Physiological responses to prolonged aquatic hypoxia in the Queensland lungfish *Neoceratodus forsteri*

Abstract

The effects of moderate and severe hypoxia on air breathing frequency and respiratory properties of the blood of the Queensland (Australian) lungfish *Neoceratodus forsteri* were measured in fish exposed to these conditions for 14-22 days at 20 °C. Haemoglobin oxygen affinity increased after exposure to moderate hypoxia (Pw_{O2} = 60 mmHg), but did not increase further after exposure to severe hypoxia (Pw_{O2} =40 mmHg). The P_{50} of whole blood (20 °C, P_{CO2} =16.0 mmHg) fell from 22.0 ±1.5 mmHg in normoxic conditions to 19.0± 1.0 mmHg in hypoxic conditions. Under both moderate and severe hypoxia, haematocrit, haemoglobin, blood lactate, and erythrocyte phosphate concentrations did not differ from normoxic values. The observed increase in haemoglobin oxygen affinity in response to aquatic hypoxia is typical of compensatory responses seen in obligate water breathers, but smaller. This suggests that the capacity of lungfish to respond to hypoxia by breathing air removes the necessity for further left-shifting of the oxygen equilibrium curve.

Keywords: Air breathing, fish; Blood, respiratory properties; Fish, Australian lungfish *(Neoceratodus forsteri);* Haemoglobin, O2 affinity, hypoxia; Hypoxia, breathing frequency, blood 02 properties; Pattern of breathing, hypoxia

1. Introduction

Fishes exhibit a range of tactics to counteract aquatic hypoxia. When escape from the hypoxic stress is not possible, a variety of physiological mechanisms may be invoked to compensate for low oxygen availability (Jensen et al., 1993; Graham, 1997; Val et al., 1998). In the short term, increases in the frequency and/or stroke volume of gill ventilation are common but, at a critical point, the energetic cost of this response may exceed the benefits of increased oxygen flow over the gills (Fritsche et al., 1993). Similarly, while suppressed metabolic rate during hypoxic episodes can reduce oxygen demand in the short term, activity must be restored eventually to allow feeding, reproduction and escape from predators (Jensen et al., 1993).

Maintenance of activity, even under sustained hypoxic challenge, can be met by a series of physiological adaptations. Particularly, both oxygen binding affinity and the concentration of haemoglobin in the blood respond to short term changes in oxygen tension and oxygen availability in the external environment (Grigg, 1969). A rapid way to preserve oxygen delivery is to increase the oxygen-carrying capacity of the blood (Wells et al., 1989). This may be achieved by the release of erythrocytes from the spleen, as in yellowtail *(Seriola quingueradiata)* (Yamamoto et al., 1985), or enhanced erythropoiesis, as in Atlantic salmon *(Salmo salar)* (Hardig et al., 1978).

An alternative mechanism to preserve oxygen delivery in the face of low ambient oxygen is to increase the oxygen affinity of the blood. Modulation of blood oxygen affinity in response to environmental factors was first demonstrated in fish by Grigg (1969), working on bullhead catfish, *Ictalurus nebulosus,* who found seasonal shifts in oxygen affinity providing compensation for the effects of seasonal changes in water temperature. Subsequently it was discovered that increased blood oxygen affinity can be induced by low ambient oxygen as well, and results from a decline in the erythrocytic concentration of organic phosphates, primarily GTP and ATP (Wood and Johansen, 1972; Weber and Lykkboe, 1978; Graham, 1983; Wells et al., 1989). Increases in blood oxygen affinity in response to low oxygen have been demonstrated in fish such as carp (Lomholt and Johansen, 1979), and tench (Jensen and Weber, 1982), which regularly encounter hypoxia in their natural habitat. However, similar responses have also been observed in species such as trout (Soivio et al., 1980) that have high oxygen demands and are usually found in well-oxygenated water bodies. Furthermore, Wells et al. (1989) found that even the Antarctic Nototheniid, *Pagothenia borchgrevinki,* which has a low oxygen demand and lives in water with a high oxygen content (MacDonald et al., 1987) responds to hypoxia by increasing both haemoglobin concentration and blood oxygen affinity, the latter modulated by lower intraerythrocytic ATP. This finding prompted them to suggest that red cell organic phosphate-modulated increases in haemoglobin oxygen affinity may be a generalised response to hypoxia in all fishes.

Air breathing fishes provide opportunities to explore this proposition further. One might expect an air breathing fish not to show these responses when exposed to low ambient oxygen in the water. When air breathing commences in response to hypoxia, the high and stable concentration of atmospheric oxygen should ensure that blood oxygen tension will remain at least as high as when fish are breathing well-aerated water (Burggren and Johansen, 1986). In this scenario, additional compensation through increased blood oxygen-carrying capacity or modification of blood oxygen affinity would appear unnecessary. However, bimodal

breathers exhibit wide interspecific variation in' the relative importance of water and air breathing. In species that rely heavily on water breathing, some adaptive adjustment in blood respiratory properties may be expected during hypoxic challenge (Johansen, 1964; Graham, 1997) and this was found to be the case in the armoured catfish, *Ancistris chagresi* (Graham, 1983).

