

## EFFECTS OF VAGAL SENSORY INPUT ON THE BREATHING RHYTHM OF THE CARP

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*Accepted 10 July 1990*

### Summary

Electrical stimulation of an epibranchial vagal ganglion, which innervates the gill region, had a marked influence on the respiratory rhythm of the carp *Cyprinus carpio*. Vagal input could initiate ventilation in fish displaying intermittent respiration. In fish breathing steadily, vagal stimuli could reset the respiratory rhythm by modifying the existing breathing cycle. An increase of stimulus intensity evoked a cough-like movement, thus delaying the onset of following cycles. Rhythmic stimulation, at intervals not more than 10% longer or shorter than the breathing cycle period, could entrain the respiratory rhythm in a one-to-one ratio. Larger differences between the stimulation interval and the period of the breathing cycle resulted in either a cyclic modification of the respiratory cycle period or entrainment patterns with coupling ratios of 2:1 or 2:3. Coughing decreased in frequency or even stopped during rhythmic vagal stimulation.

### Introduction

Transection at different levels of the brain of a variety of fishes has shown that the 'respiratory centre' is located in the rhombencephalon (Hyde, 1904; Shelton, 1959; Rovainen, 1974; Fukuda, 1978). Electrophysiological recordings have indicated that this part of the brain contains neurones that discharge rhythmically in time with respiration (Woldring and Dirken, 1951; Hukuhara and Okada, 1956; Shelton, 1961; Waldron, 1972; Ballintijn and Alink, 1977) and that remain active, although with a different rhythm, after respiratory movement has been abolished following injections of succinylcholine or curare (Von Baumgarten and Salmoiraghi, 1962; Serbenyuk, 1965; Ballintijn and Roberts, 1976; Kawasaki, 1984; Roberts and Ballintijn, 1988). Apparently, rhythmic sensory activity that would

Key words: *Cyprinus carpio*, respiratory rhythm, vagus, entrainment, phase-dependent modulation.

result from the movements is not necessary for the maintenance of the rhythm, which is presumably produced by self-sustaining generating networks.

Although the generating networks underlying respiration may be able to operate independently, it is likely that in the living animal they would be influenced by a variety of inputs. Sensory information from the gills would seem essential, not only in the regulation of the respiratory rhythm, but also for the critical adjustment of the gill arches so as to maintain a 'gill curtain' for respiration (Ballintijn and Punt, 1985). Further, the branchial system in the carp participates in feeding (Sibbing, 1984) and the gill rakers form a sieve-like structure that serves to filter food particles from the water. Sensory information would be needed in the regulation of the balance between respiratory and feeding movements and to initiate coughing movements to prevent inflowing particulate material from damaging the delicate gill surfaces.

It has been shown that afferent information originating from different peripheral regions, including the gills, has a marked influence on the breathing movements (Satchell, 1959; Shelton, 1959; Serbenyuk *et al.* 1959; Satchell and Way, 1962; Serbenyuk, 1965; Satchell and Maddalena, 1972; Ballintijn and Bamford, 1975; Ballintijn *et al.* 1979, 1983; Roberts and Ballintijn, 1988). Several types of gill receptor have been described (Satchell and Way, 1962; Sutterlin and Saunders, 1969; Shelton, 1970; Poole and Satchell, 1979; Jones and Milsom, 1982), but little was known, particularly for teleost fishes, about the activity of these receptors during breathing until it was shown in recent studies (De Graaf and Ballintijn, 1987; De Graaf *et al.* 1987) in the carp *Cyprinus carpio* that the gill receptors could be divided into two classes on the basis of their patterns of activity. In the present study we have investigated the possible significance of the sensory inputs from the gills by stimulating afferent nerve fibres, as they pass through the vagal ganglia, with electrical stimuli given in a variety of patterns designed to mimic the sensory inflow. In an accompanying paper (De Graaf and Roberts, 1991), we consider further the role of rhythmical sensory input in the control of the respiratory rhythm.

### Materials and methods

The experiments were performed on 11 carp, *Cyprinus carpio* L. (22–29 cm), maintained under light anaesthesia (MS222, 28–47 mg l<sup>-1</sup>). Each carp was held in the experimental tank by a clamp on the orbital ridges of the skull and by a plastic foam support to the body. The spinal cord was transected just behind the skull to prevent body movements. The epibranchial ganglia of the vagus nerve on one side were exposed as described in a previous paper (De Graaf *et al.* 1987). All experiments were performed in a temperature-controlled room at 20°C.

