SHORT COMMUNICATION

PERIODIC AIR-BREATHING BEHAVIOUR IN A PRIMITIVE FISH REVEALED BY SPECTRAL ANALYSIS

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The ventilatory patterns of air-breathing fish are commonly described as 'arrhythmic' or 'irregular' because the variable periods of breath-holding are punctuated by seemingly unpredictable air-breathing events (see Shelton *et al.* 1986). This apparent arrhythmicity contrasts with the perceived periodism or regularity in the gill ventilation patterns of some fish and with lung ventilation in birds and mammals. In this sense, periodism refers to behaviour that occurs with a definite, recurring interval (Bendat and Piersol, 1986). The characterisation of aerial ventilation patterns in fish as 'aperiodic' has been generally accepted on the basis of qualitative examination and it remains to be validated with rigorous testing.

The bowfin, *Amia calva* (L.), is a primitive air-breathing fish that makes intermittent excursions to the air–water interface to gulp air, which is transferred to its well-vascularized gas bladder. Its phylogenetic position as the only extant member of the sister lineage of modern teleosts affords a unique opportunity to examine the evolution of aerial ventilation and provides a model for the examination of ventilatory patterns in primitive fishes. To establish whether *Amia calva* exhibit a particular pattern of air-breathing, we examined time series records of aerial ventilations from undisturbed fish over long periods (8 h). These records were the same as those used to calculate average ventilation intervals under a variety of experimental conditions (Hedrick and Jones, 1993). Their study also reported the occurrence of two distinct breath types. Type I breaths were characterised by an exhalation followed by an inhalation, whereas type II breaths were

characterised by inhalation only. It was also hypothesized that the type I breaths were employed to meet oxygen demands, whereas the type II breaths were used to regulate gas bladder volume. However, they did not investigate the potential presence of a periodic ventilatory pattern. We now report the results of just such an analysis of ventilatory pattern that demonstrates a clear periodism to air-breathing in a primitive fish.

Time series data were collected for the duration of an 8 h video tape from each of six fish. Animals were exposed to conditions of aquatic normoxia (aquatic P_{O_2} ranged from 18.6 to 21.3 kPa) and aquatic hypoxia (aquatic $P_{O_2}=7.3\pm0.9$ kPa) (mean $\pm$ 95 % confidence interval). Under both conditions, animals were allowed access to aerial normoxia. Water temperature ranged from 20 to 24 °C but did not vary by more than 1.0 °C during a single 8 h period. The details of animal husbandry and conditions for data collection are reported elsewhere (Hedrick and Jones, 1993). Each 480 min time series consisted of 1 min bins within which a fish either ventilated or did not. This time series therefore manifests a point process and the spectral analysis of such data requires special conditioning (DeBoer *et al.* 1984). A value of zero was assigned to each 1 min bin that did not contain a breath, and a delta (δ) function with an arbitrary value of -4 was assigned to each bin that contained a breath. Because the value of δ does not influence the distribution of harmonics in the frequency domain, but rather the amplitude of those harmonics, the choice of δ is arbitrary. The resulting time series, consisting of zeros and δs, has arbitrarily sharp transitions and, therefore, the frequency domain representation contains large amounts of energy in high-frequency harmonics that are a consequence of the shape of the defined waveform and not a harmonic feature of the ventilatory pattern. For this reason, the time series were filtered with a smoothing algorithm that produces a low-pass filter before being transformed into the frequency domain. To prevent excessive attenuation in frequency bands that might include important biological information, different degrees of smoothing were applied to the normoxic and hypoxic data sets. The -3 dB cut-off point of the effective filter applied to the normoxic data occurred at a frequency of 0.082 cycles min^{-1}, which corresponds to a harmonic signal with a period of approximately 12.2 min. The -3 dB cut-off of the effective filter applied to the hypoxic data occurred at a frequency of 0.188 cycles min^{-1}, or a period of approximately 5.3 min. An estimate of the autocorrelation function was made (Statgraphics+, Manugistics, Rockville, MD, USA) and the time series were then transformed into the frequency domain with a Fast Fourier Transform (Graphtool, 3-D Visions Corp., Torrance, CA, USA). The sample length produced a 1.9×10^{-3} cycles min^{-1} bandwidth resolution in all of the estimated spectra.