On this basis, it might be expected that the Queensland (Australian) lungfish, *Neoceratodus forsteri* (Krefft), would show an increased blood oxygen affinity in response to low oxygen, perhaps attenuated when air breathing commences. This Dipnoan fish is an obligate water dweller, with well-developed gills on all branchial arches and a dorsally situated, bilobed lung (Gunther, 1871; Grigg 1965a; Johansen and Hansen, 1968). At rest in well-aerated water they breathe air rarely, respiration being supported almost entirely by the gills (Lenfant et al., 1966). During activity, they supplement oxygen requirements by air breathing (Grigg, 1965b).

When subject to acute hypoxia, *N. forsteri* responds first by increasing the frequency and stroke volume of branchial ventilation, and then by commencing aerial breathing (Johansen et al., 1967; Fritsche et al., 1993). However, previous studies have assessed individuals exposed to hypoxia for periods of only a few hours. It is unclear whether observed increases in air breathing and gill ventilation are sustained during extended periods of hypoxic exposure. It is possible that, during long-term hypoxic exposure, the short term responses of *N. forsteri* are achieved wholly or partly, by adaptive changes in the respiratory properties of the blood.

In this study we evaluated these ideas by monitoring respiratory behaviour and changes in the respiratory properties of the blood of *N. forsteri* exposed at two levels of hypoxia for up to 22 days.

2. Materials and methods
2.1. Animals
Eight Queensland (Australian) lungfish, N. forsteri with body length of 580-910 mm and body mass of 3.9-8.5 kg, were collected by electrofishing in the Brisbane River at Fernvale (27°27'S 152°40'E). The fish were transported to holding tanks at the University of Queensland, Brisbane where they remained prior to the commencement of experiments.

2.2. Experimental area
All experiments were carried out in one of two constant temperature rooms, with lights operated by a timing switch to create a 12 h day/night cycle. Water temperature (Tw) was maintained at 20 °C, by regulation of ambient air temperature in the rooms. Four animals were assigned randomly to each of two opaque plastic tanks (1220 x 600 x 600 mm^3) containing 230 L of aged tap water held at a constant level by an overflow pipe fitted to each tank. One hundred litres of fresh water were siphoned into each tank every 48 h from a reservoir positioned above the experimental tanks. Each tank was fitted with an external aquarium filter to maintain water quality. The water surface was covered by a 10 mm closed cell foam mat to reduce oxygen exchange with the air and minimise disturbance of the fish. Throughout the study, water temperature and pH were recorded at least twice daily in each tank.

2.3. Choice of hypoxic exposure levels
Preliminary tests were conducted to determine appropriate oxygen tension (Pw_{O2}) levels for moderate and severe hypoxia treatments in subsequent experiments. After vigorous aeration for a period of 48 h, nitrogen gas was bubbled through the water (one tank at a time) to lower the oxygen tension. Pw_{O2} was measured to the nearest 1 mmHg every 4 min using a T.P.S 2052A-oxygen/ salinity meter and oxygen probe.

After the mats were removed from the water surface, an observer seated next to the tank counted ventilation rate of the gills. Each fish was observed for two 1-min periods during every 10 min. Individual gill strokes were easily recognised as flowing movements of the operculum as described by Dean (1912). The number of gill strokes per minute and the occurrence of air breaths were recorded until Pw_{O2} had fallen to 50 mmHg.

Gill ventilation plateaued and air breathing commenced at approximately Pw_{O2} = 60 mmHg, so this was chosen as representative of moderate hypoxia. Oxygen tension representing severe hypoxia was determined on the basis of results from Fritsche et al. (1993) who reported continuous air breathing in *N. forsteri* in water with oxygen tension lower than 50 mmHg. This was confirmed by our observations. Accordingly, 40 mmHg oxygen tension was chosen for experiments investigating responses to severe hypoxia. Following these preliminary tests, all eight fish were allowed to recover for 3 weeks in well-aerated conditions before the study continued.

2.4. Effects of hypoxia on respiratory properties of the blood
To examine any changes in respiratory properties of the blood, four fish were maintained as controls in well-aerated conditions, while four were subjected to moderate hypoxia (Pw_{O2} = 60 mmHg) for a period of 2 weeks. A blood sample was then extracted from each fish for analysis, and the treatments were reversed so that all eight fish were sampled after exposure to both normoxic and hypoxic, conditions. After a 3-week recovery period (for all fish) in well-aerated conditions, four fish were then chosen randomly and subjected to severe hypoxia (Pw_{O2}= 40 mmHg) for a further 2 weeks.

