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THE EFFECTS OF ALTERED AQUATIC AND AERIAL RESPIRATORY GAS CONCENTRATIONS ON AIR-BREATHING PATTERNS IN A PRIMITIVE FISH (*AMIA CALVA*)

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Summary

The mechanisms and physiological control of air-breathing were investigated in an extant halecomorph fish, the bowfin (*Amia calva*). Air flow during aerial ventilation was recorded by pneumotachography in undisturbed *Amia calva* at 20–24°C while aquatic and aerial gas concentrations were independently varied. Separation of aquatic and aerial gases was used in an attempt to determine whether *Amia calva* monitor and respond to changes in the external medium *per se* or to changes in dissolved gases within the body. Air flow measurements revealed two different types of ventilatory patterns: type I air-breaths were characterized by exhalation followed by inhalation; type II air-breaths, which have not been described previously in *Amia calva*, consisted of single inhalations with no expiratory phase. Expired volume (V_{exp}) for type I breaths ranged from 11.6±1.1 to 26.7±2.9mlkg^{-1} (95% confidence interval; N=6) under normoxic conditions and was unaffected by changes in aquatic or aerial gases. Gas bladder volume (V_B), determined *in vitro*, was 80mlkg^{-1}; the percentage of gas exchanged for type I breaths ranged from 14 to 33% of V_B in normoxia.

Fish exposed to aquatic and aerial normoxia (P_{O_2}=19–21kPa), or aerial hypercapnia (P_{CO_2}=4.9kPa) in normoxic water, used both breath types with equal frequency. Aquatic or aerial hypoxia (P_{O_2}=6–7kPa) significantly increased air-breathing frequency in four of eight fish and the ventilatory pattern changed to predominantly type I air-breaths (75–92% of total breaths). When fish were exposed to 100% O_2 in the aerial phase while aquatic normoxia or hypoxia was maintained, air-breathing frequency either increased or did not change. Compared with normoxic controls, however, type II breaths were used almost exclusively (more than 98% of total breaths).

Type I breaths appear to be under feedback control from O_2-sensitive chemoreceptors since they were stimulated by aquatic or aerial hypoxia and were

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nearly abolished by aerial hyperoxia. These results also indicate that *Amia calva* respond to changes in intravascular P_{O_2}; however, externally facing chemoreceptors that stimulate air-breathing in aquatic hypoxia cannot be discounted. Type II air-breaths, which occurred in aerial hyperoxia, despite aquatic hypoxia, appear to be stimulated by reductions of V_B, suggesting that type II breaths are controlled by volume-sensitive gas bladder stretch receptors. Type II breaths are likely to have a buoyancy-regulating function.

Introduction

The bowfin (*Amia calva*) is a primitive (non-teleost) actinopterygtian fish which uses both gills and a vascularized gas bladder for respiratory exchange. Wilder (1877) first reported that *Amia calva* has the ability to exhale and inhale air and used its gas bladder as an accessory air-breathing organ. More recent studies, using light and X-ray ciné film and electromyographic (EMG) analysis of the muscles involved in air-breathing, have confirmed and extended Wilder's original observations that exhalation precedes inhalation during aerial ventilation (Randall *et al.* 1981; Deyst and Liem, 1985). The exhalation–inhalation sequence described for *Amia calva* is similar to that in gar (Rahn *et al.* 1971) and several teleost species (Kramer, 1978; Ishimatsu and Itazawa, 1981; Greenwood and Liem, 1984; Liem, 1980, 1984, 1988, 1989). A re-examination of the aerial ventilation mechanism in *Amia calva* has indicated two different patterns of EMG activity during air-breathing events (Liem, 1988, 1989), thus raising the question of whether the exhalation–inhalation sequence of air-breathing is the sole mechanism for gas bladder ventilation. This possibility was tested in the present study by directly measuring expired and inspired air flow during aerial ventilation.

The reflex control of air-breathing in *Amia calva* and other air-breathing fishes has been studied extensively, but identifying actual mechanisms involved has proved elusive (see Shelton *et al.* 1986). The gas bladder of *Amia calva* and other primitive fishes, such as gar, lungfish and polypterids, functions both as a gas exchanger and as a buoyancy organ (Liem, 1988, 1989). There is some evidence to indicate that both O_2-sensitive chemoreceptors and volume-sensitive gas bladder stretch receptors are involved in the control of aerial ventilation. For example, studies have shown that *Amia calva*, like many other air-breathing fishes, increase air-breathing frequency during periods of hypoxic or thermal stress (Johansen *et al.* 1970; Horn and Riggs, 1973; Randall *et al.* 1981; McKenzie *et al.* 1991), and that air-breathing frequency also increases after gas bladder deflation (Johansen, 1970).