Anticipating that a rhythmic pattern might be present in only one of the breath types, we created two sets of time series data. One set encoded all of the ventilatory events equally and the other only contained the type I breaths.

Fig. 1 is a representative sample of a ventilatory event time series and its power spectrum where both type I and type II breaths are represented by the same δ regardless of type. These data are for a fish exposed to aquatic normoxia. Inspection of the time series indeed suggests that the breaths are occurring irregularly or aperiodically, as has been asserted previously (Milsom, 1988; Smatresk, 1990). This description is supported by the apparently disorganized distribution of harmonics in the spectrum and by the failure of
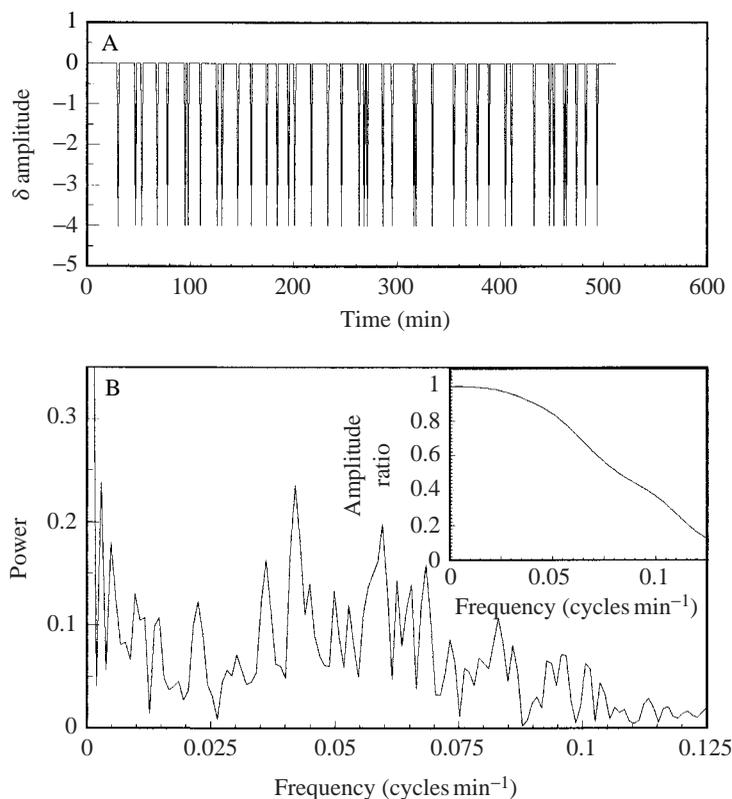
Fig. 1. (A) Unsmoothed time series of both type I and type II breath occurrence for a fish exposed to aquatic and aerial normoxia recorded for the duration of one 8 h video tape. In all cases, the data were zero-filled to produce a radix-2 length array to facilitate the application of a Fast Fourier Transform. Events were scored as −4 for each minute within which a breath occurred and as 0 for no breath. These data are smoothed prior to transformation into the frequency domain to eliminate high-frequency components that are related to the arbitrary shape of the waveform rather than to the pattern of breathing events (see text). (B) Power spectrum of the time series shown in A. The inset shows the shape of the filter function applied to the time series data by the smoothing operation. The smoothing operation was performed once in the forward direction with respect to time and repeated in the reverse direction to minimize phase shifts in the time series. The shape of the filter function was estimated by producing a time series that was the realisation of a white noise process and smoothing it with the same algorithm used for the air-breathing time series. The amplitude ratio on the ordinate of the inset is the ratio of the output (i.e. smoothed data) to the input (unsmoothed data) spectrum of the white noise time series. The −3 dB cut-off of this effective filter occurred at a frequency of 0.082 cycles min^{-1}.

