VENTILATORY BEHAVIORS OF THE TOAD *Bufo marinus* REVEALED BY COHERENCE ANALYSIS

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ABSTRACT

Breathing in amphibians is a remarkably complex behavior consisting of irregular breaths that may be taken singly or in bouts that are used to deflate and inflate the lungs. The valves at the two outlets of the buccal cavity (nares and glottis) need to be finely controlled throughout the bout for the expression of these complex respiratory behaviors. In this study, we use a technique based on the calculation of the coherence spectra between respiratory variables (buccal pressure; narial airflow; and lung pressure). Coherence was also used to quantify the effects of chemoreceptor and pulmonary mechanoreceptor input on narial and glottal valve behavior on normoxic, hypoxic, and hypercapnic toads with both intact and bilaterally sectioned pulmonary vagi. We found a significant reduction in narial coherence in hypoxic vagotomized toads indicating that pulmonary mechanoreceptor feedback modulates narial opening duration. An unexpectedly high coherence between P_l and P_b during non-respiratory buccal oscillations in hypercapnic toads indicated more forceful use of the buccal pump. We concluded that the coherence function reveals behaviors that are not apparent through visual inspection of ventilatory time series.

Key words: ventilatory control, respiratory physiology, *Bufo marinus*, time series analysis, amphibian.

RESUMO

Padrões respiratórios do anfíbio *Bufo marinus* revelados por análise de coerência

A dinâmica respiratória em anfíbios apresenta um padrão complexo e irregular de episódios respiratórios isolados ou em cadeia utilizados para inflar ou desinflar os pulmões. A dinâmica respiratória depende do controle fino e coordenado das duas saídas da cavidade bucal (glote e válvulas nasais). Neste estudo, utilizamos análise de coerência, uma técnica de análise spectral, para analisar o espectro de coerência entre três variáveis respiratórias: pressão bucal, fluxo de ar nasal e pressão pulmonar. Também quantificamos os efeitos do quimiorreceptor e do mecanorreceptor pulmonar no comportamento da glote e das válvulas nasais em sapos normóxicos, hipóxicos e hipercápnicos com os ramos pulmonares do nervo vago intactos e bilateralmente seccionados. Esta análise revela comportamentos respiratórios não aparentes pelo método tradicional de inspeção visual dos dados. Por exemplo, uma redução significativa da coerência nasal em sapos hipóxicos vagotomizados foi observada, sugerindo a existência de retroalimentação do mecanorreceptor pulmonar que modula a duração da abertura nasal. Uma coerência surpreendentemente alta entre P_l e P_b durante a hipercapnia indica uso mais intenso da bomba bucal. Concluímos que a utilização da função de coerência revela comportamentos não distinguíveis durante a inspeção visual das séries temporais ventilatórias.

Palavras-chave: controle respiratório, fisiologia respiratória, *Bufo marinus*, anfíbio, séries temporais.

INTRODUCTION

Breathing in amphibians is a remarkably complex behavior consisting of irregular breaths that may be taken singly or in bouts that are used to deflate and inflate the lungs. Control over lung inflation and deflation resides in the coordination of a positive pressure buccal pump and a pair of valves. The nares act as a valve controlling the flow of air between the buccal cavity and the atmosphere and the glottal valve controls the flow of air between the buccal cavity and the lungs (De Jongh & Gans, 1969). Buccal movements can be further characterized as respiratory or non-respiratory. Non-respiratory buccal movements are commonly observed as a long series of low amplitude buccal movements that tidally ventilate the buccal cavity, while the glottal valve is kept closed. These buccal oscillations can be interspersed by periods of inactivity or pulmonary ventilation. During pulmonary ventilation the air inside the lungs is partially replaced. A typical single breath begins with the opening of the glottal valve, which causes an initial passive expansion of the buccal cavity. This partially empties the lung and is followed by an active expansion phase in which fresh air is also drawn in through the narial valves. Compression of the buccal cavity with subsequent closure of the narial valves follows this initial phase and fills the lungs. The relative timing of valve opening and closure determines whether the breath deflates, inflates or maintains lung volume. During longer bouts of breathing, animals can combine inflation, deflation and balanced (no net change in lung volume) breaths to better wash out or exchange gases.