#### *Electrical stimulation*

Electrical stimuli were applied *via* a stimulus isolator (Neurolog NL-800) through bipolar stainless-steel electrodes inserted into the second or third vagal

ganglion. The electrodes were made of two insulated insect needles (no. 000), fixed about 250  $\mu\text{m}$  apart. Stimuli were single pulses, or trains of 2–5 pulses (pulse duration 0.5 ms, 10–20 ms interval) given singly or in series, of varying frequency (interval 300–2000 ms). The stimulus strength used during rhythmic stimulation (1.0–4.0 mA) was just below the level needed to evoke abduction of the operculum. Stronger stimuli caused coughing or erratic respiratory movements.

### *Recording*

The respiratory movements were monitored with a light-weight, pivoted arm that rested against the preoperculum, ipsilateral to the stimulated ganglion. Electromyograms (EMGs) were recorded with bipolar, enamelled copper wire electrodes (50  $\mu\text{m}$  diameter), with tin-coated tips, inserted into the main respiratory pump muscles. Earlier experiments in our laboratory (Ballintijn *et al.* 1983) revealed no significant differences between the responses of the ipsilateral and contralateral sides. All signals were stored on FM tape (Bell & Howell, Datatape 4020) and later written out on paper, using an ink-jet recorder (Siemens Oscillomink-E) or an A/D converter and a dot matrix printer.

### *Data analysis*

The frequency of the respiratory movements was determined from the transducer placed against the preoperculum. The temporal relationship between the respiratory cycle and the stimulus given to the ganglion was measured from the movement record and expressed in terms of phase, on a scale of 0 to 1. The start of active abduction was chosen as zero phase.

## **Results**

The pattern of muscle activity, as seen in the EMG recordings in our experimental fish held in the holder under light anaesthesia, was the same as has been reported previously by Ballintijn (1969). (For a detailed treatment of the cranial anatomy, see Takahasi, 1925.) The respiratory cycle can be divided into an abduction (ABD) or expansion phase, and an adduction (ADD) or contraction phase (Fig. 1). Abduction starts with contraction of the levator operculi (LO) followed, after a short interval, by the levator hyomandibulae (LH). As a result of these muscle activities the buccal and opercular cavities expand (phase 0–0.4) and water is sucked in through the open mouth. During moderate ventilation, ADD starts (phase 0.4) with a slight, passive movement, which is the result of negative pressure in the respiratory cavities that develops during ABD and of elastic forces of the respiratory system. Active ADD begins with a gradual build-up of activity in the A3 part of the adductor mandibulae during the plateau of the ADD (Fig. 1A: *p*). The A3 muscle adducts the lower jaw and thus closes the mouth. During ADD (phase 0.4–1) the volume of the buccal cavity decreases and water leaves the respiratory cavities through the opercular slits. This mainly occurs at the end of

ADD, when both the A3 and the adductor arcus palatini (AAP) are strongly active. The plateau progressively shortens as ventilation frequency increases.

Several fish, including the carp, execute a special respiratory movement called the cough (Fig. 1A: C). A breathing cycle is regarded as a cough if the adduction (contraction) phase is interrupted by an intermediate abduction (expansion) movement before the level of maximal adduction has been reached. This

