing events were counted directly from the video tape after each recording session; air breath type (type I or II) was characterized by specific ventilatory movements that were easily distinguished on video tape and were confirmed previously by pneumotachography (Hedrick and Jones, 1993). In the previous study, we found that expired ventilatory volume (V_{exp}) did not change in response to aquatic or aerial gas concentrations; therefore, V_{exp} was not measured in this study. In the present study, pneumotachography was used to confirm type I or type II breaths that were not easily distinguished by direct observations from video tape.

Gill ventilation frequency (f_G) for each fish was counted directly from the video tape with a minimum 2 min recording used as a datum. Gill ventilation frequency was measured at least once, and as many as 20 times, during each 8 h recording session; however, a single mean value for each fish in each treatment was used in the analysis. It was often not possible to observe gill ventilatory movements if the fish was facing away from the camera or if the fish was moving excessively.

Gas bladder inflation and deflation

Five fish (mass 335±99 g, mean ± S.D.) were used for gas bladder inflation/deflation experiments. These experiments were carried out during January–February when the water temperature was 6–8 °C. Each fish was brought into the laboratory and acclimated to room temperature (20–22 °C) as described above for 48–72 h; after acclimation, each fish was anaesthetized with MS-222 and placed on a surgical table and artificially ventilated as described above. The glottis was identified in the dorsal gut wall and held open with forceps. A 50 cm length of polyethylene tubing (PE90; Intramedic) was introduced through the glottis and pneumatic duct and advanced approximately 3–5 cm into the gas bladder. The cannula was secured to the palate with suture (0 silk), passed through a grommet in the frontal bone and exited through the snout. The glottal aperture of *Amia calva* used in this study is approximately 5 mm in diameter (Davies et al., 1993); therefore, it is estimated that the total area of the glottis occluded by the cannula was less than 5 %. Following surgery, each fish was allowed to recover for 24 h in an aquarium containing continuously aerated, dechlorinated water at room temperature.

Tests of gas bladder deflation and inflation were carried out with fish in normoxic (P_{O_2}=19–21 kPa), hypoxic (P_{O_2}=7–8 kPa) and hyperoxic (P_{O_2}=36–40 kPa) water on separate days. The aquarium containing the fish was bubbled with oxygen/nitrogen mixtures from a gas-mixing pump (Wösthoff) until the desired P_{O_2} was reached. The P_{O_2} of the water was measured with a Clark-type oxygen electrode (Radiometer). Gas bladder volume manipulations were achieved with minimal stimulation from catheter handling or visual and vibrational disturbances. For inflation or deflation of the gas bladder, the catheter was connected to a plastic syringe, which was firmly secured to a flat surface near the aquarium, and a volume of gas was removed from, or injected into, the gas bladder. Following each deflation or inflation, air-breathing behaviour was observed for 10 min and recorded as the number of breaths taken as well as the type (type I or II) of breath taken by the fish. The walls of the aquarium were covered with an opaque material to within 5 cm of the water surface so that the fish would not be disturbed by visual inputs, but the experimenter could observe the air breath response at the surface. At least 30 min was allowed between deflation or inflation trials.

Data analysis and statistics

Values are presented as means ± S.E.M. unless stated otherwise. For gill denervation experiments (N=17), measurements during a single 8 h recording session included f_G, the total number of air breaths, the breath type (type I or II) and the inter-breath interval (IBI, min) between type I air breaths. These measurements were obtained for each fish in the three groups (SH, PD and TD) during aquatic normoxia or hypoxia on each of the 3 days of recording. Data were analyzed using unpaired *t*-tests, and one-way and two-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls (SNK) multiple-range test where significant differences were detected by ANOVA (Zar, 1974). Where Bartlett's test revealed a lack of homogeneity of variance, data were analyzed using a non-parametric ANOVA by ranks (Kruskal–Wallis). Percentages of type I and type II air breaths for each fish were converted to their arcsine values before comparison using unpaired *t*-tests (Zar, 1974). For gas bladder deflations, least-squares linear regression was also used to examine the effects of gas bladder volume deflation on air-breathing frequency (f_{AB}). Significance was accepted at the 5 % level (P<0.05). All statistical comparisons were carried out using a commercially available statistical software package (GraphPad Prism version 2.0, San Diego, CA, USA).

