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The autonomic control and functional significance of the changes in heart rate associated with air breathing in the jeju, *Hoplerythrinus unitaeniatus*

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Summary

The jeju is a teleost fish with bimodal respiration that utilizes a modified swim bladder as an air-breathing organ (ABO). Like all air-breathing fish studied to date, jeju exhibit pronounced changes in heart rate (f_H) during air-breathing events, and it is believed that these may facilitate oxygen uptake (M_{O_2}) from the ABO. The current study employed power spectral analysis (PSA) of f_H patterns, coupled with instantaneous respirometry, to investigate the autonomic control of these phenomena and their functional significance for the efficacy of air breathing. The jeju obtained less than 5% of total M_{O_2} (M_{tO_2}) from air breathing in normoxia at 26°C, and PSA of beat-to-beat variability in f_H revealed a pattern similar to that of unimodal water-breathing fish. In deep aquatic hypoxia (water P_{O_2}=1 kPa) the jeju increased the frequency of air breathing (f_{AB}) tenfold and maintained M_{tO_2} unchanged from normoxia. This was associated with a significant increase in heart rate variability (HRV), each air breath (AB) being preceded by a brief bradycardia and then followed by a brief tachycardia. These f_H changes are qualitatively similar to those associated with breathing in unimodal air-breathing vertebrates. Within 20 heartbeats after the AB, however, a beat-to-beat variability in f_H typical of water-breathing fish was re-established. Pharmacological blockade revealed that both adrenergic and cholinergic tone increased simultaneously prior to each AB, and then decreased after it. However, modulation of inhibitory cholinergic tone was responsible for the major proportion of HRV, including the precise beat-to-beat modulation of f_H around each AB. Pharmacological blockade of all variations in f_H associated with air breathing in deep hypoxia did not, however, have a significant effect upon f_{AB} or the regulation of M_{tO_2}. Thus, the functional significance of the profound HRV during air breathing remains a mystery.

Key words: air breathing, heart rate variability, teleost.

Introduction

All air-breathing fish studied to date exhibit changes in heart rate (f_H) associated with surface air breathing (Randall et al., 1981; Graham et al., 1995; Graham, 1997). Expiration is typically associated with a bradycardia, followed by tachycardia upon inspiration. In the swamp eel, *Synbranchus marmoratus*, and the South American lungfish, *Lepidosiren paradoxa*, inspiration tachycardia is associated with large increases in cardiac output and increased perfusion of the air-breathing organ (ABO) (Johansen, 1966; Axelsson et al., 1989; Skals et al., 2006). There is some evidence to indicate that these respiration-related variations in f_H are under autonomic control, in particular of inhibitory vagal cholinergic tone (Axelsson et al., 1989; Graham et al., 1995), and it has been proposed that their role is to facilitate O_2 uptake by air breathing (Johansen, 1966; Graham et al., 1995; Skals et al., 2006).

Although air-breathing frequency is discontinuous and arrhythmic in fish (Randall et al., 1981; Graham, 1997), Graham et al. (Graham et al., 1995) proposed that the variations in f_H were a homologue of respiratory sinus arrhythmia (RSA), the cyclical variations in f_H that accompany the continuous rhythmic breathing of mammals. In mammalian RSA, f_H varies with the ventilation cycle because activity in preganglionic neurones is inhibited during inspiration so that inhibitory parasympathetic vagal tone on the heart is diminished and f_H rises. Parasympathetic tone is subsequently disinhibited, producing a bradycardia on expiration (Jordan and Spyer, 1987; Taylor et al., 1999). It is thought that these changes in vagal tone improve the efficiency of pulmonary gas exchange by matching perfusion to ventilation within each breathing cycle (Giardino et al., 2003).

How and when RSA might have evolved in vertebrates remains a topic of some speculation (Porges, 1995; Taylor et

al., 1999; Campbell et al., 2005; Campbell et al., 2006a). Indeed, there is a major difference in the association between heart rate (f_H) and ventilation rate (f_V) between fish that breathe continuously in water and mammals that do so in air. In mammals, the rate association $f_V<f_H/2$ must occur for the development of the classic RSA oscillation effect, which alternates between long and short heartbeat intervals. The presence of RSA in the instantaneous electrocardiogram (ECG) in a continuous air breather can be revealed using a mathematical technique known as power spectral analysis (PSA). If RSA is present, the PSA output spectrum reveals distinct fundamental components at the frequency of the ventilation cycle (Akselrod et al., 1981; Campbell et al., 2006a). In fish, f_V is almost always greater than half the heart rate ($f_V>f_H/2$) and therefore the sequence of cardiac intervals cannot be modulated by respiratory activity in the classic RSA pattern (Zweiner et al., 1995; Campbell et al., 2006a). The PSA spectrum of the heart rate variability (HRV) signal from water-breathing fish does not produce a fundamental component at the frequency of ventilation but, instead, shows a number of peaks scattered systematically through the spectral bandwidth, all at frequencies lower than f_V. These peaks cannot be considered to reflect RSA in water-breathing fish but, nonetheless, they exhibit an association with shifts in the length of each ventilation cycle, and a causal link between HRV and ventilation can be revealed (Campbell et al., 2005; Campbell et al., 2006a; Campbell and Egginton, 2007).

Pharmacological parasympathetic blockade abolishes the PSA peaks in the spectra of mammals, reptiles and water-breathing fish (Medigue et al., 2001; Campbell et al., 2004; Campbell et al., 2006b). This demonstrates that respiration-related modulation of activity at the sinoatrial node, exerted via the parasympathetic vagus, is an essential component of HRV in all animals examined to date (Medigue et al., 2001; Campbell et al., 2004; Campbell et al., 2006b). Thus, although the control of cardio-respiratory coupling events is still not completely understood in water-breathing fish, there does appear to be some commonality in the autonomic control of HRV between animals that breathe water and those that breathe air. Fish that respire in both air and water offer the opportunity to examine these cardio-respiratory coupling events in a single animal, and to determine the importance of each for effective oxygen extraction. This may uncover the mechanisms of cardio-respiratory synchronization that underlie the switch from water to air breathing. Despite the ubiquitous occurrence of variations in f_H associated with air breathing in fish (Randall et al., 1981; Graham et al., 1995; Graham, 1997), and its qualitative similarity with the RSA of mammals, the relative roles of inhibitory cholinergic *versus* excitatory adrenergic inputs in the generation of HRV have not been described for any species.