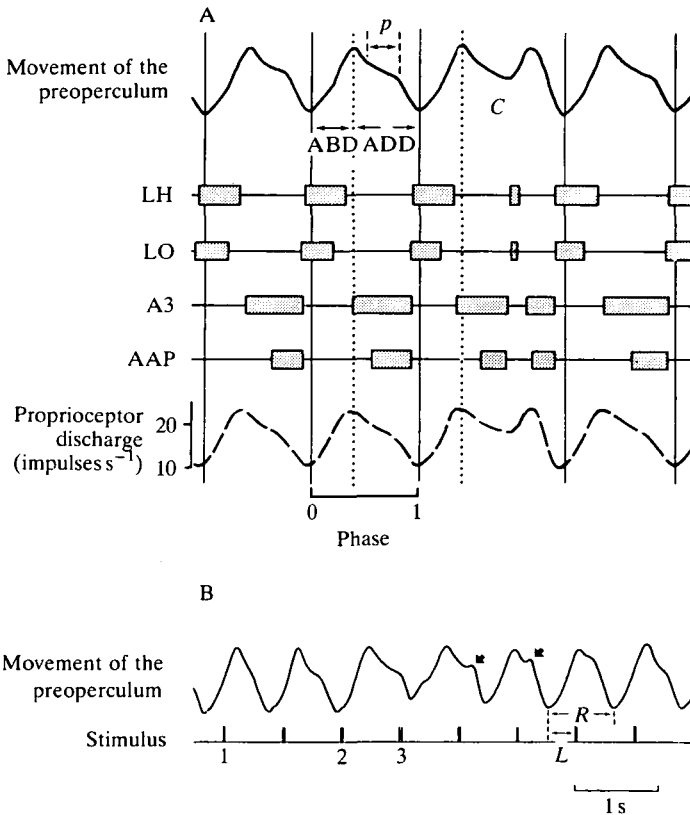


Fig. 1. (A) Diagram of the relationship between movement of preoperculum (top trace), respiratory muscle activity and gill arch proprioceptor discharge (bottom trace) during normal breathing and spontaneous coughing (C). LH, EMG of levator hyomandibulae; LO, EMG of levator operculi; A3, EMG of A3 part of adductor mandibulae; AAP, EMG of adductor arcus palatini; ABD, abduction; ADD, adduction; *p*, plateau. The pattern of gill arch proprioceptor activity is derived from a previous study (De Graaf *et al.* 1987). (B) Effect of rhythmical vagal ganglion stimulation every 700 ms on the respiratory movement. Upper trace, movement of preoperculum (ABD upwards); lower trace, stimulus marker. Arrows indicate a slight ABD following vagal stimulation during ADD. 1, 2, 3, stimuli presented at different times in a breathing cycle; for explanation see text. The figure also illustrates how the temporal relationship between the stimulus and the respiratory cycle was determined in these experiments. The stimulus position in a breathing cycle, expressed as phase, was calculated as the time (*L*) from the start of the breathing cycle (phase=0) divided by the duration (*R*) of the existing respiratory cycle.

movement is the result of a brief but intense activation of the LO and LH muscles. A cough ends with the same pattern of muscle activity as a normal cycle. As a consequence of the interpolation of the intermediate expansion movement, the cycle period of a cough is considerably lengthened compared with a 'normal' breathing cycle.

Fig. 1A also illustrates the pattern of vagal sensory nerve activity that accompanies the ventilatory cycle and a cough, based on our findings from a previous study (De Graaf and Ballintijn, 1987). A respiration-modulated discharge, which originates from gill arch proprioceptors and reflects gill arch position, can be recorded from the epibranchial vagal ganglia; the discharge rate increases during ABD and decreases during ADD.

#### *Modification of the respiratory cycle by vagal ganglion stimulation*

Electrical pulses applied singly or as a series to an epibranchial vagal ganglion modified the respiratory movement and the duration of a breathing cycle. The effect on the existing cycle is illustrated in Fig. 1B. Stimulation at intervals at least 10–15 % longer or shorter than the spontaneous respiratory cycle period resulted in a cyclic pattern of modification. In the cases illustrated in Figs 1 and 2, short pulse trains were given at intervals of 700 ms to a fish breathing with a rhythm with a cycle period of about 940 ms. As a consequence of the differences between the cycle period of the stimulus and of the ventilatory rhythm, the phase of the stimulus in the breathing cycle shifted continuously (Fig. 1B) and successive stimuli occurred progressively earlier in the breathing cycle. In this case, the respiratory cycle period shortened, but never matched, the imposed rhythm. Thus, phase locking did not occur and a cyclic pattern of lengthening and shortening of the respiratory cycle period in relation to the stimulus was observed (Fig. 2A). This phenomenon has been described as 'relative coordination' (Von Holst, 1939). Similar responses are seen for stimulation at intervals of 650 or 750 ms.

The phase position of the stimulus affects the response. A short train applied during the second half of ABD or the first half of ADD (i.e. between phase values 0.25 and 0.65) results in a shortening of the existing breathing cycle. This comes about because ABD accelerates and the plateau (*p*) of ADD is reduced (Fig. 1B: stimulus 1) as a result of increased activity of the inspiratory (LH, LO) and expiratory (A3) muscles. In contrast, a pulse train given during early ABD (phase: 0–0.25) results in inhibition of the levator hyomandibulae (LH) and levator operculi (LO) muscles, which are normally active in this phase, a slight reduction of ABD amplitude (Fig. 1B: stimulus 2) and, sometimes, a slight lengthening of cycle period (Fig. 2B). This lengthening effect is, however, less significant since the intrinsic ventilatory rhythm shifted during stimulation (Fig. 2B, cf. solid and dashed horizontal lines). A second stimulus occurring late in a cycle results in premature termination of the existing adduction movement of the preoperculum. As a consequence, the amplitude of the inspiratory phase of the following cycle is reduced (Fig. 1B: stimulus 3).

During vagal stimulation, the normal adduction of the preoperculum was sometimes interrupted by a small abducting movement (e.g. Fig. 1B: arrows) which became more pronounced, and LH and often LO discharged strongly when the stimulus intensity was increased. This response could even be evoked directly after a spontaneous cough (Fig. 3A). A cough cycle (cycle labelled C in Fig. 3A) is characterized by an intermediate expansion movement occurring during the adduction phase of the breathing cycle, and resulting from strong inspiratory muscle activity. The movement of the preoperculum and the muscle activity recorded during vagal stimulation, presented in Fig. 3A, show that the response resembles the intermediate expansion which occurs during spontaneous coughing. This view is supported by the observation that the response occurred only during ADD, even if the stimuli were given during ABD (Fig. 3B).

