

## The basis of vagal efferent control of heart rate in a neotropical fish, the pacu, *Piaractus mesopotamicus*

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### SUMMARY

The role of the parasympathetic nervous system, operating *via* the vagus nerve, in determining heart rate ( $f_H$ ) and cardiorespiratory interactions was investigated in the neotropical fish *Piaractus mesopotamicus*. Motor nuclei of branches of cranial nerves VII, IX and X, supplying respiratory muscles and the heart, have an overlapping distribution in the brainstem, while the Vth motor nucleus is more rostrally located. Respiration-related efferent activity in the cardiac vagus appeared to entrain the heart to ventilation. Peripheral stimulation of the cardiac vagus with short bursts of electrical stimuli entrained the heart at a ratio of 1:1 over a range of frequencies, both below and sometimes above the intrinsic heart rate. Alternatively, at higher bursting frequencies the induced  $f_H$  was slower than the applied stimulus, being recruited by a whole number fraction (1:2 to 1:6) of the stimulus frequency. These effects indicate that respiration-related changes in  $f_H$  in pacu are under direct, beat-to-beat vagal control. Central burst stimulation of respiratory branches of cranial nerves VII, IX and X also entrained the heart, which implies that cardiorespiratory interactions can be generated reflexly. Central stimulation of the Vth cranial nerve was without effect on heart rate, possibly because its central projections do not overlap with cardiac vagal preganglionic neurons in the brainstem. However, bursts of activity recorded from the cardiac vagus were concurrent with bursts in this nerve, suggesting that cardiorespiratory interactions can arise within the CNS, possibly by irradiation from a central respiratory pattern generator, when respiratory drive is high.

Key words: *Piaractus mesopotamicus*, neuranatomy, neurophysiology, cranial nerves, vagus, cardiorespiratory interactions.

### INTRODUCTION

This investigation sought to further our understanding of the mechanisms of vagal control of the heart. In common with other vertebrates, the heart in fish beats at a rate thought to be primarily determined by a cardiac pacemaker, which in turn operates under the influence of a number of intrinsic and extrinsic factors (Axelsson et al., 1987; Taylor, 1992). Predominant among these is tonic inhibitory control imposed by the parasympathetic division of the autonomic nervous system, *via* efferent activity in the vagus nerve (Taylor et al., 1999). This inhibitory tonus is abolished by cardiac vagotomy or injection of the muscarinic cholinergic antagonist atropine (Taylor et al., 1977). An extensive study of the elasmobranch fish *Scyliorhinus canicula* (the dogfish), summarized by Taylor (Taylor, 1992), revealed that the heart operates under an inhibitory vagal tone that varies with temperature and oxygen supply. Recordings from a cardiac branch of the vagus in dogfish revealed high levels of spontaneous efferent activity, which could be attributed to two types of unit (Taylor and Butler, 1982; Barrett and Taylor, 1985a). These units were shown to have separate origins in the CNS (Barrett and Taylor, 1985a; Barrett and Taylor, 1985b). Some units fired sporadically and increased their firing rate during hypoxia. Consequently, we suggested they may initiate reflex changes in heart rate, including a hypoxic bradycardia, as well as playing a role in the determination of the overall level of vagal tone on the heart. Other, typically larger, units fired in rhythmical bursts which were synchronous with ventilatory movements. We hypothesized that these units, showing respiration-related activity, which was unaffected by hypoxia, may serve to synchronize

heartbeat with ventilation (Taylor, 1992). As activity was recorded from the central cut end of the cardiac vagus in the decerebrated, paralysed, force-ventilated dogfish, we reasoned that it must be centrally generated. Accordingly, cardiorespiratory interactions in dogfish could be generated primarily by feed-forward control from the central nervous system (Taylor et al., 1999). However, in the intact, spontaneously breathing fish, this relationship may be reinforced by rhythmical stimulation of branchial mechanoreceptors (Taylor, 1992).

Available evidence suggests that cardiorespiratory interactions in teleost fish are predominantly under reflex control. When exposed to hypoxia, the rainbow trout, *Oncorhynchus mykiss*, showed a developing hyperventilation and bradycardia. As heart rate ( $f_H$ ) was higher than ventilation rate ( $f_R$ ) in normoxia, these changes resulted in convergence to a 1:1 ratio, synchronizing these rhythms. Both the bradycardia and the cardiorespiratory synchrony were abolished by atropine injection (Randall and Smith, 1967). These authors also demonstrated 1:1 synchrony between the heartbeat and pulses of water delivered by forced ventilation of the gills, independent of the intrinsic respiratory rhythm. This is a phenomenon clearly generated by reflex pathways. Randall (Randall, 1966) recorded bursting activity from the cardiac vagus of *Tinca tinca* and concluded that it was the efferent arm of reflex control of heart rate, generating both a hypoxic bradycardia and synchrony between heartbeat and ventilation. These results reveal an apparent fundamental dichotomy in our understanding of the genesis of cardiorespiratory synchrony in fishes. In elasmobranchs it seems to be generated primarily by central interactions in resting normoxic or hyperoxic fish, when the

overall vagal tone is relatively low. In teleosts the synchrony occurs in moderate hypoxia and it is apparently generated by reflex pathways when the vagal tone to the heart is relatively high (Taylor, 1992; Taylor et al., 1999). However, extant studies are limited to very few species and follow up studies are only available for the elasmobranchs (Taylor et al., 2006).

Evidence that bursts of respiration-related, efferent activity in the cardiac vagus could entrain the heart was obtained by electrical stimulation of the peripheral cut end of the cardiac branch of the vagus in *S. canicula* (Taylor et al., 2006). Although continuous stimulation of the cardiac vagus caused a bradycardia or even cardiac arrest, burst stimulation of the same nerve entrained the heart rate to imposed rates below or even slightly higher than the intrinsic  $f_H$  (Young et al., 1993b; Taylor et al., 2006). Similar data have been obtained from mammals (e.g. Levy et al., 1969; Levy et al., 1972; Pokvroskii, 1984), but are lacking for teleost fish.