The jeju, *Hoplerythrinus unitaeniatus*, is a facultative air breather that uses a modified swimbladder as an ABO and which exhibits the 'typical' variations in f_H during air breathing (Farrell, 1978). In the current study, we measured instantaneous beat-to-beat f_H from the ECG of jeju exposed either to normoxia or to deep aquatic hypoxia (water P_{O_2}=1 kPa), and then used PSA to examine patterns of HRV as a function of the prevailing frequencies of gill ventilation (f_V) and air breathing (f_{AB}). Pharmacological blockade was then used to investigate the roles of adrenergic and cholinergic inputs in generating HRV, and the relationship with bimodal ventilation patterns. Given the proposal that the role of the HRV is to facilitate O_2 uptake by air breathing (Johansen, 1966; Graham et al., 1995; Skals et al., 2006), we investigated the hypothesis that pharmacological abolition of HRV would reduce the efficacy with which air breathing sustained routine metabolic rate in deep hypoxia.

Materials and methods

Animals

Jeju *Hoplerythrinus unitaeniatus* (Spix and Agassis 1829) of either sex and a body mass of approximately 250 g were caught in the Miranda River, state of Mato Grosso do Sul, Brazil, and brought to Universidade Estadual Paulista (UNESP), Rio Claro, São Paulo state, Brazil, where they were maintained in outdoor 1.5 m^3 tanks provided with a flow of well-water at ambient temperature (18–26°C over the diurnal cycle) and under a natural photoperiod. The fish were fed every 2 days with chopped chicken hearts, but were fasted for at least 48 h prior to use in experiments. Experiments were performed in accordance with Home Office regulations for animal experimentation in the United Kingdom, which also complied with the guidelines for animal experimentation at UNESP, Rio Claro, Brazil.

Surgery

Jeju were anaesthetized in tricaine methane sulphonate (MS-222, 0.3 mg l^{-1}), and placed on their side on an operating table with their gills irrigated with an aerated solution of MS-222 at 0.1 mg l^{-1}. ECG recording electrodes (7-strand Teflon-coated wire, length 40 cm, diameter 0.2 mm; A-M Systems Inc., Carlsborg, CT, USA) were hooked into the end of a 24 G hypodermic needle and inserted through the opercular septum at the base of the left 4th gill arch. The wires were placed 20 mm apart and advanced 4 mm through the septum; care was taken to ensure that the pericardial membrane was not pierced. The trailing wire was attached by a single suture to the flank of the fish just dorsal to the opercular flap. A cannula (PE10 Intramedic, Clay Adams, Parsipany, NJ, USA) filled with heparinized saline (10 i.u. ml^{-1} sodium heparin in 9 g l^{-1} NaCl) was then inserted through a small puncture wound (23 G needle) made just behind the left pectoral fin, into the peritoneal cavity of the fish, and sutured to the body wall at the point of exit. This cannula and the ECG wires were then sutured together to the back of the fish, just rostral to the dorsal fin. Fish were allowed to recover in aerated water at 26°C for at least 24 h prior to placement in the respirometry chamber. The cannula was flushed gently with heparinized saline at 24 h recovery.

Respiratory metabolism and air-breathing behaviour

The instrumented jeju were transferred into clear plastic respirometry chambers (volume 3 l) at approximately 19:00 h and allowed to recover and acclimate overnight (at least 14 h) prior to experimentation. The ECG wires and peritoneal cannula were led out of a small hole in the opaque plastic lid

of the chamber, so that they could be manipulated without disturbing the fish. The respirometer was maintained at 26°C, by partial immersion in a large outer plastic reservoir tank (volume 50 l) of aerated water. Instantaneous O_2 uptake from the water (M_{wO_2}, in mmol O_2 kg^{-1} h^{-1}) was measured by intermittent stop-flow respirometry (Steffensen, 1989) as described in detail previously (McKenzie et al., 2007). Water O_2 partial pressures (P_{wO_2}) were measured with an oxygen electrode (Radiometer E5046; Radiometer A/S, Brønshøj, Denmark) and associated oxygen meter (Model 781, Strathkelvin Instruments, Glasgow, UK). The output was relayed to an automated control and data acquisition system (LoliDAQ, Loligo Systems ApS, Hobro, Denmark) with associated software (LoliResp, Loligo Systems ApS), which calculated M_{wO_2} from 10 min cycles of water recirculation through the respirometer chamber, interrupted by a flushing cycle, both generated by submerged water pumps (Eheim Gmbh & Co KG, Deizisan, Germany).