Oxygen tension in the treatment (hypoxic) tank was lowered by the respiration of the fish and then maintained at a desired level by an automatic system delivering short bursts of aeration as required. An oxygen meter (TPS ED500) monitored oxygen partial pressure. Whenever respiration by the fish caused Pw_{O2} to fall below the desired level, an external recording device (Mann Controller) activated a solenoid valve that opened airflow into the water until the desired oxygen level was restored. Using this method, Pw_{O2} was maintained within ± 2 mmHg at all times. Water flow over the oxygen probe was maintained by a submersible pump, which drew water from the tank through a short hose and into a 'T' shaped plastic holder housing the probe. Water was then passed back to the tank through a hose secured under the water surface to avoid aeration. Water temperature and pH in both treatments were measured four times daily throughout these exposure periods.

2.5. Blood sampling and analysis

After 14 days exposure, blood samples were collected and analysed from one fish each day until all eight animals had been tested. Laboratory procedures were time consuming and only one sample could be completely analysed per day. To obtain blood samples, the fish were restrained in a narrow, hessian-lined wooden trough to minimise struggling. All blood samples (2 ml) were extracted by cardiac puncture using heparinised 21 gauge needles and 2.5 ml syringes, divided equally into two plastic vials and placed immediately into crushed ice. Samples were then sub-divided for determination of haematocrit, haemoglobin concentration [Hb], oxygen equilibrium curves (including P_{50}), ATP levels, pH and blood lactate.

Haematocrit was determined using a haematocrit reader (Hawksely) after 10 µL of blood had been centrifuged for 3 min. Haemoglobin concentration was assessed from a 10 µL sample using a Pharmacia LKB ultra-spectrophotometer and methods described by Dacie and Lewis (1984). Mean corpuscular haemoglobin count (MCHC) was calculated using haematocrit and [Hb] values. An oxygen dissociation analyser (Aminco Hem-O-Scan, American Instrument Company) generated oxygen equilibrium curves. Curves were determined at 20 °C, using 4 µL samples of whole blood. P_{O2} values at 5% saturation increments were used to generate curves for both hypoxic and normoxic groups between 5 and 95% saturation at three levels of P_{CO2}. Gases for equilibrium were supplied pre-mixed and analysed by BOC Gases Australia. The mixtures were 2% CO_2 in air, 2% CO_2 in Nitrogen, 4% CO_2 in air, 4% CO_2 in Nitrogen, 6% $C0_2$ in air and 6% CO_2 in Nitrogen, allowing determination of whole blood equilibrium curves at P_{CO2} of 16, 29 and 44 mmHg. Curve data were transformed according the Hill equation and Hill's 'n' values were calculated using simple linear regression. High r^2 values were taken to indicate good fit of data to the Hill equation and thus each curve was described by the parameters 'n' to express shape and P_{50} to express haemoglobin oxygen affinity between 5 and 95% saturation. Blood pH was measured by a blood gas analyser (Radiometer BME33) in 100 µL samples equilibrated for 5 min with 2, 4 and 6% CO_2 in air. ATP concentrations were determined from 100 µL samples using a standard test kit (Sigma 366U⁻V) with slightly modified methods. Due to small sample volumes, 20% of the recommended blood quantity was used and appropriate adjustments made in subsequent calculations. Blood lactate concentration was determined from 50 µL samples using Sigma test kit 826 UV and the methods described therein.

2.6. Effects of hypoxia on air breathing

To assess the effect of prolonged hypoxia on the frequency of air breathing, four fish were filmed using a time-lapse video recorder (National WV) and video camera secured to the wall above a single tank. To ensure all air breaths were recorded, the water surface was completely covered except for a 400 x 600 mm^2 section at one end. The cover was constructed of 10 mm polystyrene foam strapped to heavy perspex so that the cover floated but was too heavy to be forced up by the fish from underneath. A 120 W red spotlight was positioned on a bench near the tank so that filming could continue both day and night, and oxygen levels in the tank were maintained using the methods previously described.

The fish were filmed continually for three successive 72 h periods, with P_{O2} maintained at 120, 60, and 40 mmHg in the three periods, respectively. At the end of each period, the controller device was reset and the oxygen tension allowed to drop to the next level. Tapes were analysed after the three trials were complete. When fish were observed rising to the surface, the tape was rewound and played back one frame at a time. An air breath was deemed to have occurred when a fish ascended, broke the surface with its snout and opened its mouth. This action was commonly, but not always accompanied by a gentle rocking of the snout. The numbers of air breaths in each period were recorded along with the time at which each breath occurred. It was assumed that all observed air breaths were related to respiratory requirements. Due to long periods between breaths, the total number of breaths per 3 h was used as the response variate for statistical analyses.

2.7. Statistical analysis

The relationships between haematocrit and [Hb] were established for each treatment using simple linear regression, and compared by analysis of covariance. Simple linear regression was used to evaluate any relationship between ATP concentration and P_{50}. Haematocrits, [Hb], MCHC, ATP and blood lactate levels were compared by single factor repeated measures ANOVA to establish dependence or independence from

the treatment groups. P_{50} values were analysed by two-way repeated measures ANOVA to establish dependence or independence from treatment groups and P_{CO_2} level. Changes in the frequency of air breathing were assessed by single factor ANOVA using the number of breaths per 3 h as the response variate. Results are presented as mean ± 1 S.D. and statistical significance was accepted at $P < 0.05$.