The neotropical teleost fish the pacu, *Piaractus mesopotamicus*, shows episodic breathing while in normoxia. This pattern is characterized by sequences of ventilatory cycles of varying amplitude separated by respiratory pauses (Leite et al., 2007). In normoxia the overall ventilation rate was similar to but slightly lower than the heart rate. In response to progressive hypoxia pacu showed increases in the frequency and amplitude of ventilation and a marked bradycardia (Leite et al., 2007), a response pattern that is typical for teleosts (Hughes and Shelton, 1962; Taylor, 1992). However, re-examination of the data provided by Leite and colleagues (Leite et al., 2007) revealed that before the development of a significant decrease in  $f_H$ , at an oxygen tension of 70 mmHg, the relationship between  $f_H$  and  $f_R$  became exactly 1:1, with very little variability in the data from 12 fish. The development of this relationship entailed an increase in  $f_H$  as well as  $f_R$ , despite the fact that the typical cardiac chronotropic response to hypoxia in fish, including pacu, is a developing bradycardia (Taylor, 1992). A follow-up study revealed that spontaneous efferent activity recorded from the cardiac vagus of pacu contained respiration-related activity in moderate hypoxia but not in normoxia, suggesting that it was generated by reflex pathways (C.A.C.L., E.W.T. and F.T.R., unpublished observations). These data raise some interesting questions regarding the nature of cardiac control in this fish. The aim of this investigation was to clarify the role of the vagus in efferent control of  $f_H$  in *P. mesopotamicus*, as a contribution to furthering our overall understanding of this relationship in vertebrates.

## MATERIALS AND METHODS

### Animals

Adult pacu, *Piaractus mesopotamicus* Holmberg 1887, of either sex (29 fish, mass  $490 \pm 75$  g) were obtained from the Pinhal fish farm, São Carlos, and from the Tropical Fish Research Centre (CEPTA/IBAMA), Pirassununga, SP, Brazil. They were maintained in tanks of aerated water at 25°C, in the animal holding facility of the Laboratory of Zoophysiology and Comparative Biochemistry, Department of Physiological Sciences, Federal University of São Carlos for at least 3 weeks, prior to experimentation. They were fed each day, *ad libitum*, until 48 h before an experiment, when food was withdrawn. The present project was carried out in accordance with the regulations of the Brazilian College of Animal Experimentation (COBEA) and was duly approved by the Ethical Committee on Animal Experimentation, Federal University of São Carlos.

### Series 1: neuranatomy

To map the distribution of neurons supplying the heart and respiratory system, nine fish had the appropriate branches of cranial nerves V,

VII, IX and X injected with a neural tracer. For the injection, the animals were anaesthetized with benzocaine ( $0.1 \text{ g l}^{-1}$ ) and transferred to a surgical table where they were artificially ventilated with a second aerated solution of the same anaesthetic ( $0.05 \text{ g l}^{-1}$ ). A binocular operating microscope (Opto SM 2001, Electronic Opto, São Carlos, SP, Brazil) was used to trace and identify selected branches of cranial nerves involved in respiratory control. Access to each nerve was as follows. The mandibular branch of the trigeminal, the Vth cranial nerve that innervates the jaws, was exposed by an incision (5 mm) at the edge of the ocular orbit, caudal to the eyeball; the opercular branch of the VIIth cranial nerve was exposed by an incision on the internal face of the operculum, at its dorso-rostral insertion on the body wall; the branchial branch of the IXth, as well as the 1st, 2nd and 3rd branchial branches of the Xth cranial nerves, which innervate the gill arches, were exposed *via* an incision at the point where the first and second gill arches join the roof of the opercular cavity. An incision made at the caudal edge of the gill chamber along its line of contact with the operculum, at the level of the medial point of the 4th branchial arch, exposed the point at which the 4th branch of the vagus divided into its respiratory and cardiac branches. A local anaesthetic (2% lidocaine; Pearson, BR, Pilot Point, TX, USA) was injected at each incision site in order to reduce the post-surgical stress.

After positive identification each nerve branch was cleared of connective tissue and then injected with 2–6  $\mu\text{l}$  of a neural tracer (Fluorogold or True Blue, Sigma, St Louis, MO, USA) as a solution in deionized water, delivered from a Hamilton syringe with the needle point inserted through the nerve sheath. The nerve was then pinched with fine forceps at the injection site to damage axons, as this promotes uptake of the tracer. The nerves, however, were not sectioned. Tracers were injected into branches of two nerves on different sides of each fish, which was then fitted with an identifying tag and recovered by irrigation of the gills with aerated water before placement in a holding tank. After recovery for 3 weeks at 25°C to enable transport of the tracer to the cell bodies of neurons supplying the injected nerve branch, each fish was terminally anaesthetized and the ventral aorta was perfused with heparinized 0.9% saline then with a 4% solution of formaldehyde in saline, buffered to pH 7.3. The brain was removed and stored in buffered fixative for 3 days before being placed in a 20% solution of sucrose in buffered saline overnight. Each brain was then frozen and sectioned (transverse sections of  $40 \mu\text{m}$ ) on a cryostat (Microm/Zeiss HM 505 E, Germany). The serial sections were mounted on gelatine-coated slides and cover-slipped in a solution of glycerine. Sections were examined under a photomicroscope (Olympus BX50 illuminator UV U-ULH, Tokyo, Japan) equipped with UV epi-illumination and a video camera attached to an image analysis system (Image-Pro Plus, Bethesda, MD, USA), enabling the images of fluorescing neuron cell bodies to be captured.

Cell bodies of labelled neurons were counted and mapped according to their proximity to the 4th ventricle and their rostro-caudal distance from obex. As there is no interneuronal transport of these tracers the technique enables identification of the cell bodies of neurons supplying axons directly to the site of labelling on the selected nerves. The proportion of axons taking up tracer is unknown so the technique enables the location of groups of cell bodies in discrete areas of the CNS but only gives approximate estimates of their relative numbers. The specific pattern of labelling for each nerve was similar in all cases and data are presented from fish that showed the largest number of labelled cells.

### Series 2: electrophysiology

For measurement of respiratory and cardiac variables, each fish ( $N=20$ ) was first anaesthetized by immersion in a solution of

benzocaine ( $0.1 \text{ g l}^{-1}$ ) until righting responses were abolished, when it was laid on its left side on the operating table. It was supported by strips of plastic sponge, so that the operculum on the underside was free to move. The fish was then supplied with a continuous flow of aerated water containing anaesthetic diluted to a concentration (approximately  $0.04 \text{ g l}^{-1}$ ) at which the animal commenced spontaneous respiratory activity, consisting of rapid shallow movements of the jaw and opercula.