The chemoreceptive sites that mediate the oxygen-related ventilatory reflexes have not been established, but there is evidence from gar that both externally and internally oriented O_2 receptors may be involved (Smatresk *et al.* 1986). One potential method for delimiting external and internal chemoreceptive sites in conscious air-breathing fish is to vary independently aquatic and aerial gas concentrations. Although forcing fish to breathe hypoxic or hyperoxic gases from the aerial environment has doubtful ecological

significance, it may be useful in discriminating between potential chemoreflexive sites used in the control of aerial ventilation.

Materials and methods

Animals

Bowfin (*Amia calva* L.) were netted by commercial fishermen in Lake Ontario and shipped by air to the University of British Columbia. There was no mortality during shipment and the fish arrived in a healthy state. They were kept indoors in large circular fibreglass tanks with continuously running dechlorinated water that ranged in temperature from 6 to 15°C during the course of the study.

Air-breathing behaviour in undisturbed fish

Eight fish, ranging in size from 246 to 940g (mean mass 503g), were used for non-invasive measurements of air-breathing behaviour. Fish were placed in separate 40l plastic containers with water at the same temperature as the main holding tank (6–15°C). They were acclimated to room temperature (20–24°C) by allowing the water to warm slowly overnight with continuous aeration. Equilibration of the water with room temperature required between 12 and 24h. After 4–5 days at room temperature, the fish were transferred to a 68l aquarium (60cm×30cm×38cm depth) filled with aerated water pre-equilibrated to room temperature.

The water surface of the aquarium was covered with a Perspex barrier containing a round hole (either 196cm^2 or 100cm^2) through which the fish could breathe air. The hole was covered with an inverted plastic funnel (volume 650ml or 300ml) with a pneumotachograph (Fleisch) attached to the spout to record changes in air flow during air-breathing events. The pressure drop across the pneumotachograph was measured with a differential pressure transducer (Validyne, model DP 103-14). A constant gas flow of 200$ml\,min^{-1}$, regulated with precision gas flow meters (Cole-Parmer), was maintained through the funnel. The differential pressure signal was fed through a voltage–frequency converter (A. C. Vetter, model 2D) and stored on the audio track of a JVC (model TU-S2U) video cassette recorder. The pneumotachograph was calibrated by the relationship between known air flow rates (0–9$l\,min^{-1}$) and the transducer voltage output. There was a linear relationship between air flow and voltage over this range. Each animal's air-breathing behaviour was recorded in 8h sessions with a video camera (JVC). Most recordings were made between 18:00 and 06:00h to minimize vibrational disturbances that normally occurred during the day. The timing of air-breathing events was determined from the videotape replay to the nearest minute using a digital clock displaying real time located within the camera field. To examine air flow changes during air-breathing, the pressure transducer signal (d.c. voltage), stored on videotape as a frequency signal, was replayed through the frequency–voltage converter and displayed on a storage oscilloscope (Tektronix model 5113). The replayed voltage signals were transferred to a Gould (model 220) chart recorder for analysis of breath volumes.

Measurement of expiratory volume

Expiratory volume (V_{exp}) was measured by integrating air flow traces recorded on chart paper, using the techniques of Glass *et al.* (1983) and Boutilier (1984), but with some modifications. Ohya *et al.* (1988) demonstrated that electronic integration of pneumotachograph signals can overestimate breath volume, owing to inertial effects, particularly at high flow rates. To allow for this, V_{exp} was calibrated by injecting known volumes of air manually through the funnel with a 30ml plastic syringe to mimic the expected V_{exp} of the fish. The standard Luer-lock tip of the syringe was replaced with a wide-bore (0.5cm) connector to minimize effects that would result from injecting air through a narrow orifice. For a given volume, air was injected at different rates, resulting in variable time intervals (T_E') that encompassed the expected expired time interval (T_E) for each fish. A typical calibration for injected volumes of 2–10ml is shown in Fig. 1A. Calibration volumes were also recorded on videotape and replayed on chart paper using the same methods as those for measuring V_{exp} for the fish. Manually injecting air resulted in oscillations that returned to baseline after approximately 200ms (Fig. 1A). The oscillatory nature of the calibrated volumes was a function of changes in the water level after injecting air and did not occur when the funnel–pneumotach arrangement was tested on a solid surface. The area under the calibration flow profiles and flow resulting from expiration for the fish (Fig. 1B) were measured with a digitizing tablet (Jandel Scientific) and associated software (Sigma Scan).

Gas bladder volume

Six fish, different from those used in the air-breathing experiments but encompassing a similar size range (217–1201g), were killed in a concentrated solution of MS-222, weighed to the nearest 0.1g and opened ventrally to remove the gas bladder. The gas bladder volume (V_B) was determined gravimetrically by allowing the maximally inflated gas bladder to displace a 0.7% saline solution from a 1500ml flask. The displaced saline was collected and weighed to the nearest 0.1g. This process was repeated three times for each gas bladder and the average of the three determinations was taken as V_B.