The water in the outer tank was level with the lid of the respirometry chamber, and this was fitted with a dome-shaped airspace (volume 150 ml) that allowed the animal to surface and gulp air. The dome had an aperture at its apex that was sealed with a rubber bung during experiments, and the moist air in the sealed space was drawn, by a peristaltic pump (MCP-E-60, Ismatec SA, Labortechnik-Analytik, Glattbrugg, Switzerland) via gas-impermeable Tygon tubing, past an O_2 electrode (model 16-730 flow-through electrode; Microelectrode Inc., Bedford, NH, USA) and then pushed back into the air dome. The electrode was connected to an O_2 meter (model OM4, Microelectrode Inc.) and the signal from this was continuously displayed and recorded on a PC with Logger Pro software (Vernier Software and Technology, Beaverton, OR, USA), via a Lab Pro data collection device (Vernier Software and Technology). Air breaths by the fish were visible as stepwise declines in the P_{O_2} signal from the air in the dome (P_{aO_2}), which were completed in between 3 and 5 s and were confirmed visually (see below). Instantaneous O_2 uptake from the air (M_{aO_2}, in mmol O_2 kg^{-1} h^{-1}) was calculated from the rate of decline in O_2 in the airspace, as described in detail by McKenzie and Randall (McKenzie and Randall, 1990). The decline in airspace P_{O_2} following each air breath was so discrete and rapid that it was not necessary to correct for potential gradual exchange of O_2 between air and hypoxic water (see below). The O_2 in the airspace was replaced at intervals by gently removing the bung and flushing the dome with an air-filled syringe (volume 60 ml). Total oxygen uptake (M_{tO_2}) was calculated by summing $M_{wO_2}$and M_{aO_2}.

To minimize any disturbance to the fish, which might influence air-breathing behaviour and therefore respiratory partitioning (Shingles et al., 2005), the whole setup was shielded from view behind sheets of cardboard on a large wooden frame. A digital video camera filmed the behaviour of the fish through the clear plastic fronts of the reservoir tank and respirometry chamber, with the images displayed on a PC. The fish could also be observed directly through a small hole cut into one of the cardboard sheets. The air breaths shown on the video signal and P_{aO_2} trace were used to calculate air-breathing frequency (f_{AB}, in breaths h^{-1}) and its reciprocal, the interbreath interval (IBI, in min).

Electrocardiograms

The instantaneous ECG was recorded using a bioamplifier interfaced with a digital recording system (PowerLab, ADInstruments, Oxford, UK). ECG was sampled at 400 Hz and both the ECG and gill f_V (opercular movements) could be observed as different frequency components transposed onto a single trace (see Campbell and Egginton, 2007). Bipolar ECG signals were recorded continuously immediately after placement in the respirometer. When the fish took an air breath, this was noticeable as a distinct artifact on the ECG trace. This was correlated with the video image of the air-breathing behaviour and the sudden drop in P_{O_2} in the air dome.

Experimental protocol

Following overnight recovery, normoxic values for respiratory and cardiac variables were collected on the undisturbed jeju for at least 1 h. Subsequently, the water P_{O_2} was reduced to 1±0.2 kPa by bubbling 100% N_2 into the outer reservoir tank. It took about 2 h 20 min for the P_{wO_2} to stabilize at this extreme degree of hypoxia, and all variables were measured throughout this period. The fish were maintained at this hypoxic P_{wO_2} for 1 h and all variables were measured continuously. While still hypoxic, the animals were then infused via their peritoneal cannula with 1 ml kg^{-1} of 10^{-5} mol l^{-1} propranolol hydrochloride (Sigma-Aldrich, St Louis, MO, USA) dissolved in saline (9 g l^{-1} NaCl) and all variables were again measured for 1 h. Subsequently, also while still hypoxic, the animals were infused with 1 ml kg^{-1} of a cocktail of 10^{-5} mol l^{-1} propranolol hydrochloride plus 10^{-5} mol l^{-1} atropine sulphate (Sigma) dissolved in saline. All variables were again measured for 1 h and then the animals were recovered to normoxia by bubbling air into the reservoir tank. This required about 1 h, and then all variables were measured for at least 40 min in normoxia.

Supporting pharmacological studies

In order to investigate the effects on hypoxic HRV of reversing the order of the pharmacological treatments, jeju (N=4) were exposed to deep hypoxia, as described, and then treated with the same doses of drugs, but with atropine sulphate given before propranolol hydrochloride. To ensure that the doses of atropine and propranolol used in the study were effectively blocking cholinergic and adrenergic control of the heart in the jeju, acetylcholine chloride (10^{-5} mol l^{-1}) and then phenylephrine (1 ml kg^{-1} of 10^{-5} mol l^{-1} solution) were infused via the cannulae 1 h after infusion of the respective antagonists and resultant changes in ECG were recorded. Oxygen uptake was not measured during these experiments.

Analysis of data

Respiratory metabolism and air-breathing behaviour

Oxygen uptake was measured for the last 40 min of each condition and mean M_{wO_2}, M_{aO_2} and M_{tO_2} were calculated. These data were then compared between conditions by one-way analysis of variance (ANOVA) for repeated measures. The percentage of M_{tO_2} met by either water or air breathing was also calculated and compared between treatments by the same method, following arc-sine transformation of the data.

Air-breathing frequency and IBI were similarly calculated and compared between treatments. In those cases where the groups failed normality (Kolgorov–Smirnov test), a non-parametric ANOVA for repeated measures based upon ranks was applied.

Calculations of autonomic tonus on the heart

To calculate the relative cholinergic (Chol, %) and adrenergic (Adr, %) tone the following equations were used, modified from Campbell et al. (Campbell et al., 2004):

$$\text{Chol} = \frac{(\text{R–R})_{\text{prop}} - (\text{R–R})_{\text{A\&P}}}{(\text{R–R})_{\text{prop}}} \times 100\,, \quad (1)$$

$$\text{Adr} = \frac{(\text{R–R})_{\text{A\&P}} - [(\text{R–R})_{\text{prop}} - (\text{R–R})_{\text{PT}}]}{(\text{R–R})_{\text{PT}}} \times 100\,, \quad (2)$$

where R–R is the instantaneous interval of consecutive R waves on the ECG trace, and the subscripts PT, prop and A&P are pre-treatment, propranolol blocked, and atropine and propranolol blocked, respectively. Data analysis was undertaken using a HRV module in Chart 5.1 (ADInstruments), and also purpose-designed computer programs in Matlab 8.1 (Mathworks Inc., Natick, MA, USA. Firstly, a continuous trace consisting of 512 consecutive R–R intervals containing no ectopic beats or artifacts was selected from each ECG trace and transferred into the computer module. This converted the raw ECG signal into an R–R interval tachogram, which contained information on the consecutive timing between each heartbeat. The tachogram waveform was then tested for stationarity using the run test, subtracting the mean f_H to normalize data. A discrete Fourier transformation (DFT) was then applied to the R–R interval tachogram, using a Hanning window to minimize spectral leakage. The DFT conveys respective frequency domain information on a time interval waveform, and creates a set of coefficients that describe the waveform (Campbell et al., 2004; Campbell et al., 2006a). The resultant output is plotted graphically, where oscillations in f_H will appear at their relative frequencies in the power spectrum.