The opercular movements were recorded by attaching to the edge of the right operculum a length of suture thread that led to a force transducer (Myograph F-2000, Odense, Denmark) inputting to a physiograph (MK-III-S, Narco Biosystems, Austin, TX, USA). A cannula (PE 50), filled with saline solution ( $0.9\%$ , NaCl plus  $100 \text{ IU ml}^{-1}$  heparin), was inserted into the caudal artery to record heart rate as blood pressure, using a standard procedure (Perry et al., 2004). The cannula was connected to a Baxter Edward pressure transducer (model PX600; Irvine, CA, USA) and the signals were amplified using a preamplifier built in-house at the University of Aarhus, Denmark. The outputs from all measuring devices were taken to a data acquisition system (either Dataq DI-194, Akron, OH, USA, during the nerve stimulation experiments, or AcqKnowledge-Biopac system, Goleta, CA, USA, during the nerve recordings).

The central cut end of the respiratory branches of the Vth, VIIth, IXth or Xth cranial nerves and either the central or peripheral cut ends of the cardiac branch of the Xth were exposed using the same operative routes described in series 1. Each branch was dissected, sectioned and placed on a pair of platinum electrodes positioned by a mechanical manipulator (Prior Scientific, Cambridge, UK), in order to either record from or electrically stimulate the nerve. The fish was then supplied with a continuous flow of aerated water containing anaesthetic, diluted until the animal commenced spontaneous respiratory activity, consisting of rapid, shallow movements of the jaw and opercula (approximately  $0.04 \text{ g l}^{-1}$ ).

#### Series 2.1: activity in the cardiac and respiratory nerves

Recordings of nervous activity were obtained from the central cut ends of respiratory and cardiac nerves in eight fish with the input to the electrodes led to a purpose-built preamplifier and then to an AC amplifier (Digitimer, Neurolog NL105, Welwyn Garden City, Herts, UK), a filter (NL 125) and an audio-amplifier (NL 120). These electrical recordings were performed inside a screened metal 'Faraday' cage to reduce electrical interference with recorded signals. Recordings of spontaneous activity were obtained from all nerves in normoxic fish supplied with aerated water (oxygen tension in the water,  $P_{\text{wO}_2}=140 \text{ mmHg}$ ) and in the case of activity in cardiac nerves from fish in which the flow rate of water was halved to induce systemic hypoxia (C.A.C.L., E.W.T. and F.T.R., unpublished). To observe the phase relationships between the bursts of respiration-related activity recorded from respiratory and cardiac nerves, the delay between both the onset and the peak of each burst and the peak of the resulting respiratory cycle, represented by opercular movements, was recorded. The onset of nervous activity was identified from integrated bursts as the time at which the waveform rose to 15% above baseline. All data were displayed and stored on a computer and are illustrated by representative traces and by data from six fish, combined to illustrate phase relationships.

#### Series 2.2: electrical stimulation of respiratory and cardiac nerves

To study the role of the cardiac vagus in the efferent control of heart rate and the role of reflexes originating in the respiratory system in modulating heart rate, the peripheral cut end of the cardiac branch of

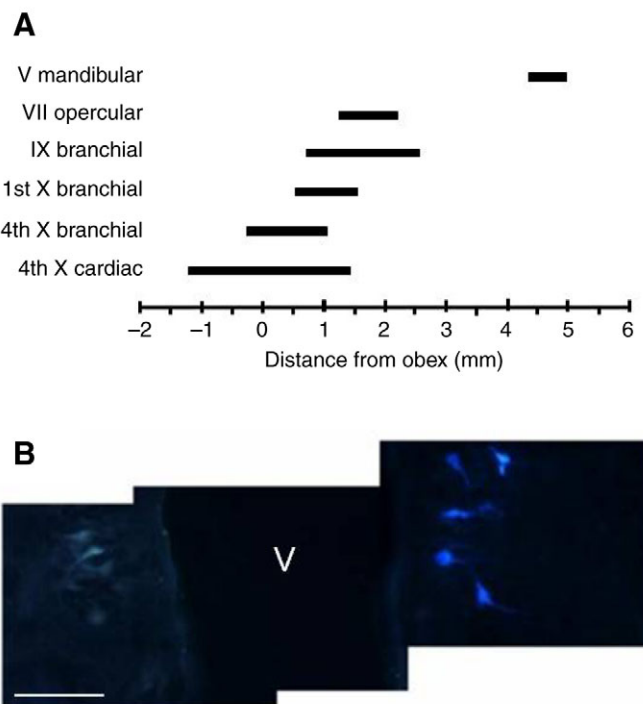


Fig. 1. (A) The rostro-caudal distribution in the brainstem of *P. mesopotamicus* of cell bodies of respiratory visceral motor neurones (RVMN) supplying axons to the mandibular Vth, opercular VIIth, branchial IXth, and 1st and 4th branchial Xth, together with cardiac vagal preganglionic neurones (CVPN) supplying axons to the cardiac vagus. Data are taken from fish with the best fills (largest number of labelled cell bodies). (B) Transverse section of the brainstem 1.33 mm rostral of obex. The cell bodies of the CVPN that supply the cardiac branch of the right vagus are stained with the fluorescent tracer True Blue and the respiratory motor neurones that supply the 4th branchial branch of the left vagus are stained with Fluorogold. They are located either side of the 4th ventricle (V). Scale bar, 100 µm.

the vagus and the central cut ends of the mandibular branch of the Vth, opercular branch of the VIIth, branchial branch of the IXth and the 1st, 2nd, 3rd and 4th branchial branches of the Xth cranial nerves were exposed for electrical stimulation in 12 fish. Each nerve was accessed as described in series 1 and placed on platinum electrodes. It was then stimulated with pulses of varying voltage and frequency using a physiological stimulator (Farnell Instruments, Leeds, UK). Initially the nerve was tonically stimulated with square-wave pulses of 1–2 ms duration, at 10–40 Hz and increasing voltages from 3 to 15 V, until the applied stimulus caused the heart to stop. The same settings were then used to deliver bursts of stimuli of 200–300 ms duration. The nerve was then stimulated at a range of bursting frequencies both faster and slower than the intrinsic heart rate. Heart rate was obtained from blood pressure pulse rate. Data are illustrated by representative traces and by the measured ranges over which the heart was recruited by the bursts of stimuli in all fish.