The valves at the two outlets of the buccal cavity (nares and glottis) need to be finely controlled throughout the bout for the expression of these complex respiratory behaviors. During single breath and bout breathing patterns, afferent feedback helps to coordinate the behavior of the buccal pump and valves to achieve effective gas exchange under a variety of ambient conditions (Baker & Smatresk, 1997). This sensory feedback comes from peripheral and central chemoreceptors, lung mechanoreceptors, and olfactory signals (assessing air quality).

Traditionally, breathing mechanics in these animals has been described by visual inspection of long time series of data with qualitative or statistical summaries of the valving behavior based on repeated measures of specific breaths (Baker & Smatresk, 1997). These analyses are most useful when describing unequivocal behaviors like the time a valve opens or closes in relation to the state of contraction of the buccal cavity. However, they are tedious, somewhat arbitrary, and cannot adequately characterize indeterminate behaviors, like the effects of incomplete valve closure. Thus, traditional techniques may not reveal subtle but important differences in mechanics arising from varying afferent input. Frequency domain analysis, on the other hand, may be readily applied to physiological time series, and is able to expose features of the signal not easily derived in the time domain. For example, recent application of these techniques suggests that heart rate and respiratory rate variability is diminished by the removal of control components and during some pathological conditions (Jacob *et al.*, 1995; Leung & Mason, 1996). These techniques have also been used to assess the interplay of sympathetic and parasympathetic control of the heart (Altimiras *et al.*, 1995; Oidas *et al.*, 1997; Pola *et al.*, 1996; Yamamoto *et al.*, 1995). Relatively little effort has been directed towards using these tools to assess other aspects of physiological time series and interactions between system components.

It has long been appreciated that amphibians challenged by hypoxia or hypercapnia breath faster and more forcefully, as judged by narial airflow or buccal pressure excursions (Macintyre & Toews, 1976; Smatresk & Smits, 1991; Wang *et al.*, 1994; Kinkead & Milsom, 1996). Neither method, however, is a reliable estimate of ventilatory airflow or mechanics. Pneumotachography alone measures airflow in and out the nares but does not distinguish between respiratory and non-respiratory airflow. Buccal pressure excursions, on the other hand, reveal the overall pattern of breathing and whether an animal is taking inflation, deflation or balanced breaths, but does not provide airflow or valving information. The behavior of the complex motor synergies of amphibian breathing can only be revealed with the mechanics measurements done in the current study, or by EMG measurements combined with flow (volume) or pressure information. Studies on toad ventilatory mechanics have shown significant differences in the roles of central chemoreceptors, peripheral chemoreceptors and pulmonary mechanoreceptors in the control of breathing pattern and timing of the glottal and narial valves (Taglietti & Casella, 1968; Jones, 1982; Baker & Smatresk, 1997), but cannot distinguish more subtle aspects of valve behavior.

In this study, we use the coherence function, derived from spectral analysis, to assess ventilatory mechanics in the toad *Bufo marinus*. The coherence function amounts to a cross-correlation performed in the frequency domain. It is a natural tool for the study of oscillatory variables (buccal and lung pressures, and airflow through the nares) that share a causal relationship. The purpose of the study was to further investigate the interplay between chemo and mechanoreceptor feedback in the control of glottal and narial valves' timing and overall pattern of operation. This afferent controlled motor pattern is a major contributor to the anuran overall ventilatory pattern. To achieve this goal, we subjected vagotomized (pulmonary vagi sectioned) and sham-operated toads to hypoxia and hypercapnia while monitoring their ventilatory variables. In this fashion we could separate the effects of mechano (lung stretch receptors) and chemoafferent signals on the ventilatory pattern.

We believe that the use of a more objective and discriminative analytical tool helped us unveil facts about the afferent modulation of the behavior of narial and glottal valves that would otherwise be hidden or unquantifiable against the background of the overall ventilatory pattern.