Results

Gill ventilation and air-breathing responses to branchial denervation

Table 1 summarizes the mean f_G values recorded for surgically treated fish (SH, PD or TD) during aquatic normoxia and hypoxia. Gill ventilation frequency was significantly affected by branchial denervation ($F_{2,31}$=9.6; P<0.002) and by

Table 1. *Mean gill ventilation frequency (cycles min^{-1}) for sham-operated (SH;* N=*4), partially denervated (PD;* N=*5) and totally denervated (TD;* N=*8)* Amia calva *in normoxic (day 1 and day 3) and hypoxic water*

	Normoxia (day 1)	Hypoxia (day 2)	Normoxia (day 3)
SH	8.9±1.0	9.7±1.1	6.7±0.7
PD	12.2±2.7	9.3±1.2	6.6±0.5*
TD	5.6±0.7‡	6.5±1.2	4.9±0.7

*Significantly different (P<0.05) from normoxia (day 1); ‡significantly different (P<0.05) from PD fish during normoxia (day 1).

Values are means ± S.E.M.

Table 2. *Mean air-breathing frequency (f_{AB}) and percentage of type I and type II breaths (of total breaths) for sham-operated (SH;* N*=4), partially denervated (PD;* N*=5) and totally denervated (TD;* N*=8) fish during normoxia (days 1 and 3) and hypoxia (day 2)*

	Normoxia (day 1)			Hypoxia (day 2)			Normoxia (day 3)		
	f_{AB} (breaths h^{-1})	Type I (%)	Type II (%)	f_{AB} (breaths h^{-1})	Type I (%)	Type II (%)	f_{AB} (breaths h^{-1})	Type I (%)	Type II (%)
SH	4.7±2.0	25.8±12.5	74.2±12.5*	9.7±0.3‡	74.5±9.7	25.5±9.7**	3.4±0.6	53.7±14.6	46.3±14.6
PD	3.5±0.9	35.0±14.4	65.0±14.4	7.4±1.2‡	61.8±5.3	38.2±5.3**	3.2±1.2	42.7±12.0	57.3±12.0
TD	9.1±2.5	28.1±6.9	71.9±6.9***	12.6±2.8‡	46.8±8.7	53.2±8.7	5.9±1.8§	40.9±8.1	59.1±8.1

*P<0.05; **P<0.02; ***P<0.001 compared with the percentage of type I breaths (unpaired t-test) for the same treatment.
‡P<0.05 compared with both normoxic treatments (one-way ANOVA).
§P<0.05 compared with normoxia (day 1) in the same treatment.
Values are means ± S.E.M.

aquatic gas treatment ($F_{2,31}$=5.3; P<0.05) owing to a reduction in f_G in the second normoxic treatment for the PD fish (Table 1). f_G in TD fish was depressed relative to that of PD fish during normoxia (day 1). In all three groups, there was no significant gill ventilatory response to aquatic hypoxia, and f_G for TD fish did not differ from that of control (SH) fish (Table 1).

Air-breathing frequency was approximately 3–9 breaths h^{-1} in all three groups during normoxia and increased significantly during aquatic hypoxia (Table 2). SH and TD fish in the first normoxic treatment (day 1) had a significantly greater proportion of type II breaths compared with type I breaths (P<0.05; unpaired t-test; Table 2), but in PD fish there was no difference between the numbers of type I and type II air breaths during normoxia. During aquatic hypoxia, SH and PD fish showed a significant change in the percentage of type I breaths relative to type II breaths (unpaired t-test; P<0.02). Both the increase in f_{AB} and an increased percentage of type I breaths during hypoxia have been noted previously in *Amia calva* (Hedrick and Jones, 1993). In contrast, TD fish showed no significant increase in the percentage of type I breaths during hypoxia (Table 2) despite the overall increase in f_{AB} compared with normoxia, and the increased frequency of type I breaths during hypoxia (Table 3). Direct