## RESULTS

### Central projections of respiratory nerves

Application of a neural tracer into the respiratory branches of the Vth, VIIth and IXth nerves and branchial branches of the Xth showed that the dorsal visceral motor neurones supplying axons to respiratory nerves (respiratory visceral motor neurones, RVMN) are distributed over a rostro-caudal extent of 6 mm in the brainstem, from 5 mm rostral

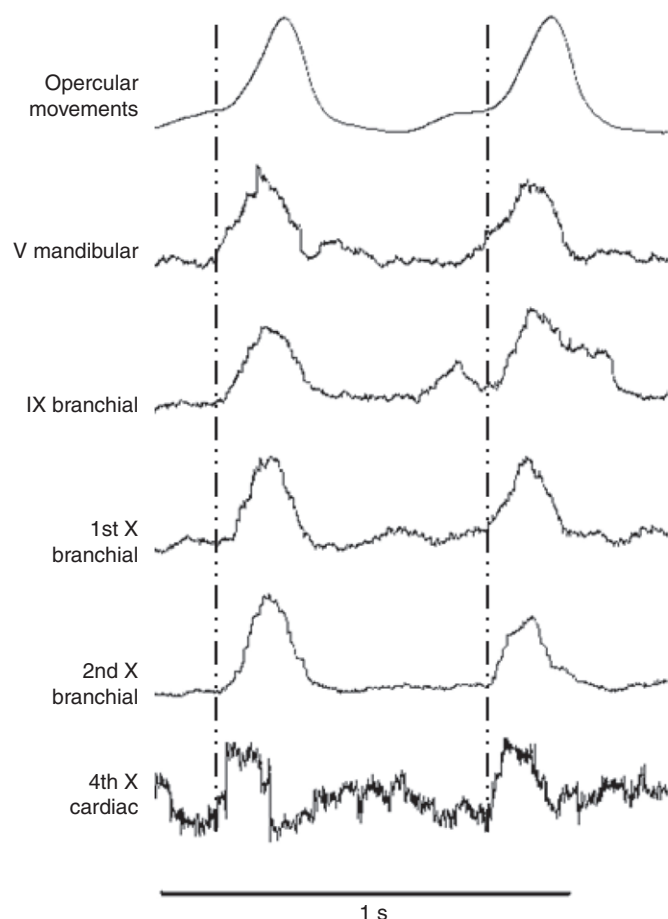


Fig. 2. A trace of opercular movements together with integrated efferent activity recorded from cranial nerves V, mandibular; IX, branchial; X, 1st and 2nd branchial, and 4th cardiac. Each is aligned with simultaneous recordings of opercular movements. The vertical lines mark the estimated onset of bursts in the cardiac nerve and reveal that this is early in the sequence of activities in all nerves. The 'noisy' trace recorded from the cardiac nerve reflects the occurrence of spontaneously active, non-bursting units.

to 1.0 mm caudal of obex. The individual rostro-caudal distributions of each respiratory branch plus the distribution of cardiac vagal pre-ganglionic neurons (CVPN) are shown in Fig. 1A. There is a large degree of overlap between all groups except the group of cell bodies supplying the mandibular branch of trigeminal Vth (Fig. 1A). The CVPN have an overlapping distribution with the dorsal motor areas of all the respiratory nerves except the mandibular Vth. This overlapping distribution of CVPN and RVMN is illustrated in the transverse section of brainstem shown in Fig. 1B.

#### Efferent activity of the respiratory and cardiac branches

Electric activity recorded from the central cut ends of the respiratory nerves of spontaneously breathing, lightly anaesthetized fish consisted of action potentials firing in bursts synchronous with the recordings of opercular movements. Respiration-related activity was also recorded from the cardiac branch of prepared fish receiving a reduced supply of water (C.A.C.L., E.W.T. and F.T.R., unpublished) and a sample of these data is included for comparison. All of these bursts anticipated movements of the operculum. Bursting activity recorded from the respiratory nerves and the cardiac vagus was integrated then aligned by matching each recording with simultaneous recordings of

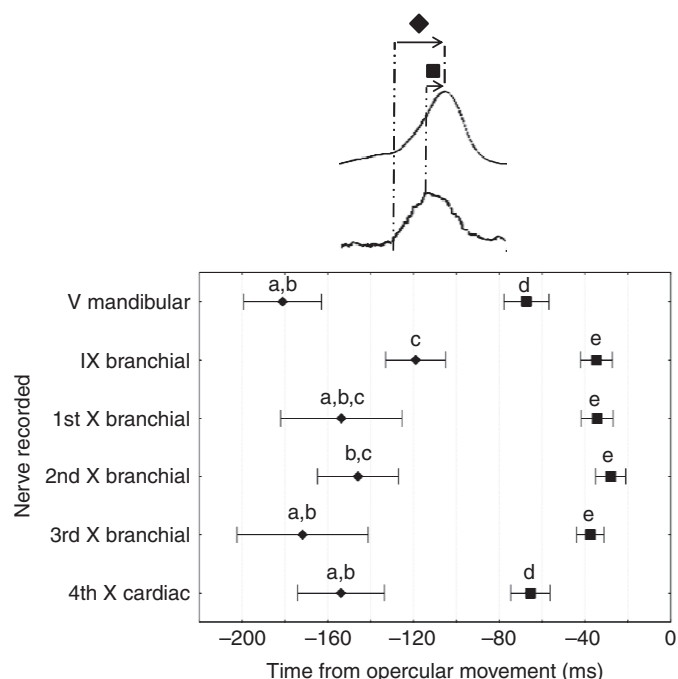


Fig. 3. Mean duration ( $\pm$ s.d.) of the interval between each onset (filled diamonds) or peak (filled squares) of the burst activity in respiratory branches of cranial nerves V, IX and X or the cardiac branch of X and the peak of the succeeding opercular movement (time=0). The cartoon at the top of the figure indicates the derivation of the points plotted below, with the upper trace being opercular movement and the lower trace a sample of activity in the Vth cranial nerve. The letters a–e denote significantly similar groups of mean values. The peaks of activity in the cardiac nerve are concurrent with activity in the Vth cranial nerve and both anticipate activity in the IXth and 1st, 2nd and 3rd respiratory branches of the Xth.

opercular movements (Fig. 2). This revealed that the cardiac bursts occurred early in the cycles of activity. Combining data from eight animals revealed the variable phase relationships between the bursting activities recorded from the respiratory nerves and the cardiac vagus (Fig. 3). The onset of the bursts was difficult to discriminate and possibly as a consequence showed a high degree of variability with the range of phases overlapping in all nerves. However, the peaks of the bursts of activity were more clearly discernible and less variable. Examination of their temporal relationships revealed that the bursts of respiration-related activity in the cardiac vagus were in phase with activity in the mandibular branch of the Vth cranial nerve and that these bursts significantly anticipated the peaks of activity in the other respiratory nerves (Fig. 3).