METHODS

The toads used in this study (*Bufo marinus*) were obtained from a commercial supplier (Sullivan Co., TN) and kept in large fiber glass tanks with unrestricted access to water on a 12/12 ligth/dark cycle. They were fed crickets twice a week. After arrival on the lab the animals were kept under observation for a couple of weeks to check for any abnormalities regarding their health status. During this period the animals did not display any reproductive behaviors. Adult animals (211-580 g) of both sexes were selected randomly for the experiments. Animals were anesthetized by immersion in a 0.3% solution of MS-222 (tricaine methanesulfonate) buffered to pH 7.0. A buccal catheter was implanted through the tympanic membrane and the lungs were catheterized with a short length of flexible tubing with numerous side holes as described by Smatresk & Smits (1991). A small incision was made in the axilla, the pulmonary branch of the vagus was identified, and separated from surrounding connective tissue. In the sham-operated group (N = 7) the vagi were manipulated but left intact, in the pulmonary denervated group (N = 7) the nerves were bilaterally sectioned. Lung and buccal pressure catheters (PE-205, Intramedic) were connected to differential pressure transducers (Validyne DP 45). Ventilatory airflow was measured by a pneumotachograph installed in a soft plastic mask that was molded to cover the nares and glued onto the face of each animal with epoxy glue (Loctite Corp.). The pneumotachograph was also connected to a differential pressure transducer (Validyne DP-103). After surgery, animals were allowed to recover for 24 hours. Catheters were then connected and animals were placed in a plastic box (12 x 19 x 13 cm, width x length x height) and allowed 1 to 2 hours to habituate to confinement. All experiments were conducted in a temperature-controlled (24°C) room. After the experiments, females that happened to be selected for the study, had their ovaries examined. Females that had well developed egg masses were discarded from the study.

Lung pressure, buccal pressure and airflow signals were digitized and recorded at 100 Hz per channel using a computerized data acquisition system (Dataq, Windaq-200). Ventilatory variables were recorded continuously for 1 hour of normoxia (20% O_2, 80% N_2), two hours of hypoxia (5% O_2, 95% N_2) followed by a 1-hour normoxic recovery period, and two hours of hypercapnia (4% CO_2, 20% O_2, 76% N_2) for both sham-operated and denervated toads. Gas mixtures were passed through the face mask at a rate of 120 ml/min via a gas-mixing flowmeter (Cameron GF-3).

Data analysis

A simplified diagram of the respiratory mechanism of the toad is shown in Fig. 1. The variables buccal pressure (P_b), lung pressure (P_l) and airflow through the nares (AF) were recorded continuously throughout the experiments. To assess the state of valves we used the coherence function of the variables on each side of the valves (P_b and P_l for the glottal valve, and P_b and AF for the narial valve).