Results

Respiratory metabolism and air-breathing behaviour

Fig. 1 shows the effects of exposure to deep hypoxia upon partitioning of respiratory metabolism between water and air breathing in the jeju, and how this varied during the pharmacological treatments and then recovery to normoxia. In normoxia, the vast majority of routine metabolism was met by O_2 uptake from water. As P_{wO_2} declined, however, there was a parallel decline in M_{wO_2}, and this fell to almost zero when the animals reached deep hypoxia (Fig. 1). At the same time, there was a reciprocal increase in M_{aO_2}, and air breathing met almost all of the demands of routine metabolic rate throughout deep hypoxia. The hypoxic P_{wO_2} beyond which air breathing behaviour was observed to increase significantly was very variable amongst the fish, ranging from about 11 kPa to below 1 kPa, with a mean (±s.e.m.) for N=7 jeju of 7.0±1.8 kPa. This value coincides well with the hypoxic P_{wO_2} in Fig. 1 at which mean M_{wO_2} started to decline steeply. During recovery, M_{wO_2} increased parallel to the increase in P_{wO_2}. Fig. 1 clearly demonstrates that M_{tO_2} remained relatively constant throughout the protocol, despite the reciprocal changes in respiratory partitioning between water and air as the animals entered and then left deep hypoxia.

The results of the effects of hypoxia and the drug treatments upon all respiratory variables are summarized in Table 1. The data confirm that exposure to deep hypoxia and then return to normoxia caused a significant decline in M_{wO_2} and a reciprocal increase in M_{aO_2}, such that M_{tO_2} did not change significantly. This increase in M_{aO_2} and the percentage of routine metabolism met by air breathing was achieved by a large and significant increase in f_{AB} and a reciprocal decline in IBI (Table 1). There was also a large increase in the amount of O_2 consumed per air breath (Table 1). Exposure to hypoxia did not produce any significant alteration in f_V, and there was no evidence of any variation in instantaneous f_V when the fish took an AB (cf. f_H results described below).

The β-adrenergic receptor blockade with propranolol caused M_{wO_2} to fall to zero but had no significant effects on M_{aO_2} or M_{tO_2}. The drop in M_{wO_2} was associated with a significant decline in f_V (Table 1). It was also associated with a significant increase in the percentage of metabolism met by air breathing when compared with the untreated hypoxic fish, although there was no significant effect upon f_{AB} or IBI (Table 1). When subsequently given a total autonomic blockade by treatment with the atropine and propranolol cocktail, M_{wO_2} and f_V returned to the rate measured in hypoxia prior to β-adrenergic receptor blockade (Table 1). There was, however, no significant effect of total

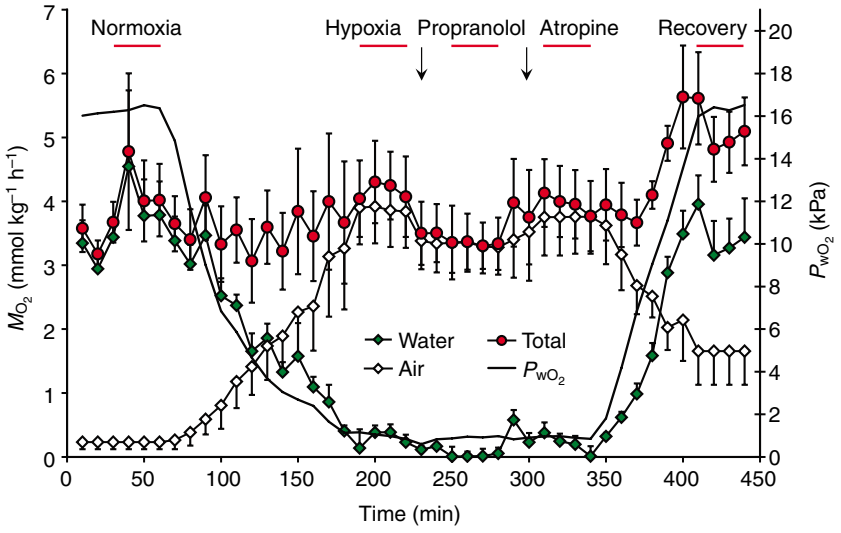

Fig. 1. Mean (±s.e.m.) rates of instantaneous oxygen uptake (M_{O_2}) from water and air, and in total, as a function of water P_{O_2} (P_{wO_2}) in seven jeju. The black arrows show the point at which either propranolol (left arrow) or atropine (right arrow) was injected (see text for details). The labelled red horizontal lines denote the periods when data were analysed for the comparisons shown in Table 1.