3. Results

3.1. Responses to acute hypoxia

Branchial ventilation rate (f_v) was monitored only during preliminary trials to determine appropriate levels of hypoxia for subsequent experiments. When nitrogen gas was bubbled through the water, Pw_{O_2} fell from 120 to 50 mmHg in approximately 80 min (Fig. 1). As oxygen levels decreased, the fish became progressively more agitated, circling their tanks repeatedly and swimming quickly from one end to the other. During this period, mean f_v (from eight fish) rose rapidly from 26 ± 3 to a peak of 43 ± 1 before reaching a plateau at approximately 70 mmHg (Fig. 1). Air breathing commenced when Pw_{O_2} had fallen to approximately 60 mmHg (Fig. 1). Three single air breaths, each taken by a different individual were observed before the experiment was terminated.
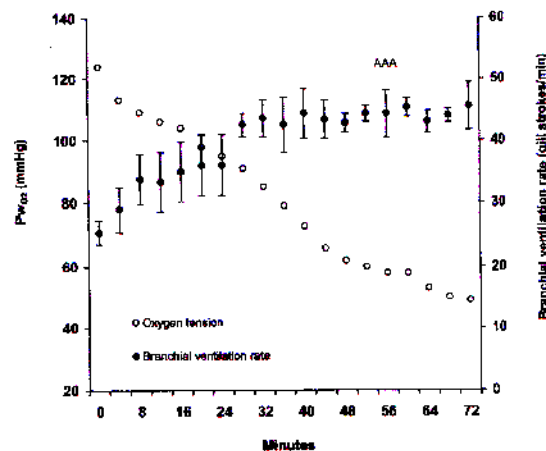

Fig. 1. Relationship between water oxygen tension (Pw_{O_2} }, branchial ventilation rate (gill strokes per minute) and the onset of air breathing. Each letter 'A' signifies a single air breath. Vertical bars are mean values ± 1 S.D.

3.2. Blood oxygen-carrying capacity

Haematocrits and haemoglobin concentrations [Hb] of the fish in this study were similar to those from well-aerated water reported by Lenfant et al. (1966). The fish did not increase blood oxygen-carrying capacity in response to either moderate (Pw_{O_2} = 60 mmHg) or severe (Pw_{O_2}_ = 40 mmHg) hypoxia (Table 1). Both haematocrit ($F=0.81$, df = 2, $P = 0.46$), and [Hb] ($F = 0.04$, df = 2, $P = 0.96$), were independent of hypoxic exposure level and, thus, there was no significant difference in mean corpuscular haemoglobin concentration (MCHC) (Table 1).

Table 1 Respiratory and haematological properties of blood from lungfish (*N. forsteri*) maintained for at least 2 weeks in normoxic, moderately hypoxic and severely hypoxic water

	Normoxia(120mmHg)	Moderate hypoxia (60 mmHg)	Severe hypoxia (40mmHg)
Haematocrit (%)	30.0±2.0	31.0±3.0	32.0± 3.0
Haemoglobin (g dL^{-1})	6.00±0.80	6.00±0.79	6.12±0.50
M(:HC (g dL^{-1})	20.18±2.17	19.56±1.55	19.33±1.03
P_{50} (mmHg) at P_{CO_2} = 16 mmHg	22.0±1.5*	19.0±1.0	20.0±1.0
ATP (mmol g^{-1}Hb)	26.34±3.14	23.67±4.13	2432±1.46
Lactate (mmol L^{-i})	1.01±0.59	0.93±0.29	0.57±0.35

Asterisk (*) indicates significantly different at $P <0.05$. Data are mean values ± 1 S.D. In normoxic and moderate hypoxia n = 8. In severe hypoxia n = 4.

3.3. Oxygen equilibrium curves

Oxygen equilibrium curves from fish in the normoxic treatment were similar to those generated by Lenfant et al. (1966), when compared at similar P_{CO_2} and water temperature (Table l, Fig. 2). Water temperature in the hypoxic treatment (20.27 °C±0.23) was slightly higher than in the normoxic treatment (19.52 °C±0.14). Mean pH values were 7.7±0.3 in hypoxic conditions and 7.5±0.3 in normoxic conditions. Analysis of variance showed