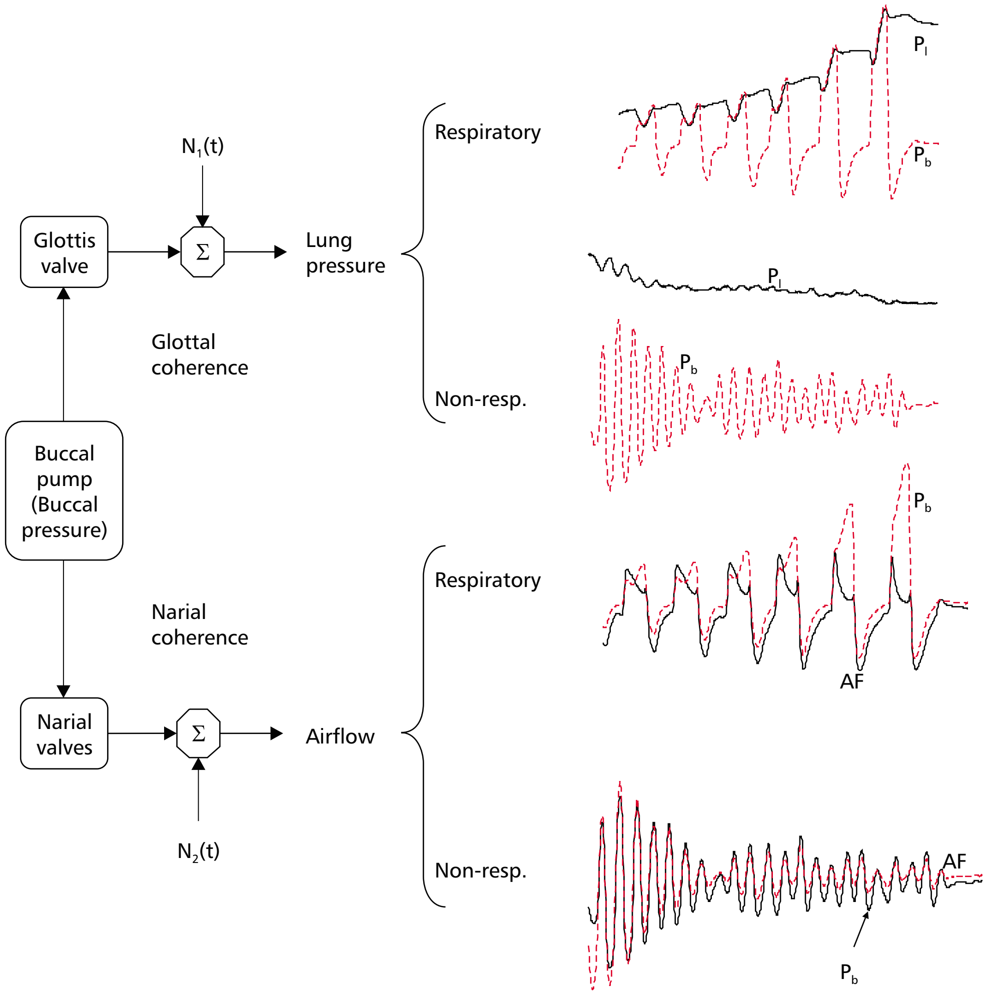

Fig. 1 — Block diagram of a simplified model of the toad respiratory system. The sample traces illustrate pressure variations (or airflow through the nares) in each compartment. Pressures are generated by the buccal pump and transferred to the lung or transformed into narial airflow depending on the states of both glottal and narial valves. The traces shown reflect the output of the system under different regimes of glottal and narial control, during respiratory and non-respiratory movements. Sample traces in the top bracket show only the two variables whose coherence will reflect glottal dynamics: P_b and P_l. Traces in the bottom bracket show the variables whose coherence reflects narial dynamics (P_b and AF). Time scales and amplitudes are not the same among the four sets of traces, although they are the same within each plot. $N_1(t)$ and $N_2(t)$ represent external noise which may diminish coherence.

One typical respiratory bout (inflation bout, sequence five or six increasingly powerful, uninterrupted lung inflations) and one typical non-respiratory bout (buccal pump is operated continuously while the glottis is kept closed) were selected from the continuous recording for each animal (6.4 seconds long, i.e. 640 points per channel at a 100 samples/second) for each gas level, and for each nerve section condition. The size of signal segments was chosen as the longest size of respiratory data that could be consistently extracted from all animals without apneas or NRBOs. The data segments were clipped and imported into MATLAB (data analysis software, Mathworks Inc). The coherence spectra between variables were calculated (see appendix). The coherence spectrum for each data segment is the average of fifty 128-point overlapping Hanning windows. Confidence intervals for the coherence estimates depend on the values of the actual parameter being estimated, decreasing its magnitude as the coherence increases. The confidence intervals for the estimates used in this study were found to be sufficiently small to be irrelevant when compared to the actual variance of the dataset. In other words, the error associated with the estimation of coherence is embedded in the variance of the data. A brief description of the coherence function and how it was implemented and its interpretation in the context of the present analysis can be found in the appendix.