Modification of aquatic and aerial gas concentrations

For experiments, a fish was placed in an aquarium and allowed 24–36h to become accustomed to the aquarium and breathing funnel. The water was continuously aerated during this period to maintain aquatic P_{O_2} (Pw_{O_2}) above 18.6kPa; air was allowed to flow through the funnel. Aquatic aeration was maintained at one end of the aquarium and the breathing funnel was placed at the opposite end; gases from aeration escaped to the atmosphere through holes in the Perspex cover over the water's surface. This arrangement minimized vibrational disturbances due to aeration that would have resulted in a noisy pressure signal from the pneumotachograph. It also helped to maintain independence of gas concentrations in either phase when different gases were used simultaneously.

The P_{O_2} and P_{CO_2} of aquatic or aerial gases were measured with Radiometer oxygen and carbon dioxide electrodes and meter (model PHM 71). The electrodes were calibrated

with precisely mixed gases from a Wösthoff gas-mixing pump (CO_2 electrode) or air-saturated water and a zero-P_{O_2} solution (O_2 electrode), or N_2-equilibrated water. The gas composition of the aquatic phase was changed by mixing gases with precision flow meters or with Wösthoff gas-mixing pumps before passing them to an aerating stone in the aquarium. Aerial gases were taken directly from an air source, from an oxygen tank (100% O_2) or from a Wösthoff gas-mixing pump (5% CO_2 in air or 8% O_2 in N_2). Pw_{O_2} was determined just prior to and immediately following a videotaping session. Any experiment where Pw_{O_2} changed by more than 1.3kPa was not included in this analysis. The P_{O_2} of the aerial phase was also measured to ensure that no changes in concentration occurred, particularly when the aquatic phase contained a different dissolved gas. There were no detectable levels of CO_2 in the aquarium with aeration or when CO_2 passed through the funnel. There was no significant drift of P_{O_2} or P_{CO_2} for funnel gases during the course of the experiments, indicating that levels of aquatic and aerial gases were maintained independently of each other.

Fish were exposed to the following six gas combinations (listed as aquatic/aerial gas): (1) normoxia/air (control); (2) normoxia/hypercapnia; (3) hypoxia/air; (4) normoxia/hypoxia; (5) normoxia/hyperoxia and (6) hypoxia/hyperoxia. Pw_{O_2} in normoxia ranged between 18.6 and 21.3kPa. 5% CO_2 (4.9kPa) in air was used for aerial hypercapnia and 8% O_2 (7.9kPa) in N_2 for aerial hypoxia. Pw_{O_2} in hypoxia/air was 7.3±0.9kPa (S.E.M.) and in hypoxia/hyperoxia it was 6.7±0.5kPa. Aerial hyperoxia was created with 100% O_2 flowing through the funnel. The fish were exposed to the six combinations of gas mixtures in random order, but were returned to control conditions (normoxia/air) after each treatment and after changing the water in the aquarium with dechlorinated water pre-equilibrated to room temperature. The fish showed no adverse behavioural effects from disturbance during this procedure.

Statistics

Values are given as mean ± 95% confidence interval (C.I.) unless otherwise stated. Data are presented as the number of observations (*n*) or the number of animals (*N*) used for experiments. One-way analysis of variance (ANOVA) and the Student–Newman–Keuls (SNK) *post-hoc* multiple-range test were used to test for significant differences between pairs of mean values of inter-breath interval (IBI) with the various gas composition treatments for each fish (Zar, 1974). Types I and II air-breaths for each treatment were expressed as percentages of total breaths. These data were arcsine-transformed and compared using an unpaired *t*-test (Zar, 1974). Least-squares linear regression was also used as appropriate. Statistical significance was accepted at the 5% level. Statistical tests were carried out with commercially available software (Systat; Evanston, IL).

Results

Behavioural observations and pneumotachograph measurements

Based on the patterns of air flow, our observations revealed two distinct types of air-breaths in every fish tested. Previous work on *Amia calva* has identified only one type of

air-breath (Wilder, 1877; Johansen *et al.* 1970; Deyst and Liem, 1985) and to differentiate the types observed in this study, the terms type I and type II breaths are used. Air-flow patterns for these breath types, as determined by pneumotachography, are illustrated in Fig. 1B. Type I air-breaths were characterized by positive and negative air flow excursions, corresponding to exhalation and inhalation, respectively. Exhalation (and calibration volumes) caused oscillations in the zero-flow baseline of the funnel–pneumotach recording system (see Materials and methods) so that zero air flow was not attained before the inhalation phase began (Fig. 1B, type I). Thus, there was a flow offset in the positive direction relative to the zero-flow baseline at the start of the inhalation phase. During inhalation, air flow invariably fell below the zero baseline.