#### *Modification of the respiratory rhythm by vagal stimulation*

Rhythmic stimulation with brief pulse trains given at intervals similar to the cycle period of the respiratory rhythm could result in synchronization ('entrainment') of the respiratory rhythm as each cycle either shortened or lengthened to match the stimulus interval. Respiration was then locked to the stimulus, which occurred at a 'preferred' phase position (Fig. 4B). Usually entrainment was achieved within 5–7 breathing cycles after the first stimulus presentation, during which time the phase position of the stimulus within the breathing cycle gradually changed (Fig. 4B). Sometimes phase-locking was unstable, and apparent only for a few (3–5) breathing cycles, whereupon respiration again ran free from the imposed rhythm. This unstable process can also be regarded as 'relative coordination'.

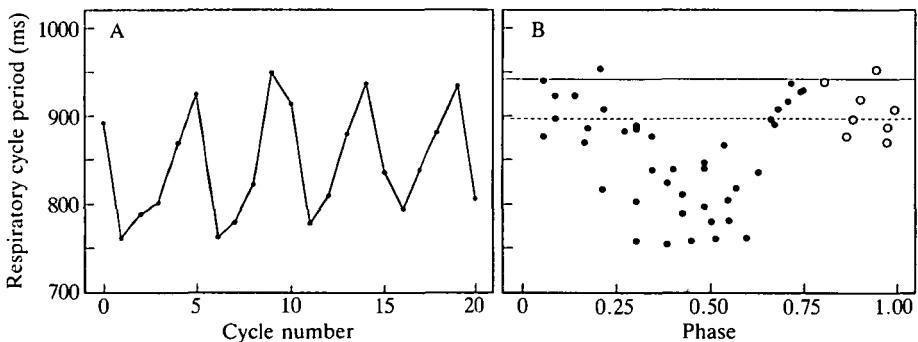


Fig. 2. Cycle modification in response to vagal stimulation. (A) Phase-dependent modification of successive breathing cycles during vagal stimulation at 700 ms interval. (B) Relationship between the phase position of a stimulus and the respiratory cycle period. The solid horizontal line indicates the mean respiratory cycle period measured before stimulation (940 ms) and the dashed line the cycle period after stimulation (896 ms). As the stimulation interval was shorter than the spontaneous cycle period, two stimuli could occur within one breathing cycle; such stimuli are indicated by open circles.

Vagal stimulation not only influenced the respiratory rhythm during the stimulation period but could also have an after-effect, as is illustrated in Fig. 5A. Here the period of the breathing cycle after stimulation was longer than the stimulus interval and only gradually returned to the pre-stimulus shorter cycle period.

Coughing decreased in frequency, or even stopped, when respiration was entrained by vagal stimulation (Fig. 5A,B). When coughing did occur during entrainment, the coupling between stimulation and respiration was disturbed and phase-locking was temporarily lost (Fig. 5C). The subsequent cycles then either gradually lengthened or shortened until respiration locked once again to the stimulus at the preferred phase position. Fig. 5C clearly shows that the phase position of the stimulus was constant before the cough.

The respiratory rhythm could be accelerated or retarded by vagal stimulation, although usually it was easier to accelerate ventilation (Fig. 2B). This preference means that the range of entrainment is asymmetrically distributed about the spontaneous cycle period, as can be seen in Fig. 6, which shows how the range of entrainment varies as the breathing rhythm changes. In the fish illustrated, the respiratory rhythm had a cycle period of about 920 ms and could be synchronized with stimulus intervals that ranged from 850 to 950 ms. The next day the spontaneous breathing rhythm had a cycle period of 880 ms and vagal stimulation now resulted in a capture range of 800 to 900 ms. Thus, although the range of

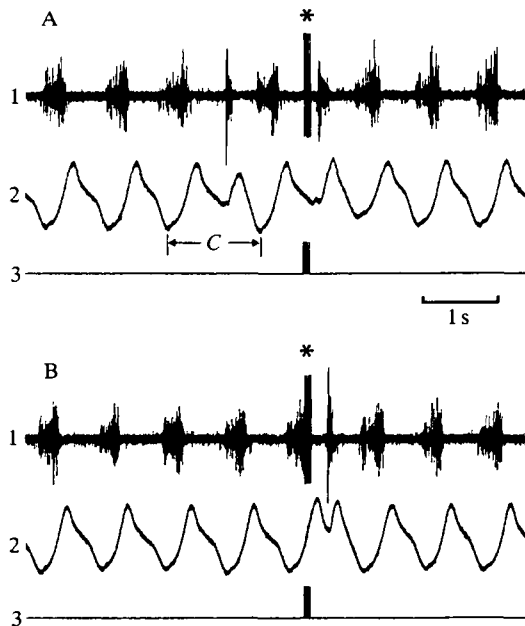


Fig. 3. Coughing evoked by pulse train stimulation. Trace 1, EMG of levator hyomandibulae; trace 2, movement of preoperculum (ABD upwards); trace 3, stimulus marker. Asterisk: stimulus artefact. The cycle labelled C indicates a spontaneous cough. (A) Stimulation during ADD; (B) stimulation during ABD.

entrainment was roughly the same, the exact periods depended on the existing ventilation rate. In all fish, the range of entrainment was usually limited to about 100 ms, which is approximately 10% of a normal breathing cycle.