#### Central stimulation of respiratory nerves

Continuous electrical stimulation of the central cut end of the respiratory branches of the VIIth, IXth and Xth cranial nerves caused cardiac arrest (e.g. Fig. 4A). When the stimuli were delivered in bursts the heart was entrained by the bursts over a wide range of frequencies lower than the pre-stimulation  $f_H$  (Fig. 4D,E). At higher bursting frequencies the heart was entrained to alternate bursts or other fixed ratios with the stimuli (e.g. 3:1, Fig. 4C). Uniquely, there was no effect on heart rate of central stimulation of the mandibular branch of cranial nerve V even at higher rates and voltages of stimulation than routinely applied (Fig. 4F). Injection of atropine abolished all effects on the heart of central stimulation of the respiratory nerves (data not shown).

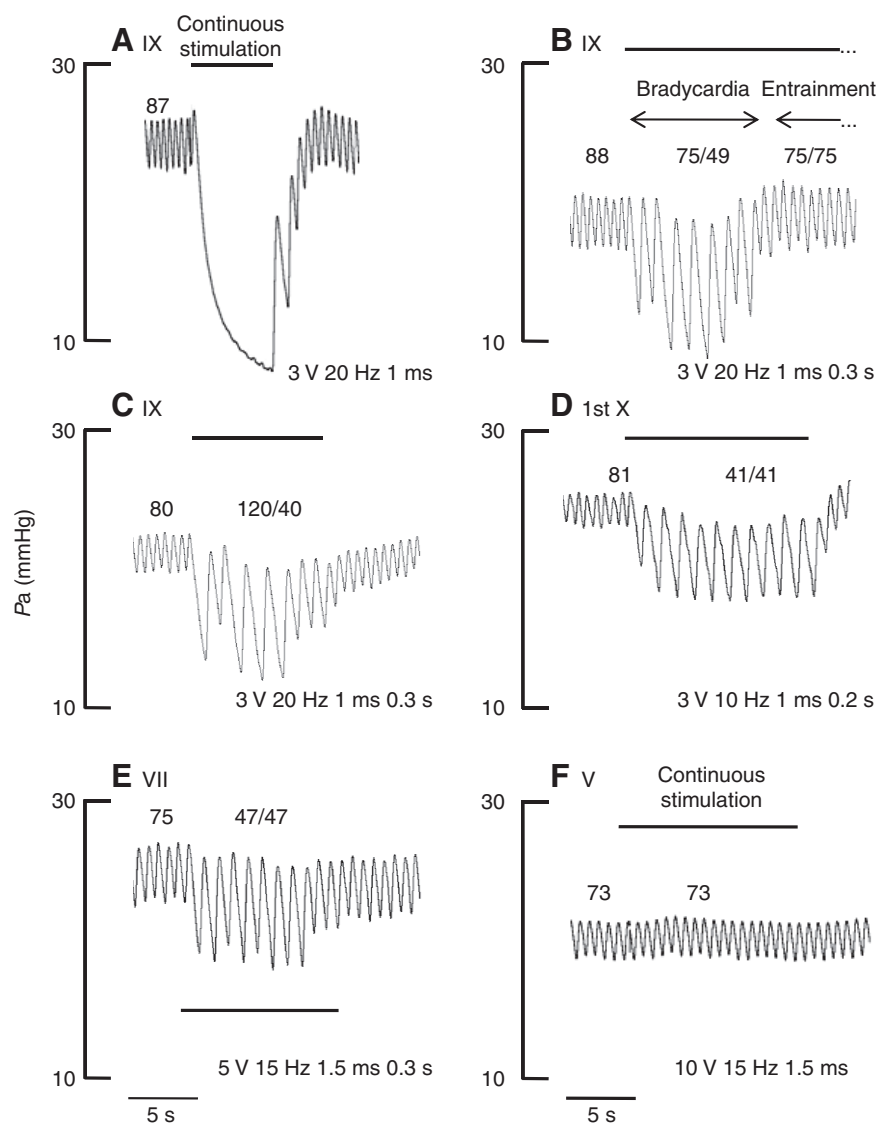


Fig. 4. Effects of central electrical stimulation of the IXth, Xth, VIIth and Vth cranial nerves on heart rate, measured as arterial blood pressure ( $P_a$ , mmHg). The period of stimulation is indicated by the horizontal bar above or beneath each trace and the stimulation parameters are given below each trace. The initial, intrinsic heart rate (beats  $\text{min}^{-1}$ ), followed by the bursting rate of the electrical stimuli and the consequent heart rate are given above each trace. (A) Continuous stimulation of the IXth cranial nerve caused the heart to stop. (B) Phasic stimulation of the IXth initially inhibited then recruited the heart. (C) At a higher bursting frequency the heart slowed to one-third of the rate of stimulation of the IXth. (D) Heart recruited by phasic stimulation of the 1st respiratory branch of the Xth. (E) Heart recruited by phasic stimulation of the facial branch of the VIIth. (F) Electrical stimulation of the Vth cranial nerve was without effect on the heart.