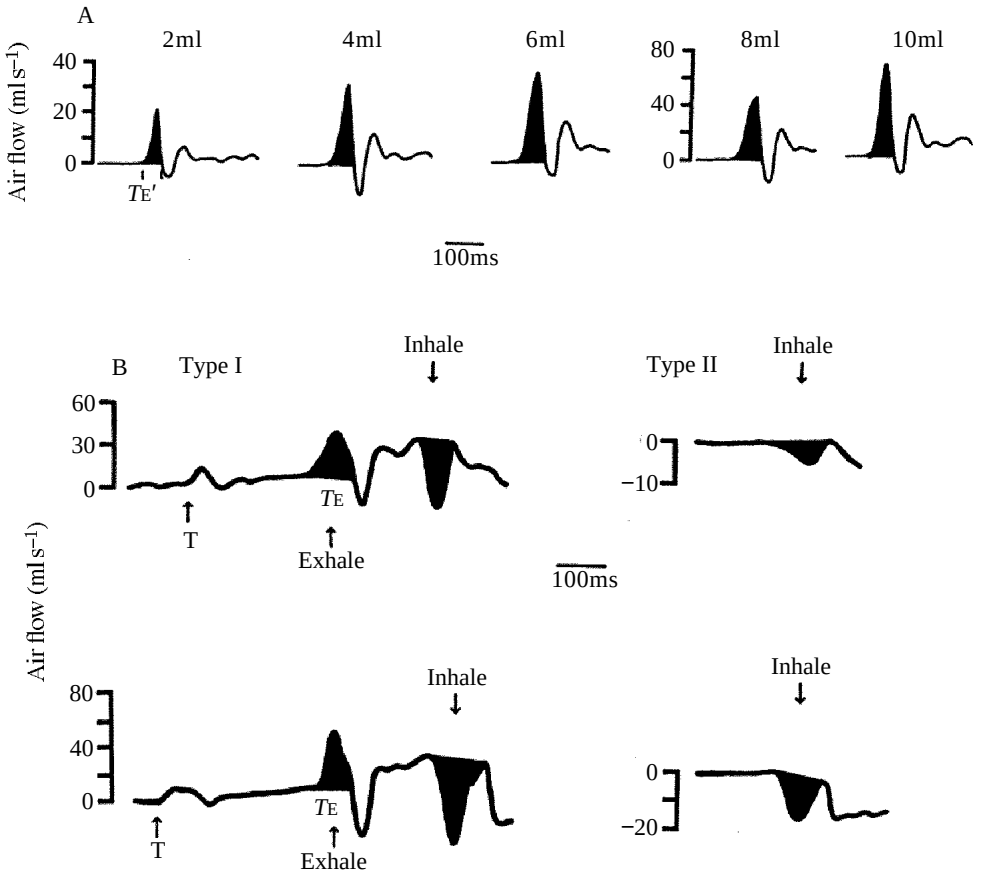

Fig. 1. (A) Calibration record of manually generated air flow measured with the pneumotachograph–pressure transducer system. Known volumes (2–10ml) were injected and the area under the flow curve was integrated. T_E' is the calibrated expiratory time interval. See Materials and methods for details. (B) Records of air flow ($ml\,s^{-1}$) for two fish in control conditions. Type I (left-hand column) and type II (right-hand column) air-breaths are illustrated. The transfer phase (T) for type I breaths is indicated, along with the expiratory time interval (T_E), analogous to T_E' in A, used to calculate V_{exp}.

Nevertheless, decreased air flow below the offset baseline was visually correlated with depression of the gular plate (see Deyst and Liem, 1985; Liem, 1988, 1989) of the fish and, therefore, the inhalation phase of the type I breathing cycle. The small positive change in air flow that occurred consistently during type I breaths approximately 200–400ms before exhalation was caused by the fish expanding the buccal cavity when approaching the surface, which altered the water level in the funnel (see Fig. 1B; T=transfer phase). This was interpreted as the fish transferring gas from the gas bladder to the buccal cavity (see Deyst and Liem, 1985). The entire sequence for type I breaths required about 0.5s, which is similar to that reported for gar (Rahn *et al.* 1971).

Type II breaths were characterized by negative air flow at the pneumotachograph, corresponding to inhalation only, with no evidence of exhalation (Fig. 1B, type II). Our observations did not reveal any loss of gas as the fish ascended to the surface; however, as in type I breaths, some loss of inspired gas from the opercular slits was usually seen during the compression phase. This loss of inspired gas precluded accurate measurements of inspiratory volume because an unknown and unmeasurable amount of gas was not transferred to the gas bladder. Measurements were, therefore, restricted to exhaled volumes for type I breaths. Because type II breaths involved a single inhalation at the

Table 1. *Expiratory volume (V_{exp}; mlkg^{-1}) of type I breaths and percentage of gas bladder exchange for six* Amia calva *in four different experimental treatments*