clearly that the lungfish responded to hypoxia by increasing haemoglobin oxygen affinity. Mean P_{50} depended strongly on both the level of hypoxia and on P_{CO2} (n = 8, P < 0.001 in both cases). However, there was no significant interaction between level of hypoxia and P_{CO2}, indicating that increasing P_{CO2} lowered haemoglobin oxygen affinity regardless of the level of hypoxia, and that exposure to hypoxia led to increased haemoglobin oxygen affinity regardless of P_{CO2}.
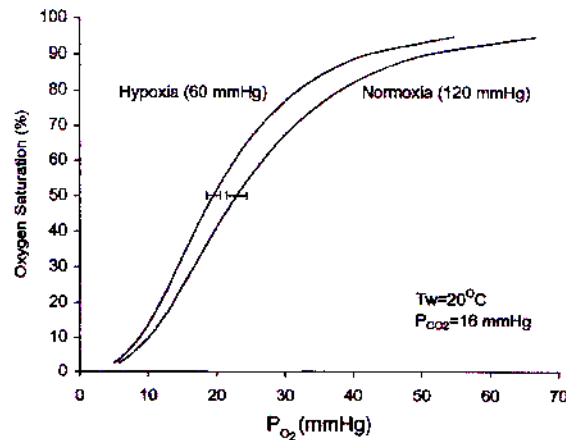

Fig. 2. Whole blood oxygen equilibrium curves illustrating the left-shifted response to moderate hypoxia in eight Queensland lungfish, *N. forsteri*, exposed to normoxic and moderately hypoxic conditions for at least 14 days. Horizontal bars indicate the mean P_{50} value ± 1 S.D., *n* = 8 for each treatment. Mean pH values were 7.7±0.3 in hypoxic conditions and 7.5±0.3 in normoxic conditions.

Mean P_{50} fell from 22.0 ±1.5 mmHg in normoxic conditions to 19.0 ± 1.0 mmHg after exposure to moderate hypoxia (Table 1, Fig. 2). Exposure to severe hypoxia did not result in any further increase in haemoglobin oxygen affinity (Table 1). Hill plots are shown in Fig. 3. At P_{CO2} = 16.0 mmHg, Hill's 'n' values were 2.27 and 2.33 for normoxic and hypoxic conditions, respectively, giving an indication of cooperative binding in both cases. Exposure to moderate hypoxia yielded no significant change in the magnitude of the Bohr effect (Δlog P_{50}/ΔpH) (F = 35.5, P = 0.42). Mean Bohr slopes were estimated at -0.48 and -0.42 for normoxic and hypoxic treatments, respectively.
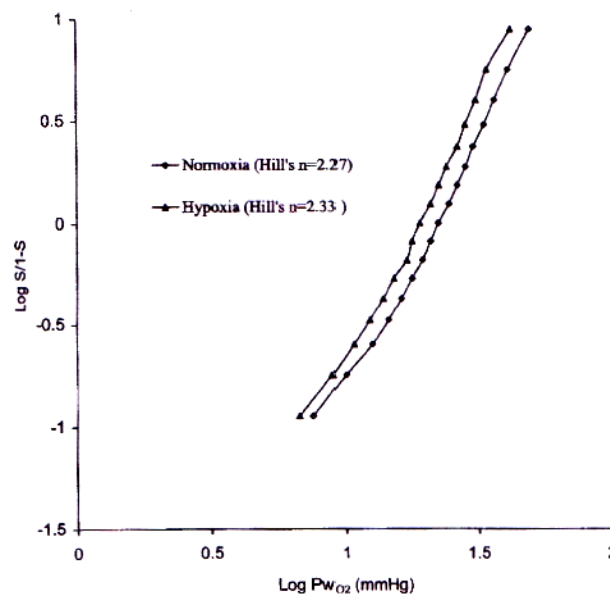

Fig. 3. Hill plots for oxygen equilibrium curves of blood from eight lungfish . *N. forsteri* exposed to normoxic and moderately hypoxic conditions for at least 14 days (P_{CO2} =16 tmmHg, Tw = 20 °C). O_2 equilibrium data are mean values plotted from 0.05 to 0.95 saturation at 0.05 increments. Mean pH values were 7.7±0.3 in hypoxic conditions and 7.5±0.3 in normoxic conditions.

3.4. Erythrocyte organic phosphates and blood lactate

The lungfish had slightly lower levels of ATP after exposure to moderate hypoxia (Table 1), however, this difference was not statistically significant (P=0.091). Blood lactate levels were highly variable, probably reflecting varying degrees of difficulty in obtaining blood samples. However, there was no significant difference in lactate levels between the treatment groups (P = 0.29).

3.5. Changes in the frequency of air breathing

Air breathing was rare in well-aerated (normoxic) conditions. A total of six air breaths were recorded from four fish held in these conditions for 72 h (Fig. 4). The frequency of air breathing increased significantly during 72 h exposure to moderate hypoxia (P = 0.012). However, of 27 air breaths recorded under these conditions, more than half of occurred within 15 h of the onset of hypoxia (Fig. 4). After this period air breathing returned to an infrequent and irregular pattern, similar to that observed from fish in well-aerated water (Fig. 4). In severe hypoxia, a further significant increase in the number of air breaths was observed (P = 0.037). Air breathing was recorded on 47 occasions in these conditions and was sustained from the beginning to the end of the 72-h trial (Fig. 4).