Table 1. *Aspects of respiratory metabolism and air-breathing behaviour in jeju exposed to deep aquatic hypoxia and then sequentially given β-adrenergic and cholinergic receptor blockers*

	Normoxia	Hypoxia	Propranolol	Atropine	Recovery
M_{wO_2} (mmol kg^{-1} h^{-1})	3.89±0.55^{a}	0.17±0.09^{b}	–0.03±0.04^{b}	0.11±0.04^{b}	3.42±0.55^{a}
M_{aO_2} (mmol kg^{-1} h^{-1})	0.23±0.11^{a}	3.88±0.58^{b}	3.34±0.45^{b}	3.74±0.59^{b}	1.66±0.53^{c}
M_{tO_2} (mmol kg^{-1} h^{-1})	4.12±0.61	4.05±0.57	3.30±0.44	3.85±0.57	5.08±0.55
Percentage air breathing	5±2^{a}	95±3^{b}	101±1^{c}	96±2^{b,c}	31±10^{d}
f_{AB} (breaths h^{-1})	2.0±1.0^{a}	19.1±5.4^{b}	18.6±2.3^{b,c}	18.9±4.2^{b,c}	6.1±2.3^{a,c}
IBI (min)	63.1±18.2^{a}	4.0±0.7^{b}	3.4±0.4^{b}	4.0±0.8^{b}	9.5±2.6^{b}
O_2 consumed per air breath (mmol)	0.14±0.03^{a}	0.25±0.05^{b}	0.19±0.03^{b}	0.25±0.06^{b}	0.32±0.06^{b}
f_V (beats min^{-1})	74.5±6.5^{a}	78.1±7.2^{a}	59.2±4.5^{b}	63.4±5.2^{a,b}	68.3±5.4^{a,b}

M_{O_2}, instantaneous oxygen uptake; w, water; a, air; t, total; f_{AB}, air-breathing frequency; IBI, interbreath interval; f_V, gill ventilation frequency. Data are means ± s.e.m. for seven jeju. Where present, a common superscript letter indicates no significant difference between means for that variable (P>0.05) by Tukey's test consequent to one-way ANOVA for repeated measures. See text for details.

Table 2. *Mean heart rate over 10 heartbeats preceding and following three air breaths under different conditions*

	Normoxia	Hypoxia	Propranolol	Atropine	Total blockade
Pre-AB	32.1±1.8^{a}	26.2±2.1^{b}	19.8±1.6^{c}	–	81.1±2.3^{d}
Post-AB	32.1±1.8^{a}	47.6±1.3^{b}	42.7±1.5^{c}	–	81.2±1.9^{d}
Pre-AB*	31.4±2.8^{a}	24.9±3.3^{b}	–	92.3± 0.5^{c}	83.6±1.9^{d}
Post-AB*	31.4±2.8^{a}	52.8±1.9^{b}	–	93.5±0.5^{c}	83.2±1.8^{d}

Heart rate data (beats min^{-1}) are means ± s.e.m. for 21 observations on seven jeju exposed to hypoxia and then infused 2 h later with propranolol and then atropine. AB, air breaths. Asterisks indicate N=4 jeju exposed to hypoxia and then infused with atropine and 2 h later with propranolol. Normoxic fish did not air breath and therefore pre- and post-AB values are mean rates taken from randomly selected sections of ECG trace containing 20 heartbeats. Means with a common superscript letter within rows are not significantly different (P>0.05) by Fisher's least significant difference procedure.

blockade upon M_{aO_2}, M_{tO_2} or f_{AB} (Table 1). Finally, recovery to normoxia was associated with a significant increase in M_{wO_2}, although there was no change in f_V. There was, however, a decline in M_{aO_2} and f_{AB} (Table 1). The slight elevation of mean M_{tO_2} that is apparent during recovery (Fig. 1, Table 1) was not statistically significant but was associated with a visible increase in the activity level of the fish in their respirometers, as observed on the video recording.

Heart rate, heart rate variability and its autonomic regulation

Overall, mean f_H was not significantly different in normoxic compared with hypoxic water. However, HRV was greatly increased in hypoxia, as a result of the pronounced increase in f_{AB}. Prior to each AB, there was a significant decline (18–21%) in mean f_H, and after inhalation of air into the modified swimbladder f_H rose by 81.5% (Table 2, Fig. 2A). The infusion of propranolol reduced overall f_H. The f_H prior to each AB was suppressed to a greater extent (24.3%) than the f_H following the AB (10.4%), and therefore adrenergic receptor blockade had the effect of causing a larger disparity in f_H prior to *versus* following each AB (Table 2, Fig. 2B). The second effect of adrenergic

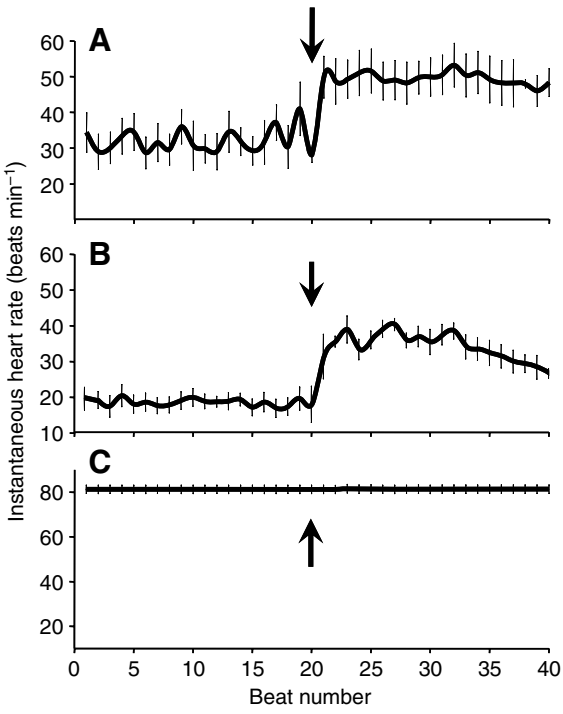

Fig. 2. Mean (±s.e.m.) instantaneous heart rate for 21 observations on seven jeju, as calculated per R–R interval over 20 heartbeats prior to and succeeding an air breath (see text for details). Each panel shows the fish under (A) hypoxia, (B) hypoxia following adrenergic receptor blockade, and (C) hypoxia following adrenergic and cholinergic receptor blockade. The black arrow denotes when exhalation was completed and inhalation initiated.

receptor blockade was to increase the time taken for f_H to return to a steady state after each AB.