Table 3. *Mean interbreath interval (IBI; min) for type I air breaths for individual sham-operated (SH), partially denervated (PD) and totally denervated (TD) fish*

	Fish	Normoxia (day 1)	P	Hypoxia (day 2)	P	Normoxia (day 3)
SH	3	21.7±14.9 (41)	0.001	7.5±5.9 (65)	0.001	19.9±12.6 (23)
	5	28.9±14.4 (16)	0.001	6.9±1.4 (69)	0.001	17.4±6.0 (27)
	9	0		9.4±3.9 (51)	0.001‡	49.5±23.3 (6)
	15	0		12.4±7.3 (39)	0.01‡	84.0±56.6 (3)
PD	1	117±6.4 (2)	0.05	12.4±3.8 (38)	0.001	52.0±13.7 (8)
	4	88.3±82.3 (10)	0.001	13.6±4.8 (30)	0.05	83.0±35.9 (3)
	6	0		25.4±5.6 (18)	0.001‡	124.7±5.5 (3)
	7	13.4±4.3 (36)***	0.001	8.7±2.6 (56)	NS	9.0±3.6 (54)
	10	46.3±28.4 (10)	0.001	13.1±3.9 (36)	NS	120±139 (2)
TD	11	40.8±21.9 (11)	0.001	8.2±2.2 (59)	0.001	40.9±29.6 (9)
	12	99.8±36.5 (4)	0.001	12.6±4.4 (36)	0.001	42.4±18.6 (9)
	13	30.6±9.8 (14)	0.001	11.6±3.2 (41)	0.01	20.4±12.5 (20)
	14	21.4±7.4 (22)*	0.001	14.2±4.3 (34)	NS	17.6±9.4 (25)
	16	114±9.2 (2)	0.001‡	27.6±7.3 (17)		0
	18	60.3±15.2 (7)	0.001	21.7±4.2 (22)	0.001	96.5±34.3 (4)
	21	15.0±10.0 (31)*	0.001	8.8±2.6 (53)	NS	10.6±5.3 (45)
	17	18.6±4.0 (26)	0.001	12.2±3.5 (37)	0.01	20.5±10.1 (10)

Significance level (P) between aquatic hypoxia IBI and both normoxic IBI values is given (Kruskal–Wallis test).
‡Unpaired t-test.
*P<0.05; ***P<0.001 compared with normoxic (day 3) value (Kruskal–Wallis test).
Values are means ± S.D. (N).
NS, no significant difference.
A value of 0 indicates that the fish did not use type I breaths during the period of observation in this treatment.

observations from the video tape analysis revealed that some TD fish had difficulty capturing and transferring inhaled air to the gas bladder during type I breaths, thus failing to fill the gas bladder sufficiently with inhaled air (see Discussion). The lack of an increased percentage of type I breaths in TD fish during hypoxia compared with SH and PD fish was 'masked' by the larger percentage of type II breaths taken by five of the eight TD fish. For example, f_{AB} in one TD fish was approximately 6 breaths h^{-1} during normoxia and increased to approximately 9 breaths h^{-1} during hypoxia; however, the majority of these breaths (>75 %) were of type II.

Table 3 shows the mean interbreath interval (IBI) for type I breaths for individual fish in each treatment. Two of the four SH fish and one of the five PD fish did not use any type I air breaths during the first normoxic treatment. IBI ranged from 13 to 117 min during normoxia and decreased to 7–28 min during hypoxia (Table 3). In all three groups, type I IBI decreased significantly during aquatic hypoxia (Kruskal–Wallis test, Table 3), indicating a strong effect of aquatic hypoxia on type I f_{AB}. In only three of the 17 fish was IBI significantly greater during the first normoxic treatment compared with the second normoxic treatment, indicating that the hypoxic exposure on day 2 had little effect on the number or frequency of type I air breaths on day 3 (normoxia).