Stimulation at intervals more than about 15% longer or shorter than the spontaneous respiratory cycle period resulted in a cyclic modification of the respiratory cycle periods, as illustrated in Fig. 2A and in the patterns shown in Fig. 7. In the latter fish, the respiratory cycle period measured before stimulation was about 775 ms, and stimulation at intervals of 800 and 900 ms led to 1:1 entrainment (Fig. 7B). However, when intervals much shorter than the lower limit of the capture range (e.g. 600 ms) were used, there was an initial 1:1 entrainment accompanied by enhancement of the general level of abduction and a 50% reduction of breathing amplitude (Fig. 7A middle section). After a few cycles, respiration synchronized with a multiple of the stimulus interval, resulting in 2:1 entrainment (two stimuli per breathing cycle) and an increase in ventilation amplitude (Fig. 7A end). However, 1:1 entrainment could be established again for a limited number of cycles if the stimulus intensity was increased. Stimulation at

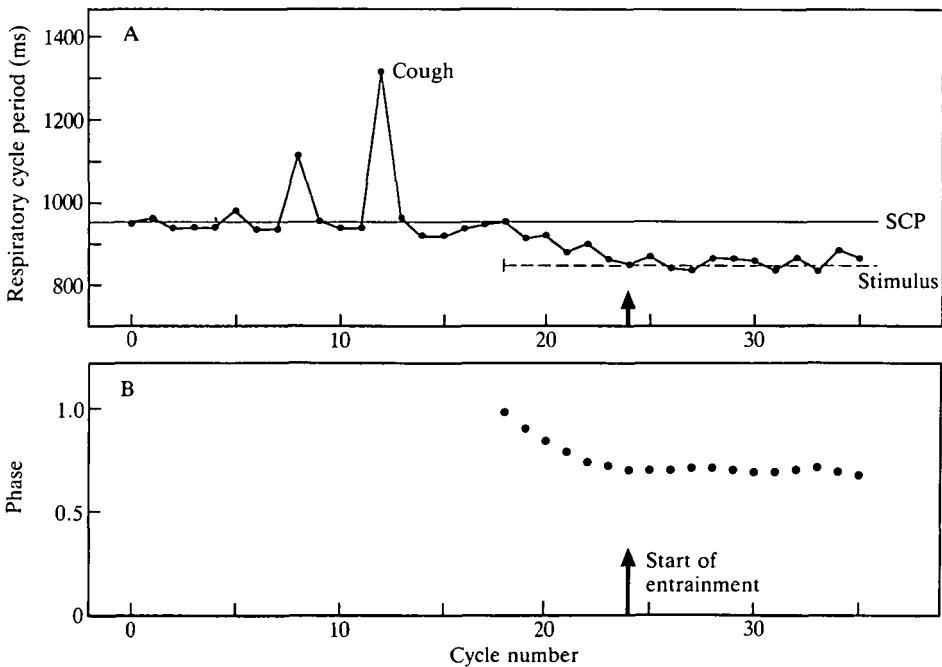


Fig. 4. Establishment of entrainment of the respiratory rhythm by vagal stimulation. (A) Respiratory cycle period plotted against cycle number. From the start of stimulation (cycle number 18) breathing cycles progressively shorten and synchronize with the stimulus after seven cycles. The solid line indicates the spontaneous cycle period (SCP) before stimulation (960 ms). The dashed line shows stimulation period and level (850 ms). Note the absence of coughing during the stimulus period. (B) Phase position of the stimulus within the respiratory cycle. The start of entrainment is marked by a vertical arrow.