	V_{exp} (mlkg^{-1})			
	T1	T2	T3	T4
B3	16.3±1.0 (19)	18.5±1.7* (6)	11.9±1.4* (37)	15.7±1.4 (10)
B4	25.4±1.8 (45)	17.1±3.3* (13)	18.1±3.7* (10)	18.1±3.4* (12)
B5	11.6±1.1 (30)	11.9±2.6 (5)	13.3±1.7 (12)	10.7±2.4 (6)
B6	26.7±2.9 (5)	20.2, 24.2 (2)	22.0±2.1 (9)	26.3±3.3 (4)
B7	15.9±1.2 (34)	16.1, 16.1 (2)	13.7±3.0 (5)	ND
B8	21.3±1.1 (24)	19.3±2.2 (5)	20.4±1.2 (19)	18.5±1.7 (9)
	Gas exchange (%)			
	T1	T2	T3	T4
B3	20.3±1.5	23.1±1.2	14.9±0.6*	19.6±1.7
B4	31.8±2.7	21.4±2.0*	22.6±2.3*	22.6±1.6*
B5	14.5±1.4	14.9±3.7	16.6±1.4	13.4±1.5
B6	33.3±2.9	25.3, 30.3	27.5±2.5	32.9±2.9
B7	19.8±1.4	20.1, 20.1	17.1±2.8	ND
B8	26.6±1.5	24.1±2.2	25.5±1.0	23.1±1.3

Values are mean ± 95% C.I. and the number of observations (*n*).

Treatments (aquatic/aerial): T1, normoxia/air (control); T2, normoxia/5% CO_2; T3, hypoxia/air; T4, normoxia/8% O_2.

*Significantly different from control (P<0.05; SNK *post hoc* comparison).

Gas exchange was calculated as V_{exp}/80×100 (see text for details).

ND indicates no data collected.

Number of observations for gas exchange are the same as for V_{exp}.

surface, the time sequence was faster (approximately 0.1s) than for type I breaths. For both breath types, *Amia calva* ascended to the surface and quickly descended after the breath. There was always a single breath at the surface, and the fish did not spend any time at the surface except for air-breathing.

Expiratory volume and gas bladder volume

Individual values of V_{exp} for type I air-breaths with the various experimental treatments are given in Table 1. V_{exp} ranged from 11.6±1.1 to 26.7±2.9mlkg^{-1} (N=6) under normoxic conditions (Table 1). Only one fish of the six showed consistent changes in V_{exp} upon exposure to hypercapnic or hypoxic gas mixtures; thus, there were no significant effects of gas composition on V_{exp} (Table 1). V_{exp} was related to body mass M (V_{exp}=3.86+0.0084M; r^2=0.27; n=323; P=0.29) but, owing to the limited size range of the fish (246–634g), and considerable variability, the dependence of V_{exp} on body mass was not significant.

V_B was determined for different fish from those used for V_{exp}, but was estimated over a similar size range as the fish used for measurement of V_{exp} by using the slope of the relationship between V_B (ml) and body mass (g) from *in vitro* measurements. V_B was linearly related to body mass: V_B=0.08M+12.2 (r^2=0.86; P<0.001). The intercept (12.2ml) was not significantly different from zero (t_5=1.05). The slope of this

Table 2. *Mean inter-breath intervals for individual* Amia calva *in six experimental treatments with altered respiratory gases (T1–T6)*

	Treatment					
Fish	T1	T2	T3	T4	T5	T6
B3	16.2±1.4 (271)	26.5±5.3* (17)	12.8±1.8 (150)	15.1±3.9 (32)	15.1±2.3 (93)	7.2±2.7* (68)
B4	24.7±1.9 (255)	24.2±4.0 (56)	13.6±2.9* (103)	9.7±4.2* (50)	26.5±7.0 (18)	12.4±4.7* (39)
B5	12.2±0.8 (390)	21.6±3.4* (22)	11.3±1.8 (85)	7.0±1.9* (69)	10.0±1.7 (88)	7.0±2.0* (63)
B6	44.2±6.7 (114)	39.1±23.9 (9)	11.4±11.3* (40)	45.7±22.7 (10)	27.6±22.7 (10)	21.3±15.7 (21)
B7	20.4±1.6 (413)	13.7±5.7 (32)	15.6±5.9 (30)	27.2±5.7 (32)	23.1±7.2 (20)	16.1±5.9 (30)
B8	29.1±3.1 (175)	32.1±10.8 (14)	15.9±5.3* (58)	11.3±6.2* (42)	14.4±7.1* (32)	21.4±9.2 (19)
B10	13.1±1.1 (254)	13.5±3.0 (34)	14.7±3.0 (33)	14.5±3.1 (33)	16.3±2.4 (56)	14.2±3.2 (30)
B12	16.6±1.5 (78)	ND	6.1±3.0* (80)	8.2±1.4* (60)	14.5±2.3 (33)	8.1±1.7* (60)

Values are mean ± 95% C.I. and the number of observations (n) for each treatment.