The data presented in Figs. 2 and 3 are the averages of glottal and narial coherences of the seven animals from each group (vagotomized and sham-operated). These averages were compared by one-way MANOVA with significance level set at 0.05. Coherence values compared correspond to 1-2 Hz frequency band (see appendix for further details)

Interpretation of ventilatory coherence

For linear systems, the coherence function can be interpreted as variation in the output (in this case lung pressure or airflow) that is contributed by the input (buccal pressure). If all the variance in the output is contributed by the input, then the coherence will be 1. The sample traces of Fig. 1 illustrate the relationship between the input (P_b, as generated by the buccal pump) and outputs (P_l or AF) during different types of breathing behaviors. If the resistances of the open glottal and narial valves are constant, AF or P_l will be linearly related to the input (P_b), and in the absence of extraneous noise, their coherence should approach unity. Stated more simply, the more similar the two traces are, the higher their coherence will be. In the sample shown in Fig. 1 (lower bracket, non-respiratory), a high coherence (P_b x AF) is seen when the nares are open, the glottis is closed, and the animal is buccal pumping. When the glottis is closed during non-respiratory buccal pumping (as shown in the upper bracket, Non-resp. traces), coherence between P_b and P_l is very low.

In addition to extraneous noise, coherence will fall if the system relating the input and the output becomes non-linear. During pulmonary ventilation (respiratory traces in both brackets), the resistance of both valves varies with time during the short transition between open and closed states. This added nonlinearity reduces the coherence between airflow and buccal pressure from unity, but will yield a coherence value consistent with normal breathing. Any significant deviations from this normal level of coherence would be an indication of altered respiratory behavior. In addition to signaling altered behavior, coherence differences between the glottal and narial valves also provide a measure of directionality of ventilatory airflow.

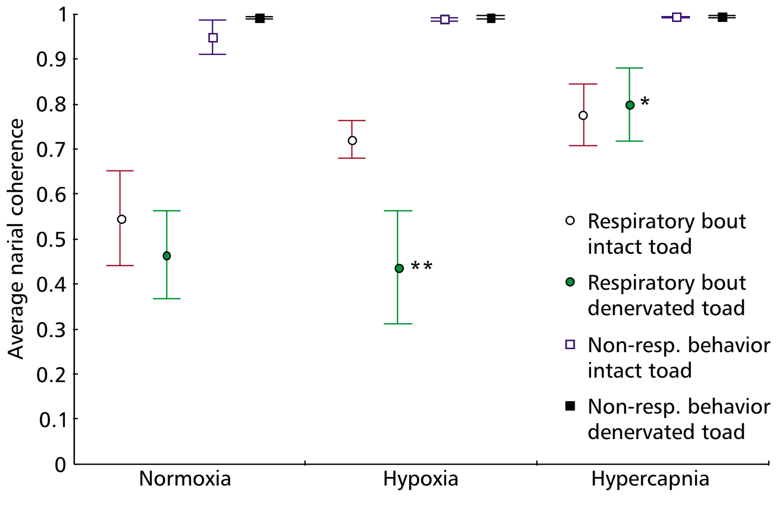

Fig. 2 — Average narial coherences, depicting narial behavior (P_b x AF) under all conditions. Mean ± SE. *: significantly different from normoxia (in this particular case, also from hypoxia). **: significantly different from intact (sham-operated) animal in the same condition.

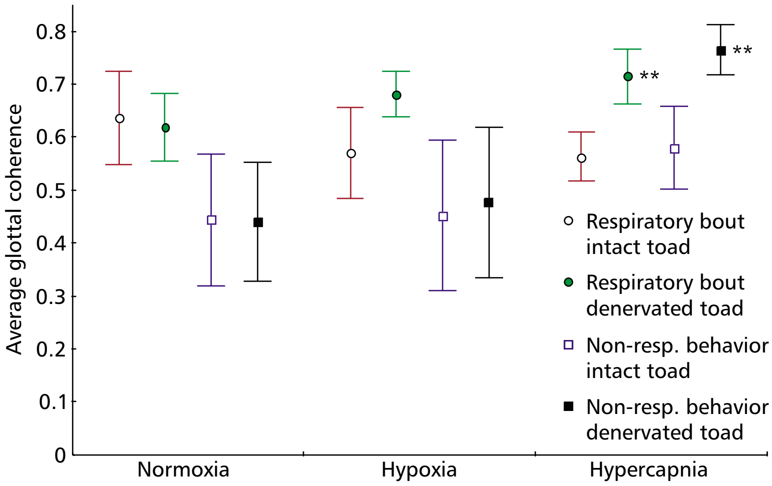

Fig. 3 — Average glottal coherences, depicting glottal behavior (P_b x P_l) under all conditions. Mean ± SE. **: significantly different from intact (sham-operated) animal on the same condition.