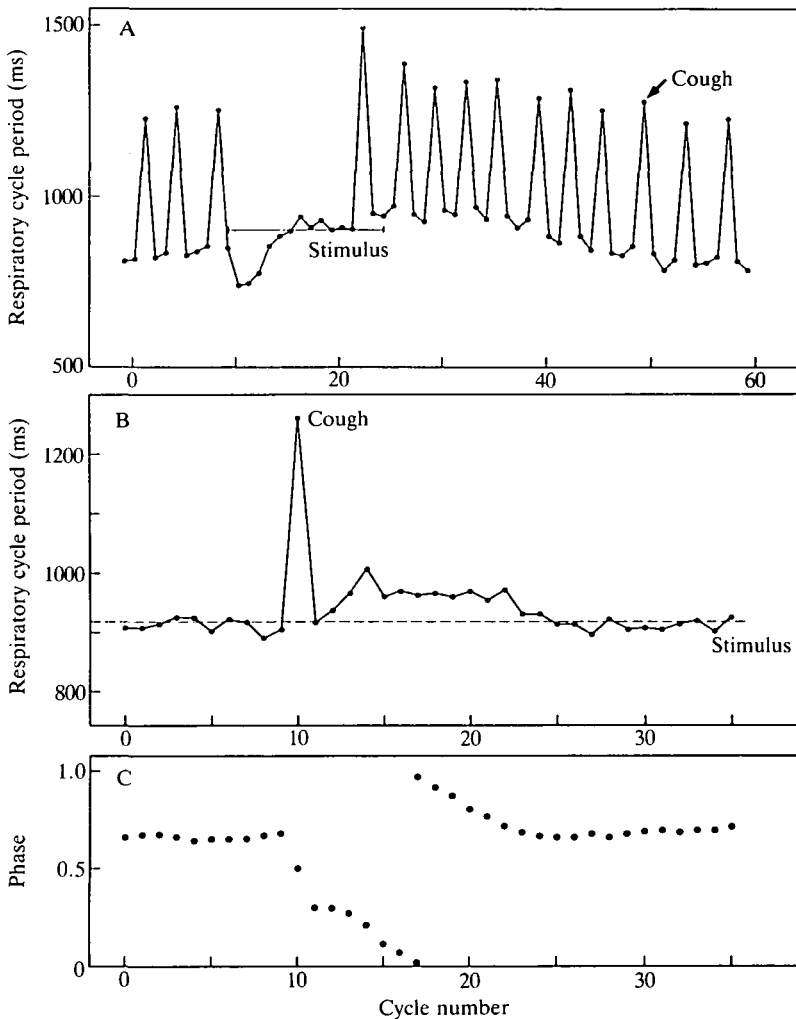


Fig. 5. Coughing and entrainment. (A) Coughing frequency decreases markedly during vagal stimulation. Note also the long-term effect of vagal stimulation seen as a progressive shortening of the cycle period back to the value measured before stimulation. All long-period events (about 1200–1500 ms) are coughs. (B, C) The impact of a cough during entrainment established by vagal stimulation every 920 ms. Phase-locking, and hence entrainment, is lost but is shortly re-established. Note the constancy of phase position before coughing.

intervals much longer than the upper limit of the range of entrainment (e.g. 1000 ms), resulted in entrainment in a 2:3 ratio (two stimuli per three breathing cycles) (Fig. 7C).

The movement pattern during the initial 1:1 synchronization illustrated in Fig. 7A could resemble that of a cough. As in a spontaneous cough, the adducting movement is interrupted by an abducting movement before the maximal level of

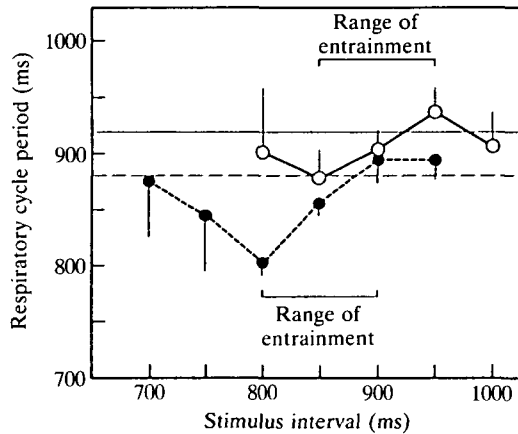


Fig. 6. The relationship between stimulus and respiratory cycle periods. Each point represents the mean (+standard deviation) of 30 cycles (coughs were excluded) from the same fish recorded on successive days. On day 1 entrainment is achieved at stimulation intervals of 850–950 ms (open circles); on day 2 the range was 800–900 ms (closed circles). Note the relatively large standard deviations beyond the limits of the capture range. The solid line (day 1: 920 ms) and the dashed line (day 2: 880 ms) indicate the mean cycle periods before stimulation. Stimulation was started at intervals similar to the spontaneous cycle period and was then lengthened or shortened in 50 ms steps.

adduction (i.e. the end of the ADD phase) has been reached. The resemblance does not hold, however, for the pattern of muscle activity underlying this abducting movement. The intermediate expansion of a cough (Fig. 7A: arrow) is mainly the result of an intense but brief activation of the levator hyomandibulae, whereas during vagal stimulation this muscle showed a much weaker, longer response. Hence, the ABD movement is executed more slowly than it is during a cough. Moreover, if stimulation with relatively long or short intervals produced coughing, then similar responses would be expected when intervals similar to the respiratory cycle period were used and Fig. 7B shows that this is not the case.

The phase position for 1:1 entrainment ranged in different fish from approximately 0.5 to 0.8, which implies that phase-locking always occurred when the stimulus was presented during ADD. However, for entrainment with a 2:1 ratio, where two stimuli were present per cycle, the phase locking took place at early and late positions in the cycle.