Experimental treatments (aquatic/aerial): T1, normoxia/air (control); T2, normoxia/5% CO_2; T3, hypoxia/air; T4, normoxia/8% O_2; T5, normoxia/100% O_2; T6, hypoxia/100% O_2.

ND indicates no data collected.

*Significantly different from control (P<0.05; SNK *post hoc* comparison).

Table 3. *Percentages of type I and type II breaths (of total breaths) in six experimental treatments (aquatic/aerial)*

Treatment	*N*	Type I (%)	Type II (%)
Normoxia/air	8	53.5±0.9	46.5±0.9
Normoxia/5% CO_2	7	47.9±2.0	52.1±2.0
Hypoxia/air	8	74.5±0.5*	25.5±0.5
Normoxia/8% O_2	8	92.0±2.1*	8.0±2.1
Normoxia/100% O_2	8	0.2±0.2*	99.8±0.2
Hypoxia/100% O_2	8	1.2±0.3*	98.8±0.3

Values are mean ± 95% C.I. and the number of animals (*N*) in each treatment.
*Significantly different from percentage of type II breaths ($P<0.001$; *t*-test).

relationship was 80mlkg^{-1} for the size range of animals used for V_B measurements, and this value was used to calculate the percentage of gas exchanged in type I breaths. Gas exchange for type I breaths was therefore calculated as $V_{exp}/80\times100$. Gas exchange ranged from 14.5±1.4 to 33.3±2.9% of V_B under control conditions and, because V_{exp} did not vary between experimental treatments, was unaffected by altered aquatic or aerial gases.

Responses to aquatic and aerial gas concentrations

The responses of individual *Amia calva* to changes in respiratory gases are summarized in Table 2. There was a large range of IBI in normoxic conditions, from 12–44min, with

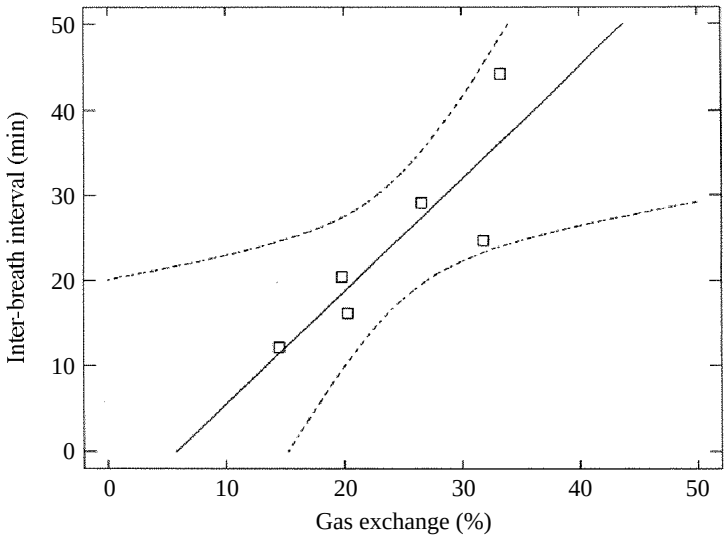

Fig. 2. The relationship between inter-breath interval (min) and the percentage of gas exchanged during a type I breath in control (normoxic) conditions for six *Amia calva*. Mean values (open squares), the regression line (solid line) and the 95% confidence limits (dashed lines) of the regression are given. The equation for this relationship is $y=1.32x-7.6$ ($r^2=0.74$; $P<0.05$).

an overall mean IBI of 22.1±9.9min (S.D.). Therefore, air-breathing frequency ranged from about 1 to 5breaths h^{-1}. There was a slightly higher percentage of type I (53.5%) than type II (46.5%) air-breaths in normoxia, but this difference was not significant (Table 3). There was a significant correlation between mean values of IBI and the percentage of gas exchanged during type I air-breaths under control conditions in six fish (Fig. 2). The regression for this relationship was, $y=1.32x-7.6$ ($r^2=0.74$; $F_{1,4}=11.2$; $P<0.05$).

Although aerial hypercapnia had no consistent effect on IBI, aquatic or aerial hypoxia significantly increased air-breathing frequency (decreased IBI) in four of eight fish (Table 2), and both treatments resulted in a significant increase in the percentage of type I air-breaths (Table 3). Type I breaths accounted for 74.5±0.5% of all breaths in aquatic hypoxia and 92.0±2.1% of all breaths in aerial hypoxia.

Aerial hyperoxia (100% O_2) combined with normoxic water exposure did not produce clear effects on air-breathing frequency; six of eight fish did not change IBI (Table 2). All fish, however, switched to the use of type II breaths; 348 of the 350 observed breaths (99.8%) with this treatment were type II (Table 3). In aquatic hypoxia, with a 100% O_2 atmosphere, air-breathing frequency increased significantly in four fish and was unaffected in the remaining fish (Table 2). Type II breaths were used almost exclusively, accounting for 98.8% of all breaths (Table 3).