the autocorrelation function to achieve statistical significance at any lag (r=0.150, s.e.m.=0.106, P>0.05; at a lag of 18 min, the correlation coefficient most closely approached significance). Power is distributed over a range of harmonics from 0.035 to
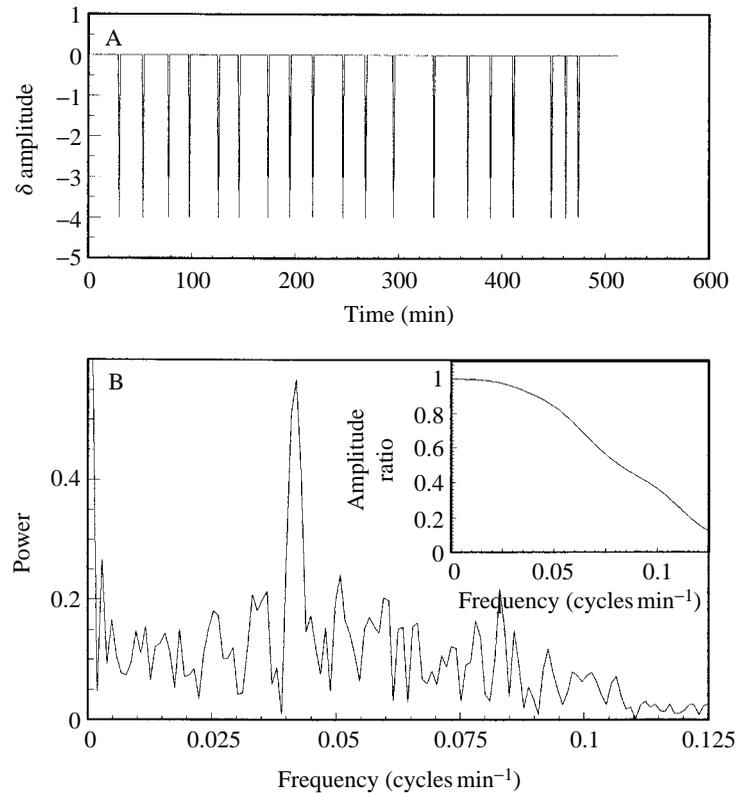
Fig. 2. (A) Unsmoothed time series of type I breath occurrence for the same data set presented in Fig. 1. These data demonstrate the role of type I breaths in manifesting the periodic pattern of ventilation that is obscured by the more irregular occurrence of type II breaths. (B) Power spectrum of the time series shown in A. The periodic nature of the air-breathing pattern is revealed by the clear dominant peak in the spectrum that occurs at a frequency of $0.042\,\mathrm{cycles\,min^{-1}}$. This indicates that a type I breath occurs approximately every 23.8 min. The inset shows the filter function applied to the time series data by the smoothing operation. The filter is the same as that shown in Fig. 1.

$0.075\,\mathrm{cycles\,min^{-1}}$, which correspond to periods of 13.3–28.6 min, respectively, with no clear dominant frequency contributing to the ventilatory time series.