#### Peripheral stimulation of cardiac nerve

Peripheral stimulation of a cardiac branch of the vagus with a continuous train of stimuli slowed or stopped the heart (Fig. 5A). When the same stimuli were delivered in bursts the heart was entrained over a wide range of burst frequencies. In all fish it was possible to entrain  $f_H$  to the frequency of stimulation over a range of frequencies lower than the intrinsic  $f_H$  (Fig. 5B), although at lower rates (e.g. at 42 bursts  $\text{min}^{-1}$ ) the heart often beat more than once for each burst of stimuli. At stimulation rates higher than the intrinsic  $f_H$  the heart was entrained either 1:1 (up to 120 bursts  $\text{min}^{-1}$ ) or by a fixed fraction of the burst frequency, from 1:2 to 1:6 up to 180 bursts  $\text{min}^{-1}$  (see Fig. 5C,D). The relationships between stimulation frequency and heart rate are plotted for four fish in Fig. 6. All four fish showed recruitment of the heart by rates of stimulation below the pre-stimulation heart rate. One fish (P2) showed recruitment by rates of stimulation well above the pre-stimulation heart rate while two fish (P3 and P4) showed recruitment by a whole number ratio of the stimulation frequency (1:2 to 1:6). The range of stimuli entraining the heart on central stimulation of the respiratory nerves is compared with the effects of peripheral stimulation of the cardiac nerve in Fig. 7. This shows that responses to peripheral stimulation of the cardiac nerve were over a wider

range of frequencies than responses to central stimulation of respiratory nerves.

The injection of atropine blocked both the slowing of the heart in response to continuous stimulation of the cardiac vagus and the entrainment by phasic stimulation, confirming that both were determined by stimulation of muscarinic cholinergic receptors (data not shown).

#### DISCUSSION

Our emphasis on control of the heart by the parasympathetic vagus is justified by the fact that control of the heart by the sympathetic nervous system and by circulating catecholamines seems of little importance in pacu. The results of a preliminary study (C.A.C.L., E.W.T. and F.T.R., unpublished) revealed a cholinergic tonus of 29% and an adrenergic tonus of 9% on the heart of pacu in normoxia. When exposed to deep hypoxia fish typically show an increased level of catecholamines in the blood (Reid et al., 1998; Reid and Perry, 2003). However, in a comparative study of three tropical teleosts Perry and colleagues (Perry et al., 2004) showed that pacu did not respond to hypoxia or injection of nicotine with a significant increase in circulating catecholamines, while the other two species showed marked increases. They interpreted this as evidence for an

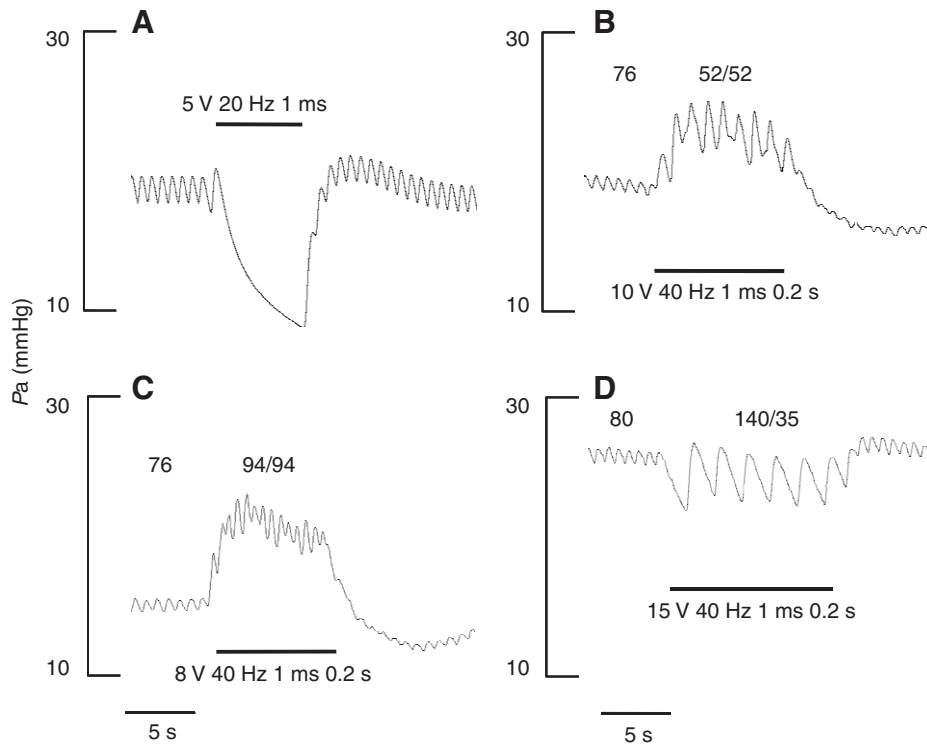


Fig. 5. Entrainment of  $f_H$  by peripheral electrical stimulation of the right cardiac vagus either with continuous trains of stimuli or with trains of brief pulses (0.2–0.3 s) of impulses (1–2 ms; 20–40 Hz). Heart rate was recorded as blood pressure (Pa, mmHg). Pre-stimulation  $f_H$  and the stimulus frequency plus the imposed  $f_H$  are given immediately above each trace (beats  $\text{min}^{-1}$ ). The periods of stimulation are indicated by the horizontal bars at the base of the traces and the stimulus parameters are given either above or below these bars. (A) Cardiac arrest during continuous stimulation. (B) Entrainment at a rate slower than the intrinsic  $f_H$ . (C) Entrainment at a rate faster than the intrinsic  $f_H$ . (D) Entrainment by every 4th burst of stimuli.

inoperative or absent humoral adrenergic response in this species. In addition, studies on mammals, reptiles and fish have revealed that sympathetic control of heart rate is generally associated with long latency control of blood pressure and changes in levels of disturbance, while tonic and rapid phasic control of the heart, which is likely to generate cardiorespiratory interactions, is predominantly parasympathetic (Bootsma et al., 1994; Taylor et al., 1999; Le Mevel et al., 2002; Campbell et al., 2004; Campbell et al., 2006).

In the elasmobranch fish *Scyliorhinus canicula*, the motor areas of the respiratory branches of cranial nerves VII, IX and X and the CVPN supplying the cardiac vagi occur in a rostro-caudal sequential series with some degree of overlap between each sequential motor group. This pattern of distribution continues rostrally with the trigeminal motor area of cranial nerve V but with no superposition (Withington-Wray et al., 1987; Taylor, 1992). *Piaractus mesopotamicus* presents a similar distribution of motoneurons but with an even higher degree of overlap between RVPN and CVPN. Each motor nucleus overlies several others and the CVPN overlap all of them except the trigeminal motor neurons, which occur in a topographically separate, more rostral position.