4. Discussion

This study demonstrates that *N. forsteri* combines at least three mechanisms to preserve oxygen delivery during periods of aquatic hypoxia. Increased branchial ventilation, air breathing and increased haemoglobin oxygen affinity act progressively and collectively to form an overall response to hypoxia that varies according to the severity of the hypoxic episode.

4.1. Short term response

The fish responded to declining Pw_{O2} by increasing the rate of branchial ventilation (Fig. 1). Further deoxygenation led to a plateau in ventilation rate and the onset of air breathing (Fig. ,1). This pattern is consistent with previous studies of *N. forsteri* (Johansen et al., 1967; Fritsche et al., 1993) and parallels the immediate response to hypoxia observed in other facultative air breathers such as the bowfin, *Amia calva* (Johansen et al., 1970). Johansen et al. (1967) showed that although *N. forsteri* increases branchial ventilation in response to hypoxia, both oxygen extraction and total oxygen uptake actually decrease during the same period. The plateau in branchial ventilation rate and subsequent air breathing by fish in this study are consistent with that conclusion. It is apparent that increased branchial ventilation alone cannot sustain oxygen requirements, even during relatively short periods of aquatic hypoxia.

4.2. Responses to prolonged hypoxiu

Consecutive air breathing has been reported in *N. forsteri* previously, at similar levels of hypoxia to those used during this investigation (Johansen et al., 1967; Fritsche et al., 1993). However; this is the first time that air breathing has been monitored on a time scale of days rather than minutes following exposure to hypoxic conditions.

The use of time-lapse video to. monitor air breathing confers distinct advantages over direct observation. Using this technique, all air breaths are recorded regardless of when they occur, thus providing an accurate picture of changes in the pattern of air breathing through time. No observers are required near the tanks, and this is important as the fish invariably showed signs of agitation when people approached their enclosure. Red lights used to illuminate tanks during the night did not appear to influence the behaviour of the fish, and more than half of all air breathing was recorded during this time.

Shortly after Pw_{O2} started to fall, the fish became visibly restless and most individuals circled rapidly around the tanks. Therefore, some air breaths observed during the initial stages of exposure may have resulted from stress or increased oxygen consumption rather than changes in external Pw_{O2} especially given the increased dependence on the lungs during activity (Grigg, 1965b). The simplest way to avoid aquatic hypoxia is, after all, likely to be by swimming to another area (Jensen et al., 1993). The reactions of the fish in this study indicate that *N. forsteri* would be likely to respond in this way.

Frequent air breathing persisted for approximately 15 h after the onset of moderate hypoxia (60 mmHg) (Fig. 4). After this period the frequency of air breathing diminished rapidly and assumed an irregular and infrequent pattern similar to that seen in normoxic treatments. This suggests that, in moderate hypoxia, *N. forsteri* breathe air to supplement oxygen uptake only until alternative compensatory mechanisms can be invoked. Graham and Baird (1982) found a similar result in both *Ancistris chagresi* and *Hoplosternum plecostomus,* in which air breathing rates declined over a period of exposure.
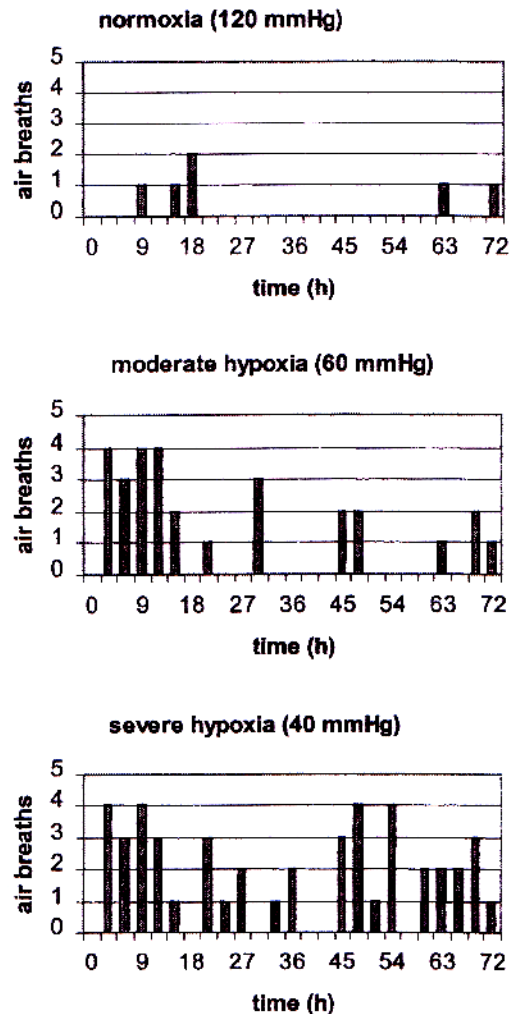
Fig. 4. Total number of air breaths (per 3 h) from four lungfish, *N. forsteri* subjected to three successive 72-h periods of exposure to three increasing levels of hypoxia. The levels were; normoxia (120 mmHg) moderate hypoxia (60 mmHg) and severe hypoxia (40 mmHg).