Effects of gas bladder deflation and inflation

Gas bladder deflation had a highly significant effect on f_{AB} in the five *Amia calva* used; f_{AB} was significantly correlated with the volume removed (V_R) from the gas bladder: $f_{AB}=0.5V_R+4.3$ (Fig. 1; $F_{1,88}=35.7$; $P<0.001$; $r^2=0.29$). The overall slope of the regression (0.50±0.08) was significantly different from zero ($t_{88}=5.98$; $P<0.001$). The effects of V_R on f_{AB} during aquatic normoxia and hyperoxia were highly significant (normoxia: $F_{1,49}=16.2$; $P<0.001$; hyperoxia: $F_{1,23}=15.0$; $P<0.001$), with slopes of 0.38±0.1 and 0.54±0.14, respectively. During aquatic hypoxia, however, V_R did not have a significant effect on f_{AB} ($F_{1,11}=3.6$; $P<0.1$) with a slope of 0.56±0.3. In every instance except one, regardless of aquatic P_{O_2}, the initial response to deflation was a type II air breath, and the majority of these breaths (>50 %) were taken less than 1 min following deflation. Type I breaths were occasionally observed during the 10 min following deflation, but these occurred during aquatic hypoxia and were never the first or second breath in a series of air breaths. There appeared to be a volume threshold of approximately 3 ml kg^{-1}; that is, changes to the volume of the gas bladder below this threshold volume did not elicit air-breathing. Large deflations typically caused the fish to sink to the bottom of the aquarium, and they then struggled to reach the surface to take an air breath, thus indicating qualitatively that deflation caused the fish to become negatively buoyant. Gas bladder inflation above the apparent volume threshold typically prevented an air breath attempt if the animal was approaching the water surface. Large inflations caused fish to rise rapidly towards the surface because of increased positive buoyancy and usually resulted in excess air being expelled from the mouth before the fish reached the air–water interface. This allowed the fish to attain neutral or negative buoyancy and to return to the bottom of the aquarium.

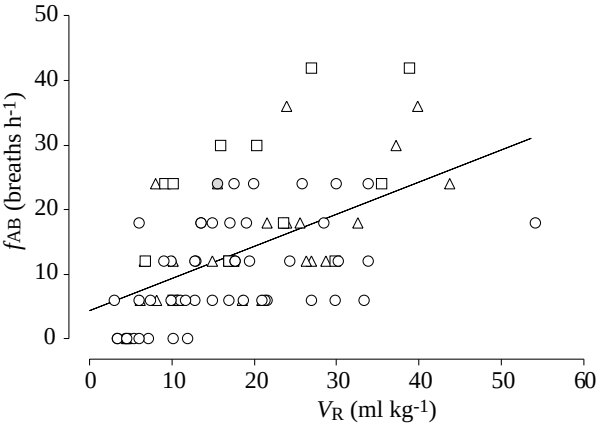

Fig. 1. Air-breathing frequency f_{AB} (breaths h^{-1}) as a function of the volume of gas removed V_R (ml kg^{-1}) from the gas bladder in *Amia calva* during aquatic normoxia (circles), hypoxia (squares) and hyperoxia (triangles). The solid line is the line of best fit using least-squares linear regression analysis for all values (see Results). The relationship is significant at $P<0.001$.

Discussion

The important findings from this study are (1) that f_G is depressed by complete chronic branchial denervation, but is not significantly altered by exposure to aquatic hypoxia; (2) that chronic total or partial branchial denervation does not abolish or attenuate type I breaths during aquatic hypoxia, suggesting that other branchial sites, such as the pseudobranch, may be responsible for the hypoxic ventilatory responses after complete branchial denervation; and (3) that deflation of the gas bladder resulted in type II breaths almost exclusively, and f_{AB} was positively correlated with the volume of gas removed from the gas bladder, suggesting a role for volume-sensitive gas bladder stretch receptors in regulating type II air breaths.