In Fig. 2, the time series is the same as that in Fig. 1, but the type II breaths have been removed. This produces a time series with a much more regular appearance. The periodic nature of these ventilatory events is demonstrated by the dominant peak in the power spectrum that occurs at a frequency of $0.042\,\mathrm{cycles\,min^{-1}}$, which corresponds to a periodic ventilatory interval of 23.8 min. The arbitrary nature of the δ function makes it difficult to establish statistical significance for the magnitude of the peak in the spectrum in Fig. 2, but the observation that over 36 % of the total power in all harmonics resided under this peak is a strong indication that the pattern of type I breaths was indeed periodic. The autocorrelation function also indicated that the perceived periodism in the
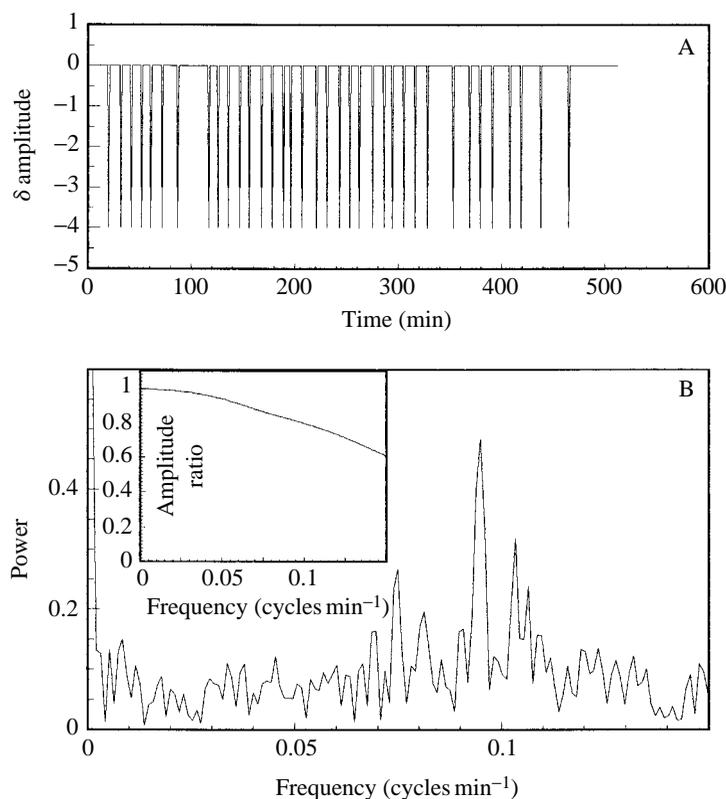
Fig. 3. (A) Unsmoothed time series of type I breath occurrence for a representative fish exposed to aquatic hypoxia and aerial normoxia. (B) Power spectrum of the time series shown in A. This spectrum shows a dominant periodism that occurs at a frequency of 0.095 cycles min^{-1}, indicating that this fish takes a type I breath approximately every 10.5 min under these conditions. The inset shows the shape of the filter function applied to the time series data by the smoothing operation. In this case, the filter cut-off was shifted to a high frequency to reduce attenuation at frequencies that contained physiological information (-3 dB at 0.188 cycles min^{-1}).

spectrum was significant ($r=0.353$, s.e.m.$=0.127$, $P<0.05$).

The response of the animals to aquatic hypoxia is demonstrated by the time series of type I breaths and the associated power spectrum shown in Fig. 3. Inspection of these data suggests that while this ventilatory pattern is somewhat more irregular than the normoxic pattern there is still a strong periodicity. This assessment is supported by a large peak in the spectrum at a frequency of 0.095 cycles min^{-1}, which corresponds to a period of 10.5 min. The increased variability of the hypoxic data is demonstrated by this harmonic, which contains a lower proportion of the total power in all harmonics (17%) than did the dominant harmonic in the normoxic data. In any case, the presence of a large peak in the power spectrum and the statistically significant value of the autocorrelation function

(r=0.270, s.e.m.=0.096, P<0.05) indicate that the pattern of type I breaths in hypoxic conditions is periodic.

Similar time series analysis of the pattern of type I breaths for all of the fish studied produced a dominant harmonic with a mean frequency of 30.5±8.67 min (s.e.m.) during normoxia. Under conditions of aquatic hypoxia, the dominant frequency of type I breaths shifted to a mean of 15.6±4.66 min.

These results show that aerial ventilation in *Amia calva* is indeed periodic and that this periodism is demonstrated by the type I breaths. This is the first study to demonstrate periodic aerial ventilation in any air-breathing fish. This finding is made possible by the use of relatively long-term observations. Indeed, the finding that the primary periodic component of the type I air-breath time series was approximately 30 min indicates that an observer would have to record ventilation for several hours to identify a periodic character in the presence of variations in behaviour. The characterisation of the pattern as periodic is an important finding for two reasons. First, the use of words such as 'periodic' or 'arrhythmic' to describe a given time series in the absence of rigorous characterisation can impart an ambiguous understanding with respect to both the pattern demonstrated in the time series and any underlying mechanisms that may be operating to generate that time series. Second, our observations that the air-breaths of *Amia calva* are periodic suggests that there is a physiological determinism at work that produces this pattern and that is itself periodic.