4.3. Blood oxygen-carrying capacity

Both the oxygen binding affinity and the concentration of haemoglobin in the blood may be responsive to short term changes in the oxygen tension and oxygen availability in the external environment. Soivio et al. (1980) reported that trout exhibit large shifts in blood oxygen-carrying capacity within 6 h of exposure to hypoxia. Similar rapid increases have also been found in other teleosts (Yamamoto et al., 1980) and amphibians (Tufts et al., 1987) stressed by severe exercise.

The haematocrit and haemoglobin concentrations [Hb] of *N. forsteri* from normoxic treatments in this study compared well with fish maintained in similar conditions by Lenfant et al. (1966). This was interpreted as an indication of sound methodology and established confidence in comparisons made between the treatment groups. We found no evidence to suggest that *N. forsteri* supplements oxygen uptake during hypoxia by increasing blood oxygen-carrying capacity. Both haematocrit and [Hb] remained stable in normoxic and hypoxic treatments throughout the study (Table 1). Even when exposed to severe hypoxia, no change was evident in either value. Unlike obligate water breathers, *N. forsteri* has the option of breathing air to compensate immediately when oxygen levels in the water are low. This simple alternative may obviate the need for rapid adjustments in the blood oxygen-carrying capacity.

4.4. Whole blood oxygen affinity

Oxygen equilibrium curves from fish in normoxic trials compared well in both shape and position with those reported by Lenfant et al. {1966}, when compared at similar P_{CO2} and temperature. Whole blood oxygen affinity (based on P_{50}) clearly increased in response to moderate hypoxia (Rig. 2). While all eight fish had lower P_{50} values after exposure to hypoxic conditions, the exact time course of this response remains undetermined. If whole blood oxygen affinity increased within 15 h, this would provide a possible explanation for the rapid

decline in the frequency of air breathing after this period. However, there are of course alternative explanations. A decrease in routine oxygen consumption during hypoxia has been observed in many fish species (Jensen et al., 1993). At constant temperature, decreases in oxygen consumption are achieved primarily by avoiding activity. One such example, the Sacramento blackfish *Orthodon microlepidotus,* exhibits significant metabolic depression over a wide range of temperature when exposed to hypoxic conditions (Cech et al., 1979). We made no measurements of metabolic rate during this investigation. Therefore, the possibility exists that the observed decline in the frequency of air breathing corresponded with a similar decline in metabolic rate. While reducing metabolic rate may be a feasible solution to avoid hypoxia in the short term, requirements for feeding, spawning and avoidance of predators mean that during prolonged hypoxic episodes other alternatives must be found (Jensen et al., 1993).

By analogy with previous studies (e.g. Babiker, 1985; Graham, 1983; Wells et al., 1989), increased whole blood oxygen affinity would be expected to be associated with a decrease in the concentration of erythrocytic phosphates such as ATP and/or GTP. Indeed, seven of the eight fish had a lower ATP levels after exposure to moderate aquatic hypoxia. Furthermore, the mean concentration of ATP was also lower in the hypoxic treatments. Despite this, the changes in ATP concentration were not statistically significant at $P < 0.05$ (Table 1).

Increased whole blood oxygen affinity in hypoxic treatments could not be attributed to increased pH, decreased P_{CO2} or lower temperature. Mean pH in the normoxic treatment was similar to the mean pH in both moderate and severe hypoxia. The spasmodic nature of aeration in hypoxic treatments meant that evaporation was lower in these tanks, and hence, they also experienced less evaporative cooling. Therefore, water temperature was slightly higher in hypoxic treatments than in the controls. This would tend to decrease oxygen affinity of the haemoglobin, and so serves only to emphasise the left-shifted response observed in hypoxic treatments. It is also unlikely that differences on Pw_{CO2} led to the observed results. Wells et al. (1989), using techniques similar to those used in this study, found that the intermittent nature of aeration in hypoxic treatments led to a slight build up of CO_2 in those tanks. Elevated Pw_{CO2} would also tend to reduce rather than increase haemoglobin oxygen affinity, thus further emphasising the left-shifted response in hypoxic treatments.

Taking all of these factors into consideration, modulation of organic phosphates remains the most likely candidate to explain the observed increase in haemoglobin oxygen affinity after the fish had been exposed to aquatic hypoxia. The lack of statistical significance in ATP concentration may derive from a combination of imprecision in measurements, the small shift in the curve and/or insufficient replication. Alternatively, the observed decrease in P_{50} may have resulted from a decrease in the concentration of other organic phosphates, particularly GTP. Several previous studies (reviewed in Graham, 1997) have concluded that GTP is a more potent modifier of haemoglobin oxygen affinity than ATP in a range of fish species. As GTP concentrations were not measured during this study, the exact mechanism underlying the observed left shift in the oxygen equilibrium curve remains unclear.