Discussion

Since Wilder (1877) first established that *Amia calva* used its gas bladder as a respiratory organ with active exhalation and inhalation of atmospheric gas, subsequent studies have confirmed this double-pulse mechanism of ventilation (Johansen *et al.* 1970; Randall *et al.* 1981; Deyst and Liem, 1985; Liem, 1988, 1989). Liem (1988, 1989) has described air-breathing in *Amia calva* as a four-phase process involving initial transfer of gas from air bladder to buccal cavity, exhalation of buccal gas, inhalation of atmospheric gas and compression (or transfer) of inhaled gas from buccal cavity to gas bladder. Using pneumotachography, we have also confirmed this four-phase exhalation/inhalation sequence in *Amia calva*, called type I in this study, but, in addition, a new breathing type was identified. This new breath type, called type II, involves a single inhalation with no expiratory phase. In the context of Liem's description, type II breaths appear to involve the third and fourth phases of the breathing cycle: inhalation of atmospheric gas and subsequent transfer of inhaled gases to the gas bladder.

It is not clear why previous studies with *Amia calva* have not reported type II breaths. Deyst and Liem (1985) used X-ray ciné film, EMG analysis and buccal pressure recordings to examine air-breathing mechanisms in *Amia calva*, but only described one breathing mechanism (i.e. type I). Their fish, however, were placed in hypoxic water to stimulate air-breathing and because type I breaths predominate in hypoxia, very few type II breaths, if any, would have been recorded.

We attribute our success in finding type II breaths in *Amia calva* to two factors. First, this study directly measured the patterns of air flow during air-breathing events which

allowed a clear interpretation of the mechanisms of aerial ventilation. Our observations of the air-breathing behaviour of *Amia calva* were also instrumental in corroborating the air flow measurements. Second, our measurements were made with undisturbed fish over relatively long (8h) periods. Air-breathing patterns in fish are known to be extremely labile and sensitive to disturbances at the surface, which may affect air-breathing behaviour and frequency (Gee, 1980; Smith and Kramer, 1986).

Expiratory volume and gas exchange

V_{exp} for *Amia calva* ranged from about 12 to 27 $ml\,kg^{-1}$, which is similar to values of 24.9 and 31.7 $ml\,kg^{-1}$ for gar obtained by direct collection of expired gases (Rahn *et al.* 1971; Smatresk and Cameron, 1982), indicating that pneumotachography is a useful, non-invasive method of measuring V_{exp} in *Amia calva*. Wilder (1877) also measured expiratory volume by direct collection of gas and obtained values ranging from 18 to $105\,ml\,kg^{-1}$ for a single fish. Although the values in this study were generally lower, it is clear that V_{exp} varies considerably within and between fish. Owing to this variability in V_{exp}, there is no evidence for the control of V_{exp} on a breath-by-breath basis in *Amia calva* breathing normoxic, hypercapnic or hypoxic gas mixtures (Table 1). The lack of change in V_{exp} with increased hypoxic ventilatory drive differs from the pattern seen in other aquatic and terrestrial vertebrates, where tidal volume generally contributes to increases in minute ventilation (Milsom, 1990). The results from this study indicate that increased respiratory frequency, but predominantly a switch to type I breaths, modulates aerial ventilation in response to increased oxygen demand. In hypoxia, therefore, minute ventilation increases as a result of both the interaction of increased respiratory frequency and an increase in the exchange of gas bladder contents through type I air-breaths.

Aerial hypercapnia had no clear effect on either V_{exp} or ventilatory frequency in *Amia calva*. If, as evidence from gar suggests (Smatresk and Azizi, 1987), gas bladder stretch receptors were inhibited by high levels of CO_2, we would have expected *Amia calva* in this study to have initiated air-breaths at higher than normal gas bladder volumes. This would probably have resulted in lowered V_{exp} upon exposure to aerial hypercapnia. This did not occur, supporting the evidence of Milsom and Jones (1985) that slowly adapting pulmonary stretch receptors in *Amia calva* do not possess any CO_2 sensitivity, unlike those found in gar. There is also no evidence for central CO_2 chemoreceptors that could potentially mediate air-breathing reflexes in *Amia calva* (Hedrick *et al.* 1991).

V_B values for *Amia calva* (this study) and gar (Rahn *et al.* 1971) are about 8% of body mass, which is within the theoretical (Alexander, 1966) and measured ranges (Jones, 1957) for freshwater fishes. From our measurements of V_{exp} and V_B, we calculate that, on average, *Amia calva* exchange about 25% of V_B during a type I breath, which is sufficient to account for the range of gas bladder P_{O_2} values that have been measured (Johansen *et al.* 1970; Hedrick, 1991). These results also support the direct observations of Deyst and Liem (1985) that a substantial (approximately 75%) residual volume remains following an air-breath.