The infusion of the β-muscarinic acetylcholine receptor blocker atropine significantly increased f_H and abolished all of the marked changes in f_H that accompanied each AB (Fig. 2C). It also abolished all the other HRV components.

Calculation of relative tonus using Eqns 1 and 2 showed that adrenergic tonus was 75.51% before the AB and 47.28% after the AB, and cholinergic tonus was 304.3% before and 81.5% after the AB.

Fig. 3 shows representative results of PSA on R–R intervals of a jeju in normoxia then hypoxia with the pharmacological treatments. In normoxia, when the fish did not exhibit air breathing, the f_H showed high frequency beat-to-beat variation (Fig. 3Ai). The R–R intervals formed two discrete groups in period length with centre frequencies of 1650 and 2150 ms (Fig. 3Aii). The switch between each R–R interval length was oscillatory in nature and the frequency of these oscillations was observed as fundamental components in the power spectrum between 0.147 and 0.189 Hz (Fig. 3Aiii). Calculated in the time domain (1/Hz) the length of each of these oscillations was between 6.8 and 5.2 s, and corresponds to a beat frequency of two to three heartbeats.

Air breathing in hypoxia failed to abolish the short-term beat-to-beat variability pattern observed in normoxia when no AB were taken (Fig. 3Bi,ii). When plotted on a frequency histogram the R–R intervals still formed two discrete groups. This HRV was oscillatory and showed fundamental

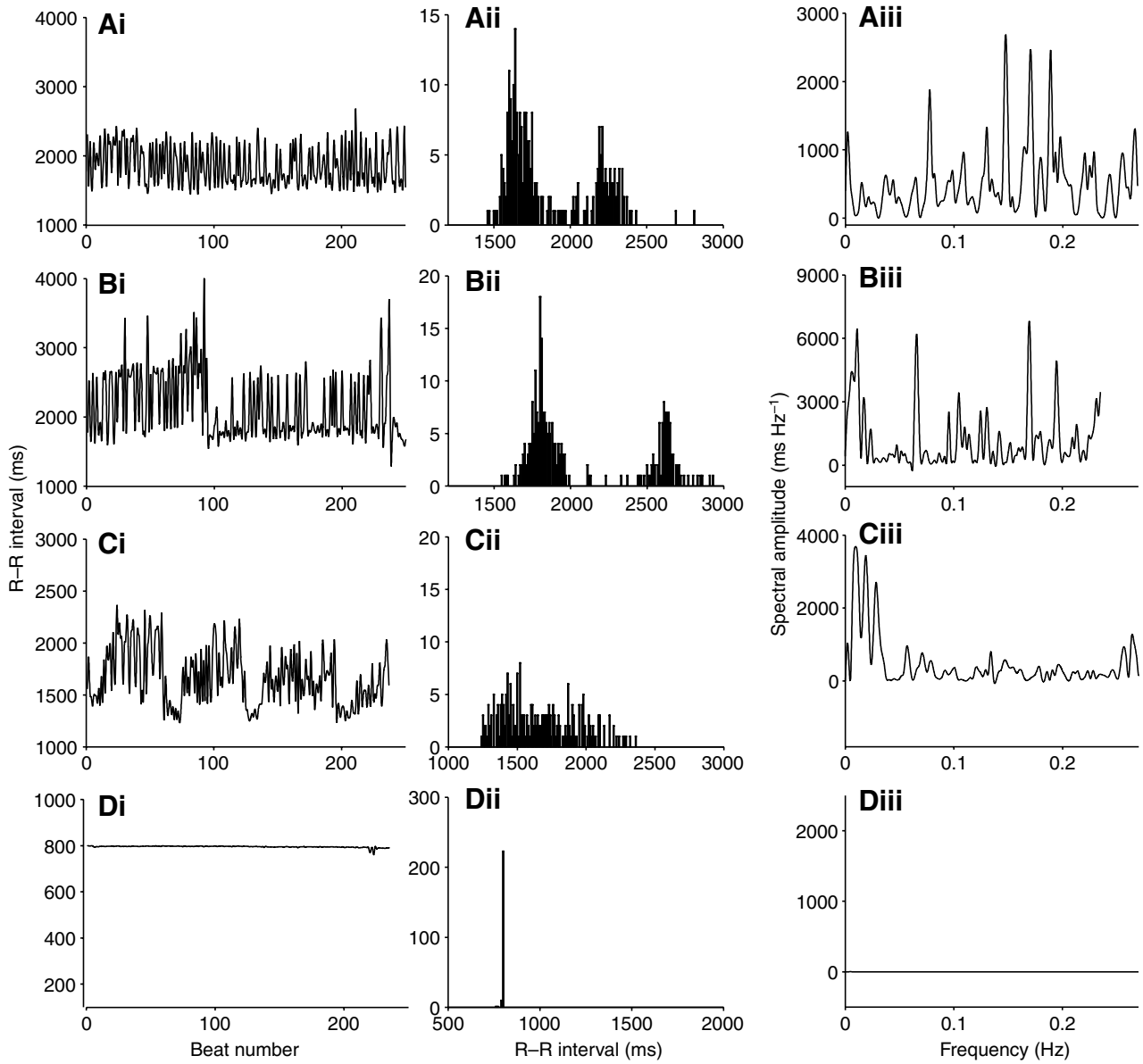

Fig. 3. The pattern of heart rate variability (HRV) within 250 consecutive heartbeat intervals (R–R interval) of a single *H. unitaeniatus* in (A) normoxia, (B) hypoxia, (C) hypoxia and blockade of adrenergic receptors, and (D) hypoxia and total (adrenergic and cholinergic receptor) blockade. The 250 consecutive R–R intervals are displayed as (i) a tachogram plot, (ii) a frequency histogram in 5 ms bins, and (iii) a power spectrum of the Fourier transform that shows the frequency and power of oscillatory components of the R–R interval tachogram.

components in the power spectrum at a frequency similar to that observed in normoxia (Fig. 3Biii). The changes in f_H associated with AB could also be observed in the power spectrum at a frequency of 0.0021 Hz. This corresponds to around 476 s in the time domain and was synchronized with IBI for this fish. Because the timing of the AB was irregular, there was a large smearing of the spectrum across the IBI range (Fig. 3Biii).