Barrett and Taylor (Barrett and Taylor, 1985a) showed that respiratory branches of cranial nerves V, VII, IX and X in *S. canicula* fire in a temporal sequence according to the rostro-caudal distribution of their motoneurons in the brainstem. Similar patterns of sequential firing were recorded from pacu. In both fish this respiration-related efferent activity was also recorded from the cardiac vagi. However, in dogfish this activity was recorded from normoxic animals that were pharmacologically paralysed (Barrett and Taylor, 1985a) suggesting that it was generated by central interactions between the RVMN and CVPN that are sited together in the dorsal vagal nucleus (DVN). In contrast, bursting efferent activity was only recorded from the cardiac vagus of pacu during periods of increased ventilatory effort in fish exposed to moderate hypoxia (C.A.C.L., E.W.T. and F.T.R., unpublished), suggesting that it may be generated by reflexes derived from branchial

receptors. Previous work on pacu (Leite et al., 2007) revealed that mean heart rate increased from  $63 \pm 6$  beats  $\text{min}^{-1}$  in intact fish to  $82 \pm 3$  beats  $\text{min}^{-1}$  following denervation of the gill arches supplied by the IXth and Xth cranial nerves. This suggests that an important element of vagal tone on the heart of pacu is generated reflexly, possibly by stimulation of branchial receptors.

Central stimulation of the respiratory branches of cranial nerves VII, IX and X with bursts of stimuli that may simulate stimulation of mechanoreceptors recruited the heart in pacu (Fig. 4). This supports the possibility of a role for afferent activity derived from branchial receptors in recruiting the heart. Phasic central stimulation of a respiratory branch of the vagus in the dogfish induced bursts of efferent activity in the cardiac vagus (Young et al., 1993a; Taylor, 1992) and this is likely to be the case in pacu. So, an increase in ventilatory effort during hypoxia, by stimulating branchial mechanoreceptors, may generate respiration-related activity in cardiac nerves that can cause the heart to beat at a rate corresponding to the ventilation rate. This is in accordance with the recruitment of the heart by imposed pulses of water flow, described for the trout (Randall and Smith, 1967). This model is clearly based on reflex feedback control of cardiorespiratory interactions. So, the current study provides support for the generation of cardiorespiratory interactions by a reflex route. However, there is also the possibility that during the current experiments RVMN were stimulated antidromically by electrical impulses during central stimulation of their efferent fibres and that direct interactions between these neurones and CVPN in the DVN led to induced activity in the cardiac nerves and subsequent cardiac recruitment. These putative relationships were summarized for dogfish in a schematic diagram (Taylor, 1992).

The alternative possibility that cardiorespiratory interactions may be generated centrally in the brainstem is supported by the apparent concurrence of bursting activity in the cardiac nerve with activity in the Vth cranial nerve (Figs 2 and 3). In contrast to the other respiratory nerves, central electrical stimulation of the Vth

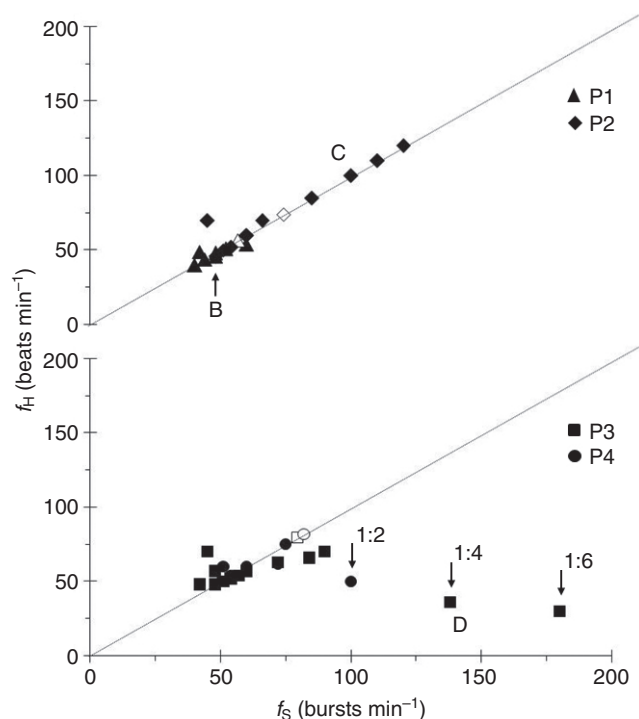


Fig. 6. Graphs showing the range over which heart rate ( $f_H$ ) of *P. mesopotamicus* (four fish, P1, P2, P3 and P4) was entrained by brief (0.2–0.3 s) bursts of electrical stimuli (1–2 ms pulses at 20–40 Hz) delivered down the peripheral cut end of the right cardiac vagus at rates ( $f_S$ ) between 40 and 180 bursts  $\text{min}^{-1}$ . Open symbols denote the pre-stimulation  $f_H$  of each fish and filled symbols the entrained rates. Points deviating from the central line denote different ratios between  $f_H$  and  $f_S$ , which are indicated near each point. Points labelled B, C and D are taken from the traces illustrated in Fig. 5A–D. Values for fish P1 and P2 are plotted separately from those for P3 and P4 for greater clarity.

was without effect on heart rate (Fig. 4) and the motor neurones supplying the Vth cranial nerve do not overlap with the CVPN (Fig. 1). Accordingly, the concurrence of activity between the Vth and cardiac nerves is likely to be generated centrally, possibly by inputs to both groups of neurons from a central respiratory pattern generator when respiratory drive is high, as in hypoxia. Consequently, it is still not possible, on the basis of the present data, to determine how much of the efferent activity delivered by the cardiac vagus originates reflexly from stimulation of branchial chemo- and/or mechanoreceptors and how much comes from central interactions between respiratory and cardiac motor areas or a central respiratory rhythm generator. There is evidence for both sources of interaction and their relative importance may vary with the level of central respiratory drive.