Air-breathing in *Amia calva* may be classified as periodic behaviour, along with gill ventilation in some fish and lung ventilation in birds and mammals. Does this imply that similar mechanisms are operating in all of these systems? There is experimental evidence that gill ventilation in fish and breathing in mammals have an intrinsic periodism that arises from a central rhythm generator (CRG) located in the brain stem (Rovainen, 1974; Suzue, 1984; Richter *et al.* 1986; Smith *et al.* 1991). Current findings indicate that aerial ventilation in fish occurs in response to changes in blood and/or aquatic P_{O_2}. This evidence has led to the conclusion that aerial ventilation in fish is an 'on-demand' response and has resulted in the characterisation of the breathing pattern as arrhythmic (Smatresk, 1990). If the pattern of ventilation is periodic, and the behaviour arises from peripheral feedback from oxygen-sensitive chemoreceptors, then we might hypothesize either that the aerial ventilation pattern in *Amia calva* is dependent on an unrecognized CRG or that there is periodic fluctuation in peripheral chemoreceptor activity. In the light of the available experimental evidence (Shelton *et al.* 1986; Burleson and Milsom, 1993), we believe the most reasonable conclusion is that periodic fluctuations in peripheral feedback are responsible for the pattern of aerial ventilation. Therefore, the periodism that is manifest in the ventilatory pattern of *Amia calva* represents a type of rhythmic ventilatory pattern distinct from those that arise from a CRG.

Given that the aquatic P_{O_2} remained constant for the duration of each experiment, we can conclude that fluctuations in the P_{O_2} of the blood compartment led to the observed breathing pattern. How do periodic fluctuations arise? It seems obvious that, during the breath-holding period, blood P_{O_2} will fall from a locally high level following the preceding breath as oxygen diffuses from the gas bladder into the pulmonary blood, which subsequently admixes with the venous return (see Johansen *et al.* 1970). The

presence of a minimum blood P_{O_2} threshold that, when reached, triggers a type I air-breath could provide a mechanism for refilling the gas bladder and raising the blood P_{O_2} back above the threshold; in effect, a 'reset' in blood P_{O_2}. The presence of a threshold for triggering type I breaths is supported by experimental data showing that these breaths were not initiated in fish exposed to aerial hyperoxia (Hedrick and Jones, 1993). This type of oscillation mechanism is a variety of relaxation oscillator (see Winfree, 1990) known as an 'integrate and fire' model (Glass and Mackey, 1988) that can produce periodic behaviour. With this mechanism, any change that alters the time constant of the fall in blood P_{O_2} following an air-breath will change the inter-breath interval, and this is precisely what is observed in experimentally induced hypoxia (Fig. 3).

The presence of both breath types in our initial analysis obscured the fundamental periodic nature of the type I breaths (Fig. 1), so the type II breaths were subsequently removed from the data sets before further analysis. If we invoke an integrate and fire model for the type I breaths and observe periodicity (a similar mechanism has been hypothesized to account for the volume regulation function of type II breaths, Hedrick and Jones, 1993), then why do the type II breaths not appear periodic as well? We believe the answer to be that there is a certain degree of variability, or noise, in the volumes captured in each breath regardless of type, introducing a variability in the degree of 'reset' following a breath. It has been observed that, during the transfer of captured air from the buccal cavity to the gas bladder, some unknown volume is lost from the operculum (Hedrick and Jones, 1993). It is this loss of captured air that we feel is likely to provide a source of variability in the size of the breath. Because type II breaths are smaller in volume, they may be subject to greater relative variability and, therefore, greater variability in inter-breath interval.

We have invoked an integrate and fire model for the control of aerial ventilation in *Amia calva* and have explained how loss of air volume during inhalation and transfer may explain some of the variability in our data, but this is not the only possible explanation for the observed pattern. The data presented here do not allow us to distinguish between the linear model we have used to analyse our data or a non-linear model that produces similar periodicity or quasi-periodicity (Winfree, 1990). Various embeddings of the air-breath intervals in two-dimensional space produced no clear geometric structure suggestive of anything more complicated than the linear model we have invoked in this paper (Casdagli *et al.* 1991). However, our sensitivity is low for a non-linear approach because of the relatively small amount of data collected over the 8h period for this type of analysis. Therefore, it may prove instructive in the future to perform this type of undisturbed recording of ventilation over longer periods in order to examine the possibility that more complicated, non-linear mechanisms are at work in the control of ventilatory patterns in these fish.

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