A major effect of propranolol was to abolish the oscillation in short-term beat-to-beat variability observed in non-blockaded fish. Although short-term HRV occurred it did not form distinct groups and the R–R intervals were instead spread between 1250 and 2400 ms with no centre frequencies (Fig. 3Cii). The loss of the oscillatory component was confirmed by the absence of discrete high frequency components in the power spectrum (Fig. 3Ciii). The further infusion of atropine abolished all HRV, and with total blockade the heart beat at a very steady rate and did not vary at all with the AB (Fig. 3Di,ii) and, therefore, no components whatsoever were observed in the power spectrum. (Fig. 3Diii).

Although the HRV results are only provided for a single representative jeju, the patterns described were similar for all individuals, with the mean rate changes in f_H shown in Table 2 and Fig. 2. Reversing the order of the autonomic blockades, i.e. atropine prior to propranolol, did not change the resulting total blockade effect, although f_H changes around the AB induced by propranolol could not be observed if atropine was infused first. When acetylcholine and phenylephidrine were infused 1 h after blockade, they had no significant effect on f_H, which confirmed that the doses of propranolol and atropine applied had caused total effective blockade of adrenergic and β-muscarinic receptors (data not shown).

Discussion

The results reveal complex patterns of beat-to-beat variation in f_H in the jeju, which were influenced significantly by the partitioning of bimodal respiration between water and air. The pharmacological studies demonstrated that these patterns were under autonomic control, with a contribution from both adrenergic and cholinergic systems. The results provided no evidence, however, that this complex autonomic regulation of f_H facilitated M_{O_2} by air breathing, inasmuch as total pharmacological blockade had no effect on f_{AB} and therefore the efficacy with which air breathing regulated M_{O_2} in deep hypoxia.

Video recordings of un-instrumented jeju, made over 24 h in the same respirometer chambers as used in the current study, revealed that they did not air breathe any more frequently in normoxia than the instrumented animals described here (D.J.M., unpublished observations), indicating that air-breathing behaviour had not been influenced by the presence of the ECG wires and peritoneal catheter. The almost total absence of air breathing in aquatic normoxia in the current study is consistent with other reports for this species at a similar temperature (Oliveira et al., 2004; Perry et al., 2004), but the associated minimal proportion of M_{tO_2} that was met by air breathing differs from that reported by Randall et al. (Randall et al., 1978), where M_{aO_2} represented up to 20% of routine metabolic rate in normoxia. When the jeju was in normoxia and air breathing very infrequently, mean f_V was slightly more than twice f_H. A situation where $f_V > f_H/2$ is typical of unimodal water-breathing fish species (Stevens and Randall, 1967; Campbell et al., 2004; Campbell et al., 2005; Campbell et al., 2006a) and the sequence of cardiac intervals cannot be modulated by respiratory activity in the classic RSA pattern (Zweiner et al., 1995; Campbell et al., 2006a). Indeed, in normoxia the PSA spectrum of HRV in the jeju was qualitatively similar to that of unimodal water-breathing fish, with a number of peaks scattered systematically throughout the spectral bandwidth, all at frequencies lower than f_V (Campbell et al., 2005; Campbell et al., 2006a).

When exposed to deep aquatic hypoxia, the jeju was able to maintain aerobic metabolism at routine levels through a large increase in f_{AB}. This increase was presumably a result of the stimulation of oxygen-sensitive chemoreceptors (Smatresk et al., 1986; McKenzie et al., 1991), and the P_{wO_2} threshold for the increase in M_{aO_2} coincides well with the hypoxic P_{wO_2} at which previous studies have noted a stimulation of f_{AB} in this species (Oliveira et al., 2004; Perry et al., 2004). Perry et al. (Perry et al., 2004) exposed jeju to a level of deep aquatic hypoxia similar to the current study (P_{wO_2} ~1.3 kPa) and demonstrated that air breathing allowed them to maintain arterial blood O_2 content unchanged from normoxia, indicating that they were not experiencing systemic hypoxia. It is interesting that the jeju did not exhibit any significant changes in f_V in hypoxia. In unimodal water-breathing fish, hypoxia elicits gill hyperventilation via a chemoreceptor-driven reflex (Burleson et al., 1992) but air-breathing fish exhibit a diversity in gill ventilatory responses that reflects the complex nature of physiological strategies for bimodal respiration (Graham, 1997), with some species showing a reflex decrease (Smatresk et al., 1986) but others a reflex increase (McKenzie et al., 1991) in f_V. Perry et al. (Perry et al., 2004) reported that the *in vivo* P_{50} for whole blood of this species was approximately 1 kPa (7.7 mmHg). Such a high affinity Hb might explain why the jeju in the current study managed to retain net O_2 uptake from water in very deep hypoxia. Additionally, the jeju did not show any change in mean f_H in hypoxia, with no evidence of the hypoxic bradycardia reported by Oliveira et al. (Oliveira et al., 2004). The reason for this disparity in response is not clear; Farrell (Farrell, 2007) proposed that air-breathing fish should not exhibit hypoxic bradycardia if their air-breathing strategy allowed them to maintain O_2 delivery to the myocardium, but this remains to be tested.