### Discussion

The present experiments in which electrical stimulation was used to mimic afferent input to the hindbrain demonstrate that sensory signals from the gills can have a significant influence on the neuronal network that generates the respiratory rhythm. Stimulation of the vagus affected individual respiratory cycles, the

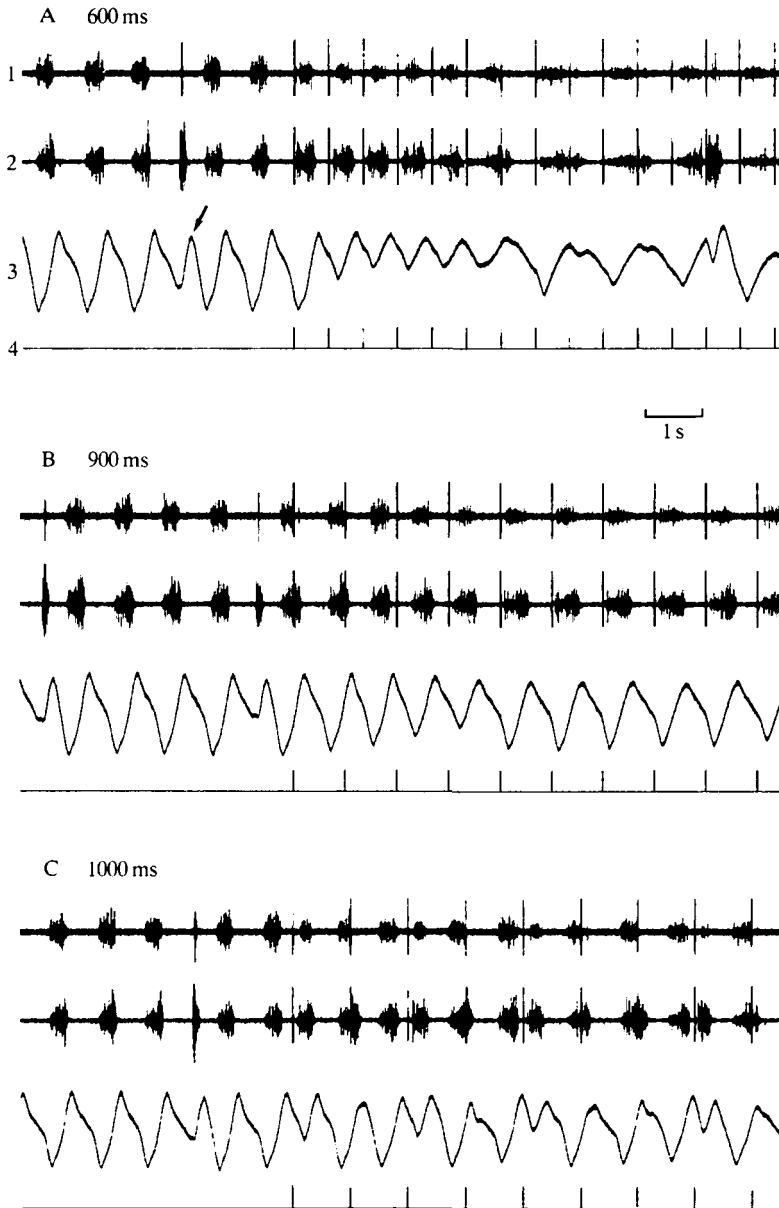


Fig. 7. Respiratory patterns in response to vagal stimulation at different intervals. Trace 1, EMG of levator operculi; trace 2, EMG of levator hyomandibulae; trace 3, movement of preoperculum (ABD upwards); trace 4, stimulus marker. (A) Stimuli given every 600 ms initially resulted in 1:1 entrainment, which then switched to synchronization in a 2:1 ratio (two stimuli per breathing cycle). Arrow, intermediate expansion of spontaneous cough. (B) Stimuli given at 900 ms intervals led to complete 1:1 entrainment. (C) Stimuli applied every 1000 ms resulted in 2:3 entrainment (two stimuli per three cycles).

respiratory rhythm and coughing. The impact was evident even though only one of the eight epibranchial ganglia that innervate the branchial region was stimulated.