Tonic peripheral electrical stimulation of the cardiac vagus in pacu caused cardiac arrest but when the stimuli were delivered in bursts they were shown to recruit the heart over a wide range of frequencies. This entrainment was apparently a form of cardiac pacing, with the heart caused to contract by each burst of electrical stimuli rather than the bursts increasing the duration of each cardiac interval by phasic inhibition of the pacemaker. This relationship is suggested by examination of Fig. 5C, which shows the heart being driven faster than its intrinsic rate, and more particularly by Fig. 5D, which shows the heart responding to every 4th burst of stimuli. Fig. 6 shows examples of whole number ratios between

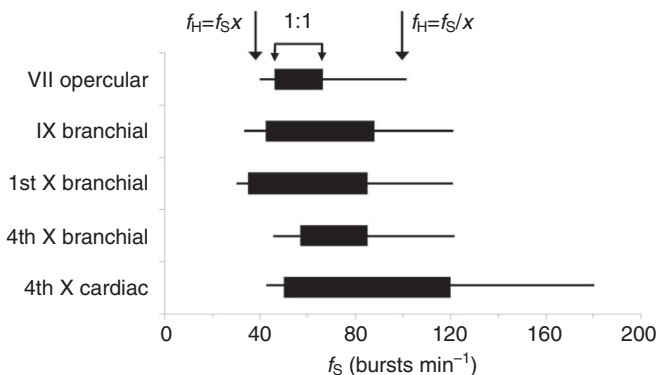


Fig. 7. Ranges of stimulation frequency over which the heartbeat was recruited by peripheral stimulation of the cardiac nerve (from the 4th branch of the Xth cranial) or by central stimulation of the opercular branch of the VIIth, branchial (respiratory) branch of the IXth or the 1st and 4th branchial branches of the Xth cranial nerves. The thick bars denote 1:1 recruitment, while the thin extensions denote recruitment to rates higher or lower than the stimulation frequency, with  $x$  being a whole number between 2 and 6. In the cardiac denervated fish (lower trace) intrinsic heart rate was  $79 \pm 3$  beats  $\text{min}^{-1}$ .

stimulation and heart rates varying from 2:1 in fish P4 to 6:1 in fish P3. The range of frequencies over which central stimulation of respiratory nerves recruited the heart was narrower than reported here for direct peripheral stimulation of the cardiac vagus (see Fig. 7). Unlike central stimulation of respiratory nerves, recruitment by peripheral stimulation of a cardiac nerve could sometimes be to rates higher as well as lower than the intrinsic heart rate. The potential cardio-acceleration resulting from phasic efferent stimulation of the cardiac vagus was impressive, with the heart driven to beat at almost twice its intrinsic rate. This requires a drastic reinterpretation of the role of the cardiac vagus in determining heart rate in fish, which has historically been described as tonic inhibition (Taylor, 1992).

A central question raised by these data is how cardiac contractions can be driven by bursts of efferent activity derived from peripheral electrical stimulation of the cardiac vagus when its role is primarily inhibitory, due to the release of acetylcholine onto muscarinic receptors on the heart. If the inhibitory effect of the vagus related directly to the intensity of its efferent output then increased rates of bursting should slow the heart rather than recruiting it to a faster rate. Bursts of stimuli delivered at rates considerably faster than the intrinsic  $f_H$  (higher than 120 bursts  $\text{min}^{-1}$ ) did indeed have an overall inhibitory effect (i.e. caused the heart to slow) but again there was evidence of cardiac pacing, because the heart was observed to beat at fixed whole number ratios of the applied stimulus (see Fig. 5D), implying that it was being paced by the imposed stimulus, even though not by each individual burst. A partial answer to this apparent paradox was provided by Thompson and O'Shea (Thompson and O'Shea, 1997). They showed that in an elasmobranch fish, acetylcholine had an atypical excitatory effect on cardiac ventricular muscle that was blocked by atropine. However, their data implied that the effect was of long latency and probably mediated by indirect effects on the release of catecholamines so it is unlikely to play a role in the rapid beat-to-beat control exhibited in the present investigation.

A direct relationship between efferent vagal activity and heartbeats has been reported in mammals (Levy et al., 1969; Levy et al., 1972). The pattern of the vagal electrical activity to the heart has been

described as non-constant, having pulses or bursts of activity occurring with variable frequencies (Jewett, 1964; Katona et al., 1970; Kunze, 1972; Taylor et al., 1999). The effects of bursts of efferent vagal activity on the heart have been investigated in several groups of mammals. In all of them it was possible to pace the heart with burst activity over a range of stimulation frequencies lower than the intrinsic  $f_H$  (e.g. Pokrovskii, 1984; Pace et al., 1984) and in some species at rates higher than the intrinsic  $f_H$  (Brown and Eccles, 1934; Levy et al., 1981). The neurological basis of recruitment of the heart at rates higher than its intrinsic rate by bursts of electrical stimuli delivered peripherally down the cardiac vagus has long been of interest to mammalian physiologists. Pokrovskii (Pokrovskii, 1984; Pokrovskii, 2003; Pokrovskii, 2006) reported that increasing the intra-burst frequency improved the effectiveness of the stimulation in pacing the heart. This implies that as more acetylcholine is delivered to efferent vagal synapses the heart is driven to beat rather than inhibited. Brown and Eccles (Brown and Eccles, 1934) described a complex relationship between the chronotropic effect of efferent stimulation of the cardiac nerve and the phase of the cardiac cycle in which stimuli were delivered. Levy and colleagues (Levy et al., 1972) and Spear and colleagues (Spear et al., 1979) confirmed that a pulse of stimulation could be effective in causing two inhibitory phases separated by a brief phase of cardio-acceleration, depending on the phase of the cardiac cycle in which it was delivered. Martin (Martin, 1977) noted that atrio-ventricular conduction time in the heart of the dog could be shorter in the presence of vagal stimulation. Similar complex relationships were reviewed by Levy and colleagues (Levy et al., 1981). Pace and colleagues (Pace et al., 1984) noted that the maximum R-R intervals were triggered when the efferent stimulus was delivered to the post-ganglionic terminal of the vagus, on the sino-atrial node, during the phase of slow depolarization of the pacemaker cells, while minimum R-R intervals occurred when the stimulus was delivered before this phase.

These observations imply that the effect of each burst depends on the phase of the cardiac cycle at which it is applied. Thus, the vagal effect on the heart cannot be measured merely in terms of the amount of acetylcholine delivered per unit time. The overall effect of vagal stimulation will be related to the summation of the effects of stimuli delivered in the inhibitory and stimulatory phases. Similar detailed information is not yet available for fish but the present study does provide clear evidence that the cardiac vagus imposes beat-to-beat control of heart rate in fish rather than merely imposing an inhibitory tone.

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