On the basis of change in mean P_{50}, whole blood oxygen affinity in *N. forsteri* increased by approximately 14% after exposure to moderate hypoxia. When compared to other fish species, this increase is relatively minor. Trout subject to hypoxia have been found to decrease P_{50} by between 24 and 30% (Soivio et al., 1980; Tetens and Lykkboe, 1981, 1985). The Antarctic fish *Pagothenia borchgrevinki* showed a decrease in P_{50} of approximately 35% (Wells et al., 1989), while similar changes of up to 50% have been demonstrated in carp subject to hypoxic conditions (Weber and Lykkbae, 1978). Nevertheless, even an increase in whole blood oxygen affinity of 14% would provide a considerable benefit in oxygen loading at the gills. At 60 mmHg, the predicted benefit of increased haemoglobin oxygen affinity is subtle (Fig. 2). However, below this point, the advantage becomes clear. A small increase in the efficiency of oxygen loading at the gills would also minimise the trade off with decreased oxygen unloading at the tissues.

4.5. Severe hypoxia

When exposed to severe hypoxia, the lungfish surfaced to breathe air on a more frequent and more sustained basis than in moderate hypoxia (Fig. 4). However, whole blood oxygen affinity did not change from the levels measured in. moderate hypoxia.

Air breathing provides access to a high and stable supply of oxygen. In severe hypoxia, frequent air breathing would act in tandem with increased haemoglobin oxygen affinity to preserve oxygen uptake. Increased whole blood oxygen affinity favours greater uptake of oxygen during each air breath (Weber et al., 1979). In addition, loss of chemically bound oxygen across the gills to the water is minimised by having blood with a higher affinity for oxygen (Johansen et al., 1967). In *N. forsteri,* this combination of frequent air breathing and increased whole blood oxygen affinity appears able to preserve oxygen delivery even at very low levels of Pw_{O2}.

4.6. Exposure of lungfish to hypoxia in the field

The levels of PW_{O_2} used during this investigation may have limited or infrequent physiological significance for *N. forsteri* in the wild. Grigg (1965b) reported only high concentrations of oxygen at several sites along the Burnett and Mary Rivers, and concluded that it seems unlikely that *N. forsteri* would experience hypoxia routinely in its natural habitat. He demonstrated that use of the lung was associated with activity, and suggested that its functional significance is as a supplementary organ during periods of increased activity such as spawning, feeding and during flood conditions. Our data shows that lungfish are, nevertheless, well able to adapt to hypoxic conditions, even though they may encounter them only infrequently.

4.7. Comparisons with other species

The comparatively small increase in whole blood oxygen affinity observed during this study should not be interpreted as an indication that *N. forsteri is* incapable of larger shifts. Rather it provides further insights into the wide range of options open to air breathing fish during hypoxic challenge. The armoured catfish, *Hypostamus sp.,* is a facultative air breather that responds to moderate hypoxia by increasing the frequency of air breathing (Weber et al., 1979). In this respect it is similar to *N. forsteri.* However, when exposed to extreme hypoxia (Pw_{O_2} = 20-25 mmHg), *Hypostamus sp.* exhibits increases in haemoglobin oxygen affinity in the order of 30% (based on decrease in P_{50}), associated with a similar decrease in organic phosphates, principally GTP (Weber et al., 1979). Weber et al. (1979) concluded that the air breathing organ (intestine) could not maintain high internal oxygen tension in severe hypoxia, despite frequent air breathing. Therefore, when air breathing alone cannot compensate for low Pw_{O_2}, *Hypostamus sp.* reacts in the same way as an obligate water breathing species. Thus, the extent of *N. forsteri's* ability to increase whole blood oxygen affinity may become apparent only if access to the surface were denied during hypoxic exposure.

Another facultative air breather, *Ancistrus chagresi,* which uses its stomach as an air breathing organ, also responds to hypoxia by increasing blood oxygen affinity from about 20 to about 10 mmHg on exposure to Pw_{O_2} <30 mmHg (Graham, 1983). It, however, also increases both haematocrit and haemoglobin content by about 25%.

The structure and relative efficiency of air breathing organs varies widely between species of air breathing fish (Johansen, 1969; Graham, 1997). Similar variation also exists in the oxygen-carrying capacity and whole blood oxygen affinity of blood from the air breathing species (Johansen et al., 1978; Graham 1997). This variation should be matched by similar differences in responses of air breathing species to prolonged periods of aquatic hypoxia. While *N. forsteri* has some ability to compensate for hypoxia by increasing whole blood oxygen affinity, only minor adjustments were observed, and other mechanisms such as increased air breathing were also invoked. The progression from water breathing to predominantly air breathing fish species may be marked by a decline in the tendency to compensate for hypoxia by altering the respiratory properties of the blood. Unfortunately, there is still insufficient comparative data to draw this conclusion with confidence.

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