This arises because greater airflow though an open valve will diminish airflow through the other outlet. Thus, there should be a reciprocal relationship between glottal and narial airflow and we would expect that the average narial coherence between P_b and AF would fall as gas is directed towards the lungs during pulmonary ventilation (lower bracket, respiratory).

RESULTS

Narial valving (P_b x AF coherence)

As predicted, the coherence between P_b and AF approached unity during non-respiratory buccal oscillations (NRBO), because the nares were kept open while the glottis was closed (Fig. 2). During respiratory breaths the narial and glottal valves opened and closed in a coordinated fashion, and coherence fell, as a result of the dynamic changes in their states. Exposure to hypoxia and hypercapnia increased narial coherence in sham-operated animals, suggesting that the nares remained opened longer when peripheral or central chemoreceptors were stimulated, but this effect was not statistically significant. Elimination of afferent feedback from pulmonary mechanoreceptors had little effect on normoxic or hypercapnic animals, but significantly diminished narial coherence in hypoxic toads (Fig. 2), suggesting a significant interaction between peripheral chemoreceptors and pulmonary mechanoreceptors in control of the narial valve.

Glottal valving (P_b x P_l coherence)

Prior to conducting the study, we predicted that glottal coherence would approach zero during non-respiratory movements when the glottal valve was closed. Interestingly, there was a moderately high coherence between P_b and P_l during NRBOs. Visual inspection revealed that the flanks and lungs of the animals moved synchronously with NRBOs (as shown in Fig. 1, panel 2 of NRBO), which could be significant to animals that breath hold on lung inflation. Respiratory coherence was significantly greater than NRBO coherence for the glottal valve, as expected since the valve opens during pulmonary ventilation. The only exception to this is during hypercapnia where, due to the more forceful operation of the buccal pump, non-respiratory buccal oscillations are transmitted to the lower part of the body mechanically (via body wall). This is the only case where glottal coherence does not reflect gas exchange between the buccal cavity and the lungs. This phenomenon appears to be especially prominent in hypercapnic denervated toads, in which it significantly augmented not only non-respiratory glottal coherence but also respiratory glottal coherence. In the case of respiratory glottal coherence it is possible the increased coherence reflects the combined effects of (previously described) mechanical coupling and longer glottal opening. Hypoxia, hypercapnia or pulmonary denervation per se, however, did not significantly affect glottal valve behavior.

DISCUSSION

The current study contributes two major findings to our understanding of amphibian ventilatory mechanics. First, the significant reduction of narial coherence following vagotomy in hypoxic animals was not predictable from visual inspection of the data, or previous mechanics studies. This loss of coherence suggests that the role of pulmonary mechanoreceptor feedback was amplified during the high amplitude lung inflation cycles of hypoxic animals. The practical result of this may be that when animals are inflating their lungs vigorously due to hypoxic stimulation of peripheral afferent activity, a "full lung" signal results in longer narial opening, thus increasing buccal cavity washout. The reason why these changes in narial dynamics cannot be detected by simple visual inspection is linked to the fact that narial valve dynamics do not consist of two well defined and discrete states, open and closed. Instead, they never fully close, they only increase resistance to the passage of air. Therefore minor modifications on their *modus operandi* can affect significantly the amount of air that is exchanged through them at any given moment without changing the overall shape of the airflow curves. A detailed study based on careful measures of respiratory mechanics might be able to reveal these changes but would require a long, tedious and error-prone breath-by-breath analysis of the traces.