The typical cycling of bradycardia then tachycardia with each AB cycle has been reported previously in *H. unitaeniatus* (Farrell, 1978) and, in the current study, this was associated with a significant increase in HRV in hypoxia. The quantitative relationship between f_{AB} and f_H was in a range similar to that of animals which only utilize air breathing (Akselrod et al., 1981; Campbell et al., 2006a) and, indeed, a relationship where $f_{AB} < f_H/2$ clearly prevailed. This was associated with a pronounced fundamental component in the power spectrum that was coherent with the IBI for air breathing. For the jeju, therefore, the tachycardia and marked decline in HRV during inspiration may have reflected an inhibition of preganglionic neurones (e.g. the baroreflex), while the bradycardia and associated increase in HRV on expiration (immediately prior to surfacing) may have reflected disinhibition of cardiac vagal

tone, in a manner similar to what is known for mammals (Jordan and Spyer, 1987; Taylor et al., 1999). However, the overall HRV pattern observed in the jeju was quite different from the RSA effect as documented for mammals (Akselrod et al., 1981; Giardino et al., 2003) and recently in a reptile (Campbell et al., 2006b). This is because, although there were marked changes in f_H directly associated with the irregular AB events, in the intervals between each event a situation where $f_V > f_H/2$ prevailed, and the HRV pattern was the same as had been observed in the fish relying exclusively upon gill ventilation in normoxia. Indeed, the PSA demonstrated that the jeju in hypoxia still exhibited the same higher frequency peaks that had been observed scattered through the spectrum in normoxia. These peaks are, therefore, entirely independent of any effects of air breathing upon f_H.

The effects of autonomic blockade on air breathing have never been investigated in fish; the absence of any effect of propranolol on f_{AB} indicates that β-adrenergic receptors play no role in regulating these reflexes in hypoxia. On the other hand, the significant decline in f_V following treatment with propranolol has been reported in other fish (McKenzie et al., 1995). The significant decrease in M_{wO_2} following β-adrenergic receptor blockade may have reflected this inhibition of gill ventilation. It is particularly interesting, indeed, that the propranolol abolished the discrete higher frequency components of the HRV signal, as these are qualitatively similar to spectral components that have been causally related to gill ventilation in unimodal water-breathing fish (Campbell et al., 2005; Campbell et al., 2006a). It is tempting to speculate that there may be a causal link between these components of the spectrum and gill ventilation patterns in the jeju, although this clearly requires experimental verification. In any case, it is unusual that the adrenergic system should play a role in the high frequency beat-to-beat oscillation of f_H (Jordan and Spyer, 1987; Taylor et al., 1999).

Respiratory sinus arrhythmias are classed as high frequency oscillations caused by vagal stimulation of the sinus node, with adrenergic tonus being responsible only for low and mid-frequency changes in f_H (Jordan and Spyer, 1987; Taylor et al., 1999). The results of the cholinergic blockade revealed that inhibitory vagal tone was indeed responsible for the greatest proportion of the HRV in the jeju, as is the case for mammals and fish (Jordan and Spyer, 1987; Taylor et al., 1999; Campbell et al., 2004; Campbell et al., 2005), and that this inhibitory tonus modulated the greatest part of the changes in f_H around each AB event. Vagal tone in the jeju was very high by comparison with most unimodal water-breathing fish species (Altimiras et al., 1997). The jeju, however, exhibited a very unusual pattern in autonomic regulation because the adrenergic and cholinergic components appeared to work in concert rather than antagonistically. That is, the bradycardia prior to each AB was associated with a pronounced increase in HRV that was caused by a simultaneous rise in both adrenergic and cholinergic tone. Following the AB, the tachycardia was associated with a drop in cholinergic and adrenergic tone and a severe reduction in HRV. In all fish studied to date, and in the vast majority of all other vertebrates, the parasympathetic and sympathetic branches of the autonomic nervous system work reciprocally, and thus their effects are compounded (Taylor et al., 1999). The only exception of which the authors are aware is the bearded lizard (*Pogona barbata*) (Seebacher and Franklin, 2001). It is not clear whether this apparently paradoxical pattern of autonomic regulation in the jeju might also be found in other fish species with bimodal respiration. Furthermore, given the existence of this profound but unusual autonomic control of the heart in the jeju, and its clear relationship with air breathing, it is perhaps somewhat surprising that pharmacological blockade of all HRV had no effect whatsoever upon f_{AB} and the regulation of routine metabolism by air breathing in deep hypoxia.

It is possible that the changes in f_H that accompany air breathing might facilitate oxygen uptake from the ABO under conditions where metabolic demand is high, for example during aerobic exercise (Farmer and Jackson, 1998). There is also a large behavioural component to surfacing and gulping air, with fear of predation being a significant factor that can influence patterns of surfacing activity in air-breathing fish (Smith and Kramer, 1986). Fear of predation can also cause a reflex bradycardia in fish, and this can influence the timing and intensity of cardiac chemoreflexes during surfacing behaviours (Shingles et al., 2005). Thus, it is conceivable that some element of the changes in f_H during air breathing might reflect higher order behavioural inputs linked with approaching the water surface.

In conclusion, the jeju exhibits HRV patterns that are qualitatively similar to those of both water-breathing and air-breathing vertebrates, and these patterns may be controlled independently of each other. The expiration bradycardia and the inspiration tachycardia with each AB are qualitatively similar to mammalian RSA, but after each AB the fish quickly return to the HRV pattern of a water-breathing fish. The adrenergic and cholinergic tone rose and fell together around each AB event, which is very unusual and appears paradoxical. Nonetheless, pharmacological abolition of all these HRV patterns was without effect upon f_{AB} or the efficacy with which the jeju regulated metabolism by air breathing in deep hypoxia. This may indicate that the HRV patterns associated with air breathing do not function to facilitate O_2 uptake from the air-breathing organ. It should be kept in mind, however, that it is very difficult to detect the role of RSA in optimizing gas exchange in mammals (Giardino et al., 2003), and so this is an interesting area for future research in air-breathing fish.

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