The considerable variability of air-breathing frequency in normoxic conditions is due to the amount of gas exchange during type I breaths. There was a significant correlation

between IBI and the percentage of gas exchanged (Fig. 2). As V_{exp} increased, and therefore the amount of gas bladder contents exchanged with each breath, IBI also increased. Larger amounts of gas exchange should raise the P_{O_2} of the gas bladder and increase the diffusion gradient between the gas bladder and blood. If type I breaths are triggered in response to lowered blood P_{O_2} (see below), an increased P_{O_2} gradient should prolong the interval between type I air-breaths (Hedrick and Katz, 1991).

Physiological control of air-breathing

The responses of *Amia calva* to changes in respiratory gases in the aquatic or aerial phases showed changes in both frequency and pattern. Air-breathing frequency increased significantly in some fish in aquatic hypoxia, a response typical of *Amia calva* (Johansen *et al.* 1970; Randall *et al.* 1981; McKenzie *et al.* 1991) and many other air-breathing fishes (see Shelton *et al.* 1986). There was also a shift to a predominance of type I air-breaths in hypoxia, suggesting that these breaths respond to changes of P_{O_2}. Moreover, type I breaths were stimulated by aerial hypoxia, in the presence of aquatic normoxia, indicating that O_2-sensitive chemoreceptors respond to lowered blood P_{O_2} and reflexly stimulate type I breaths. These results are, therefore, consistent with studies on gar (Smatresk and Cameron, 1982) and lungfish (Johansen and Lenfant, 1968) showing that internal hypoxia stimulates air-breathing. An important difference between this study and previous work is the finding that a specific air-breathing mechanism (type I breaths) is preferentially stimulated by hypoxia. Aquatic hypoxia caused a similar shift in the frequency and pattern of air-breathing as aerial hypoxia, so chemoreceptors monitoring either aquatic P_{O_2}, presumably at the gills, or internal P_{O_2} may be responsible for the stimulation of type I breaths.

Although it seems clear that type I breaths respond to internal and, possibly, external P_{O_2}, a question remains as to the control and function of type II breaths. *Amia calva* exposed to aerial hyperoxia clearly switched to the use of type II breaths. These breaths were used almost exclusively despite aquatic hypoxia, a condition that resulted in predominantly type I breaths with aerial normoxia. Compared with fish in normoxia, air-breathing rates were maintained or increased, in contrast with other studies where aerial hyperoxia reduced air-breathing frequency (Garey and Rahn, 1970; Lomholt and Johansen, 1974; Burggren, 1979; Pettit and Beitinger, 1985; Smatresk *et al.* 1986). This indicates that type II breaths in *Amia calva* are probably not controlled by O_2-sensitive chemoreceptors.

The most likely explanation for the function of type II breaths is the regulation of V_B. Our observations revealed that when fish lost inhaled air during the compression phase of the type I breaths, type II breaths followed within a few minutes or less. This indicates that air-breathing is not 100% efficient; that is, there is some error in the capture and transfer of inhaled air. Type II breaths might therefore be viewed as playing a role in adding to V_B when a volume 'threshold' is not reached. In addition, gas bladder volume would also be expected to decline during inter-breath intervals as O_2 diffuses from gas bladder to blood, without being replaced by other gases, making the fish less buoyant. Because *Amia calva* do not have the ability to secrete gas into the gas bladder and are dependent upon air-breathing for buoyancy regulation, the simplest response to

reductions of V_B would be to replace the lost volume by inhalation. Thus, our working hypothesis is that type II breaths function to regulate V_B and, therefore, buoyancy.

In support of this view of buoyancy regulation, Gee and Graham (1978) reported that air-breathing frequency was maintained in *Brochis splendens*, an air-breathing catfish, while breathing 100% O_2 from the atmosphere at rates similar to those in normoxia. Their study indicated that buoyancy regulation, rather than external or internal P_{O_2}, was responsible for the maintenance of air-breathing. An increased oxygen diffusion gradient from the air-breathing organ (ABO) to blood resulted in a faster decline in the volume of the ABO than in fish breathing air. Thus, a clear hydrostatic function, in addition to the respiratory function, was indicated for the ABO of *Brochis splendens*.

Johansen (1970) demonstrated that *Amia calva* do increase air-breathing in response to gas bladder deflation. Moreover, gas bladder deflation results in exclusively type II breaths being taken by the fish (M. S. Hedrick and D. R. Jones, in preparation), supporting the hypothesis that type II breaths regulate V_B. Therefore, the switch to type II breaths in aerial hyperoxia probably results from an increased rate of gas bladder deflation, through an increased O_2 diffusion gradient, and subsequent feedback from gas bladder stretch receptors (Milsom and Jones, 1985). Because type I and type II breaths appear to regulate two different functions, gas exchange and buoyancy, respectively, these breaths are likely to be controlled by different neural pathways.

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