#### *Impact of vagal stimulation on the respiratory cycle*

In our experiments vagal ganglion stimulation exerted the same effect on the breathing cycle as was reported previously ('cycle modifying effect', Ballintijn *et al.* 1983), i.e. it was shortened if the stimulus was given during adduction, but changed little when the stimulus was delivered during abduction. As a result, vagal stimulation could reset the breathing rhythm in a phase-dependent manner. A phase-dependent modulation of rhythmic motor systems by sensory stimulation has been reported now for a range of preparations (e.g. Rossignol *et al.* 1988). Vagal stimulation during inspiration in mammals resulted in abrupt termination of inspiration and initiation of expiration (Iscoe *et al.* 1979). Vagal input during expiration resulted in prolongation of that phase, which is in contrast with our results in the carp. Phase-dependent modulation of the respiratory cycle was also demonstrated in the dogfish *Scyliorhinus canicula* (Roberts and Ballintijn, 1988), where the effect of vagal nerve stimulation on the cycle period was different from that seen in the carp because the cycle period lengthened when stimuli were given during abduction. These differences among species may reflect differences in central organization or may result from the experimental activation of different types of afferent fibres. It has been observed in the rabbit, for instance, that expiration is shortened when lung irritant receptors are activated, whereas stretch receptor stimulation lengthened expiration (Davies *et al.* 1978).

#### *Impact of vagal stimulation on the respiratory rhythm*

In the carp, the respiratory rhythm could be entrained by repetitive vagal stimulation. The range of frequencies over which entrainment was possible was limited, which is probably because only one of the eight vagal ganglia is stimulated. The establishment of entrainment, however, illustrates the importance of this type of sensory input. It was always easier to drive the rhythm faster than the existing rate rather than to retard it. This agrees with the observation that in response to vagal stimulation an individual cycle shortens considerably, but lengthens only slightly. Indeed, entrainment can be regarded as being a series of 'cycle-modifying effects'. Stimulation at intervals of at least 10–15% longer or shorter than the spontaneous respiratory cycle period could result either in a cyclic pattern of modification, similar to the phenomenon described by Von Holst (1939) as 'relative coordination', or in entrainment patterns with coupling ratios of, for example, 2:1 or 3:1. Even at these stimulation frequencies, however, 1:1 synchronization could be obtained if the stimulus intensity was increased, presumably because more afferent fibres were recruited.

#### *Impact of vagal stimulation on coughing*

Although coughing can be evoked as a reflex response to mechanical stimulation of the gills (e.g. Hughes, 1975), it does occur spontaneously, it persists after the

glossopharyngeal and vagal nerves have been cut (Shelton, 1959) and it may be dependent on a central generating mechanism separate from the generator network that produces 'normal' respiratory movements. Vagal stimulation has one of two effects on coughing, depending on the stimulus intensity used. Low-intensity or moderate-intensity vagal stimulation does not elicit a cough, but instead a proprioceptive reflex involving abduction of the preoperculum and changes in the timing of respiration. Repeated stimulation at moderate intensity reduces or even abolishes spontaneous coughing. In contrast, stimulation at high intensity evokes a cough-like movement (Fig. 3) similar to the response elicited by mechanical stimulation of the gills (carp: De Graaf *et al.* 1987; tench: Young, 1971).

The different effects of electrical vagal ganglion stimulation on coughing may be due to the activation of afferent fibres that innervate different types of receptors.

#### *Sensory receptors supplying the vagus nerve*

Two types of mechanoreceptor, the afferent fibres for which run in the vagus nerve, have recently been described in the gills of the carp (De Graaf and Ballintijn, 1987; De Graaf *et al.* 1987). Gill arch proprioceptors, which are present in or around the cartilaginous tissue between the epibranchial and ceratobranchial of each gill, provide the central nervous system with detailed information about the degree of expansion of the branchial basket; thus, they could signal whether an adequate level of ABD and/or ADD has been reached. These proprioceptors may be involved in phase switching and in entrainment of the respiratory rhythm, because we have shown in the present experiments that stimuli given about the transition from ABD to ADD had the strongest effects on the respiratory cycle (Fig. 2B). A second type of receptor, inactive during normal breathing, is present in the gill filaments and gill rakers and has a relatively high threshold. When stimulated mechanically, this receptor discharges phasically and evokes a motor reaction in the gill. When this stimulus is strong, a cough is evoked (De Graaf *et al.* 1987) and so we suggest that the coughs evoked by vagal ganglion stimulation are the result of activation of the afferent fibres that originate from these mechanoreceptors.

Some of the differences seen between fishes in their responses to vagal stimulation result, we believe, from the unselective nature of electrical stimulation and because it was given to only a small percentage of the sensory fibres that convey information from the gills into the hindbrain. We have argued that the gill arch proprioceptors, responding to movement of the branchial basket, are most likely to be involved in regulation of ventilation in the carp. In an accompanying paper (De Graaf and Roberts, 1991) we investigate further the role of these receptors in respiratory motor control by imposing movements onto the gill arches that emulate natural breathing movements.

The authors are indebted to Mrs C. A. M. Oomen for help with the experiments, and Drs F. W. Maes and S. Daan for their criticism and valuable

discussions during preparation of the manuscript. This research was supported by the Foundation for Fundamental Biological Research (BION), which is subsidized by the Netherlands Organization for Scientific Research (NWO).

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