Role of branchial reflex pathways in ventilation

Afferent nerve activity from the first gill arch of teleosts has been shown to increase in response to hypoxia, low pH or chemical stimulants such as sodium cyanide (NaCN) (Laurent and Rouzeau, 1972; Milsom and Brill, 1986; Burleson and Milsom, 1990, 1993; Burleson et al., 1992). The afferent activity from the gill arches, carried in glossopharyngeal (IX) and vagal (X) pathways, is presumed to mediate the cardiovascular and ventilatory responses to hypoxia in conscious fish; however, denervation of these afferent branchial pathways has generally failed to abolish the ventilatory responses to hypoxia (Hughes and Shelton, 1962; Saunders and Sutterlin, 1971; Randall and Jones, 1973), suggesting that extrabranchial sites, possibly in the central nervous system (CNS), initiate hypoxia-induced ventilatory responses (Bamford, 1974; Jones, 1983). At present, there is no evidence to support a role for CNS chemoreceptors in

mediating hypoxic or hypercapnic ventilatory reflexes in air-breathing or non-air-breathing fish (Graham et al., 1990; Hedrick et al., 1991). More recent experiments have shown that gill ventilation and air-breathing responses to hypoxia can be eliminated by complete branchial denervation combined with pseudobranch ablation (Burleson and Smatresk, 1990; McKenzie et al., 1991). Burleson and Smatresk (1990) reported that complete branchial denervation in channel catfish *Ictalurus punctatus*, a species that lacks a pseudobranch, was necessary to abolish hypoxic ventilatory reflexes, supporting the hypothesis that O_2-sensitive chemoreceptors are localized peripherally. Their study also indicated that multiple branchial chemoreceptor sites are present because failure to denervate catfish completely resulted in an intact hypoxic ventilatory response. A similar study with *Amia calva* indicated that complete branchial denervation combined with pseudobranch ablation could abolish air-breathing responses to relatively brief (15 min) exposures to aquatic hypoxia and attenuate increases in gill ventilation frequency and opercular amplitude (McKenzie et al., 1991).

Gill ventilation frequency was similar for fish in the present study to that in the study of McKenzie et al. (1991), despite differences in methodology. The present study used a non-invasive measurement of f_G, whereas McKenzie et al. (1991) used direct opercular pressure measurements. Both studies also indicate that f_G during normoxia is reduced by complete branchial denervation. We did not observe any changes in f_G for control or denervated fish upon exposure to hypoxia, which contrasts with the significant increase in both f_G and opercular amplitude in sham-operated fish in the study of McKenzie et al. (1991). Their study also showed that f_G and opercular amplitude were attenuated, but not abolished, by branchial denervation. One drawback of the present study is that we were only able to measure f_G and could not measure an index of ventilation volume, such as opercular pressure. It is therefore possible that fish in the present study increased their ventilation volume in response to hypoxia or relied more heavily on ventilation volume than on changes in opercular amplitude during hypoxia under the experimental conditions in this study.

There are other important methodological differences between the present study and the study by McKenzie et al. (1991) that may also explain differences in air-breathing responses to hypoxia in sham-operated and denervated fish. One major difference between the two studies is the type of apparatus used to hold fish for measurements. The fish in the study by McKenzie et al. (1991) were placed into a smaller, shallow box with a forward air space for air-breathing similar to the type used in a previous study on the role of central chemoreceptors in air-breathing *Amia calva* (Hedrick et al., 1991). Although this type of apparatus is necessary for studies in which measurements of opercular and blood pressures are made *via* indwelling catheters, there is little room for movement by the fish. This might affect the type of air breaths used by the fish, particularly if buoyancy control is unnecessary in shallow water. For example, SH fish in our study had an overall f_{AB} of 3–5 breaths h^{-1} during aquatic normoxia and of approximately 10 breaths h^{-1} during aquatic hypoxia; these values are approximately twice the f_{AB} values reported for SH fish by McKenzie et al. (1991) or Hedrick et al. (1991) under similar conditions. A significant percentage of these breaths were of type II in the present study, particularly during normoxia (Table 2), indicating that, when fish are allowed more access to movement in a larger container, overall f_{AB} may be higher. If we remove type II breaths from our analysis, f_{AB} values in these studies are similar.