The second phenomenon revealed by this analysis, was the unexpectedly high coherence between lung and buccal pressure during non-respiratory buccal oscillations. That lung pressure clearly oscillated in phase with NRBOs has not been previously noted. Visual inspection of toads confirmed that the flanks of the animals moved whenever the buccal pump was active, despite a closed glottal valve. Previous studies have found rhythmic oscillations in lung pressure even in the absence of buccal movements in gar and salamander lungs (Luckhardt & Carlson, 1920; Azizi & Smatresk, 1986). This phenomenon has been termed lung automatism and arises from pulmonary smooth muscle contractions. It is believed that such movements mix lung air in animals that breathe infrequently, to augment gas exchange. It is possible that the mechanical coupling of NRBOs to lung pressure oscillations serves a similar gas-mixing role in toads. The most direct explanation for the increased coherence during NRBOs in hypercapnia is that buccal oscillations were more forceful during hypercapnia, particularly following vagotomy. On visual inspection of toads in this condition, it becomes clear that the extremely high amplitude oscillations of the buccal pump get transmitted to the lower parts of the body making the animal "sides" oscillate in phase with the buccal cavity. We believe that the pressure waves are transmitted by mechanical action of the buccal pump muscles on the surrounding structures. The fact that the toad's head is completely fused with the lower part of the body also facilitates the transmission of pressure.

The high level of buccal and lung pressure coherence during pulmonary ventilation was not surprising, since glottal opening leads to pressure equalization between the buccal cavity and lung. Glottal valve coherence was not modulated significantly by afferent feedback, however, suggesting that there was relatively little variability in the overall opening time of the glottal valve. The current study assessed coherence only for inflation breaths. Baker & Smatresk (1997) assessed the full spectrum of breathing behaviors, and found that the timing of glottal valve relative to buccal pump activity and opening duration varied in animals taking inflation *vs.* deflation breaths.

In these studies, the coherence function augments conventional analysis of ventilatory mechanics and may reveal behaviors that were not apparent through visual inspection of ventilatory data sets. The technique may also be used to quantify indeterminate states, like partial valve closure. For example, in the current data set it is clear that narial valving is complex and is modulated by afferent activity. Previously the nares were not considered to play an active role in ventilatory mechanics, thus their afferent modulation had not been assessed. Similarly, most investigators working on amphibians have noted that their body wall moves during NRBOs, but the major function of buccal oscillating movements was thought to be for olfaction or washing out buccal cavity gas in preparation for pulmonary ventilation. This analysis focused attention on the mechanical coupling of NRBOs and lung pressure changes, suggesting an additional role for these movements in mixing pulmonary gas. Thus, coherence analysis is not a substitute for conventional ventilatory mechanics studies, but is useful adjunct for revealing the details of valving behaviors. Since amphibian breathing depends on the complex interactions of narial and glottal valving, this tool is certainly one that can contribute to our understanding of ventilatory control in these and other animals under various environmental and physiological or pathological conditions.

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APPENDIX

The coherence between two time series ($\gamma^2_{xy}(f)$) is the Fourier transform of their cross correlation function. This amounts to a frequency-wise comparison of the series spectra by means of their cross spectrum ($S_{xy}(f)$) normalized with respect to the geometric mean of the spectra of the two time series,

$$\hat{g}^2_{xy}(f) = \frac{\left|\hat{S}_{xy}(f)\right|^2}{\hat{S}_{xx}(f)\cdot\hat{S}_{yy}(f)} \qquad (1)$$

Instead of using the whole coherence spectrum, only the frequency components related to the buccal pump's fundamental frequency were considered when comparing different treatments. This pumping rate is highly conserved among animals and varies from 1 to 2 Hz. These values were confirmed by filtering out the 1-2 Hz frequency components from the spectrum and reconstructing the time series with an inverse Fourier transform. The time series thus reconstructed would no longer display the characteristic features associated with respiratory behavior. The average coherence in the buccal pump operating frequency-range (1-2 Hz) for each data segment was calculated, and transformed using Fisher's Z transformation (Naidu, 1996):

$$z = \tanh^{-1}(\gamma^2) \qquad (2)$$

to obtain a normal distribution. The transformed coherences were then compared by one-way MANOVA, with the significance level-set at $p < 0.05$. Specific hypotheses were tested by post-hoc comparisons using Tukey's HSD statistic.

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