The fish in the present study were allowed a longer recovery period (3–4 weeks) following surgery than the 48 h recovery time allowed in the study by McKenzie et al. (1991), which may have enabled alternative pathways (i.e. metabolic) or other cues to develop which could regulate hypoxic ventilatory responses. For example, McKenzie et al. (1991) found that branchially denervated *Amia calva* exposed to aquatic hypoxia showed a significant increase in plasma catecholamine concentration and that catecholamine infusion increased f_G, but not f_{AB}. Although we did not measure catecholamine levels in the present study, there may be differences in the amount of 'stress' (as indicated by catecholamine levels) experienced by the fish in the two studies, and this may in turn have had some impact on hypoxic ventilatory responses. Exposure to hypoxia was relatively brief (15 min) in the study by McKenzie et al. (1991), which may not have allowed full expression of air-breathing responses to hypoxia. In the present study, the mean IBI for type I air breaths for hypoxic TD fish was 14.6±2.8 min (mean ± S.E.M., N=8). Thus, relatively short hypoxic exposures may not allow observations of air-breathing responses in denervated fish, particularly if type I air breaths are used predominantly. Finally, and perhaps most importantly, we did not remove the pseudobranch from the *Amia calva* in our study, although the innervation from cranial nerve IX to the pseudobranch was cut. There is some evidence that the pseudobranch in some species is innervated by cranial nerve VII (Nilsson, 1984); thus, the possibility exists that some peripheral chemoreceptive tissue remained intact in our fish. Given the lack of evidence for CNS sites that could modulate air-breathing responses to hypoxia (Hedrick et al., 1991), it is likely that an intact pseudobranch allows expression of hypoxic ventilatory reflexes.

The denervation procedure in our study appeared to have a detrimental effect on air-breathing behaviour and ventilatory mechanics that has not been noted previously. Our observations from video recordings of air breathing indicated that some TD fish could not capture and transfer inhaled air to the gas bladder in a normal fashion. More specifically, as inhaled air was transferred from the buccal cavity to the gas bladder, most was lost through the opercular cavity. Under normal circumstances, a small amount of gas is lost in this way, and this small loss of inhaled gas appears to be an important source of variation that masks an underlying periodicity present in type I air breaths (Hedrick et al., 1994). The failure to transfer inhaled gas adequately in denervated fish is probably due to the loss of afferent information from the gill arches. A significant amount of mechanoreceptive information

is contained in gill arch afferents that transduce information about gill position and deformation (see Burleson et al., 1992). Our observations suggest that some aspect of this afferent information is necessary for the normal expression of the motor control involved in air-breathing behaviour; however, this remains to be tested experimentally. The failure to transfer inhaled air adequately to the gas bladder in TD fish resulted in a larger number of type II breaths (inhalation only) and a lack of an increase in the percentage of type I breaths during hypoxia (Table 2). In TD fish, stimulation of type I breaths by hypoxia (Table 3) caused qualitatively greater losses of inhaled gas compared with SH or PD fish, resulting in an increased frequency of type II breaths to restore gas bladder volume. This lends indirect support to our hypothesis that type II breaths function to maintain gas bladder volume within normal limits (see below).

Expired ventilation volume (V_{exp}) was not measured for type I air breaths in the present study, but in a previous study on this species we found that V_{exp} was unaffected by aquatic or aerial hypoxia (Hedrick and Jones, 1993). Thus, f_{AB} and the relative proportions of type I or type II breaths make a more important contribution to minute ventilation than does V_{exp}. However, inhaled volume is the more important parameter with respect to both gas exchange and buoyancy regulation. Our observations that TD fish lost substantial amounts of inhaled gas through the opercular cavity would not be confirmed by measurements of V_{exp}. From our observations, it was clear that large losses of gas resulted in decreased buoyancy and an increased proportion of type II air breaths during hypoxia (Table 2).

Gas bladder volume regulation of air-breathing

It is known that gas bladder deflation stimulates, and inflation inhibits, air-breathing responses in *Amia calva* (Johansen et al., 1970), gar *Lepisosteus osseus* (Smatresk and Cameron, 1982) and lungfish *Protopterus annectens* (Pack et al., 1992). The response is analogous to the Breuer–Hering inspiratory-terminating reflex in mammals. These species have been shown to have slowly adapting and/or rapidly adapting mechanoreceptors that respond to volume changes in the gas bladder (DeLaney et al., 1983; Milsom and Jones, 1985; Smatresk and Azizi, 1987). Because these primitive air-breathing species are dependent upon gas bladder volume for buoyancy regulation, in addition to the role of the gas bladder as a gas exchanger, it is reasonable to assume that volume-sensitive gas bladder stretch receptors provide information that is integrated into the overall air-breathing behaviour. The present study demonstrated a clear functional role for volume-sensitive feedback in the regulation of air-breathing in *Amia calva*: reductions in gas bladder volume result in an increased frequency of type II breaths and a restoration of gas bladder volume (Fig. 1). Afferent information from gas bladder stretch receptors in *Amia calva* is carried in the ramus intestinalis branch of the vagus nerve; nerve fibre discharge is slowly adapting, increases in response to inflation and decreases or ceases in response to deflation (Milsom and Jones, 1985). Moreover, afferent nerve discharge is proportional to gas bladder volume in *Amia calva*; therefore, our observation that the increased frequency of type II breaths is also proportional to the reduction in gas bladder volume can be explained by the characteristics of gas bladder mechanoreceptor discharge.

Our interpretation of the role of type II breaths in regulating gas bladder volume and thus playing an important role in buoyancy control is supported by a number of observations. First, *Amia calva* with bilateral denervation of the ramus intestinalis nerve fail to control buoyancy (Hedrick, 1991). Second, *Amia calva* exposed to aerial hyperoxia (100 % O_2) switch to exclusive use of type II breaths (Hedrick and Jones, 1993). Our interpretation of the latter result is that, when the contents of the gas bladder have a very high P_{O_2}, there is greater diffusion of O_2 into venous blood, resulting in a high blood P_{O_2} downstream from the gas bladder. Because O_2 from venous blood would diffuse across the gills into the water, there would be a continuous flux of O_2 from the gas bladder to the venous blood. The high blood P_{O_2} under these conditions presumably 'silences' the oxygen chemoreceptors that regulate type I breaths, yet diffusion of O_2 from the gas bladder to the blood will still result in a reduction in gas bladder volume. Third, we have estimated, using gas bladder and tidal volume data from Hedrick and Jones (1993), that a typical type II air breath would increase gas bladder P_{O_2} by approximately 0.5 kPa during aerial normoxia. This small change in P_{O_2} is unlikely to be detected by intravascular oxygen-sensitive chemoreceptors (Burleson et al., 1992); therefore, type II breaths are unlikely to contribute significantly to gas exchange. Alternatively, type I breaths, which are of larger volume and exchange approximately 30 % of the gas bladder contents with each breath, are sufficient to maintain the P_{O_2} of approximately 7–11 kPa measured in the gas bladder of *Amia calva* (Johansen et al., 1970; Hedrick, 1991). Finally, results from computer model simulations (Hedrick and Katz, 1991) with 'firing' thresholds for P_{O_2} and gas bladder volume that regulate type I and type II breaths, respectively, produce realistic air-breathing periodicities that are similar to those observed from *Amia calva* during aquatic normoxia and hypoxia (Hedrick et al., 1994). Taken together, these observations indicate that the proximate function of type II breaths is to regulate gas bladder volume; volume regulation of the gas bladder is clearly important for maintaining buoyancy in the aquatic environment.

Evolutionary implications

Our current view of air-breathing in *Amia calva* is that this species evolved two air-breathing mechanisms for coping with the conflicting demands of buoyancy and gas exchange that occur when a single organ is used for both functions. This conflict arises because an effective gas exchanger requires high rates of diffusional exchange across its respiratory surface, whereas the effectiveness of a buoyancy-control organ relies on minimizing diffusional gas flux. Any primitive lung, or gas-filled structure, automatically imposes a change in buoyancy on the animal whether or not the inhaled gas is involved with gas exchange and metabolic demands. *Amia calva* appears to

have evolved a strategy in which gas exchange is regulated by type I breaths, whereas the buoyancy/volume function of the gas bladder is regulated by type II breaths which simply replace the gas lost by diffusion across the respiratory surface to the blood. Although it is not known whether other air-breathing fishes have similar mechanisms to cope with the conflicting demands of gas exchange and buoyancy, it appears that buoyancy regulation has been a strong selection pressure for the development of type II air-breathing behaviour in *Amia calva*.

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