The Journal of Experimental Biology 202, 817–827 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
JEB1703

CHANGES IN MOTOR NETWORK EXPRESSION RELATED TO MOULTING BEHAVIOUR IN LOBSTER: ROLE OF MOULT-INDUCED DEEP HYPOXIA

STEFAN CLEMENS[1,*], JEAN-CHARLES MASSABUAU[2], PIERRE MEYRAND[1] AND JOHN SIMMERS[1,‡]

[1]*Laboratoire de Neurobiologie des Réseaux, Université Bordeaux I and CNRS, UMR 5816, Avenue des Facultés, 33405 Talence Cedex, France* and [2]*Laboratoire d'Ecophysiologie et Ecotoxicologie des Systèmes Aquatiques, Université Bordeaux I and CNRS, UMR 5805, Place Peyneau, 33120 Arcachon, France*

*Present address: Georgia State University, Department of Biology, Atlanta, GA 30302, USA
‡Author for correspondence (e-mail: simmers@lnpc.u-bordeaux.fr)

Accepted 7 January; published on WWW 9 March 1999

Summary

The well known rhythmically active pyloric neural network in intact and freely behaving lobsters *Homarus gammarus* was monitored prior to and following ecdysis. Despite long-lasting hormonal and metabolic alterations associated with this process, spontaneous pyloric network activity remained largely unaltered until the last 12–48 h before exuviation. At this time, the most notable change was a progressive lengthening of pyloric cycle period, which eventually attained 500–600 % of control values. It was only in the very last minutes before ecdysis that burst patterning became irregular and the otherwise strictly alternating motor sequence broke down. After the moult, coordinated rhythmicity was re-established within 10 min. Concomitant with these final changes in motor network expression at ecdysis was a drastic reduction in blood oxygen levels which led to a temporary near-anoxia. By imposing similarly deep hypoxic conditions both on intermoult animals and on the pyloric network *in vitro*, we mimicked to a large extent the moult-induced changes in pyloric network performance. Our data suggest that, despite major surrounding physiological perturbations, the pyloric network *in vivo* retains stable pattern-generating properties throughout much of the moulting process. Moreover, some of the most significant modifications in motor expression just prior to ecdysis can be related to a substantial reduction in oxygen levels in the blood.

Key words: Crustacea, lobster, *Homarus gammarus*, moulting, oxygen, hypoxia, stomatogastric system, central pattern generator, pyloric network.

Introduction

Decapod crustaceans have been subject to intensive biological studies throughout the last 100 years, and the events associated with moulting (ecdysis) have been generally appreciated and documented for much of this time (Drach, 1939; Passano, 1960). Few aspects of crustacean physiology are as important as moulting since, as a means of growth for an animal with an exoskeleton, it is necessarily a process that dominates the animal's life. Most work on this issue has focused on the physiological events that accompany ecdysis (for a review, see Aiken, 1980). For example, the flux and time course of Ca^{2+} uptake as a function of the moult cycle (Greenaway, 1985; Cameron, 1989; Neufeld and Cameron, 1992, 1994) along with the weight gain of animals by fluid absorption (Dall and Smith, 1978; Mykles, 1980) have been studied intensively. Respiratory (Mangum et al., 1985; Burtin and Massabuau, 1988; Wheatly and Ignaszewski, 1990) and ionoregulatory (Wheatly, 1985) aspects of ecdysis have also been investigated, as well as hormonal titres in the haemolymph (Hepper, 1977; Durliat et al., 1988; Fadool et al., 1989). However, until now, only a few studies have addressed behavioural aspects of moulting (Cromarty et al., 1991) and, to our knowledge, only a single investigation has monitored ongoing motor activity in a moulting animal (Kuramoto, 1993). In contrast, a number of such studies have been performed in insects (e.g. Levine and Truman, 1982; Mesce and Truman, 1988). Thus, despite a large quantity of information concerning ecdysis, little is known about the performance and dynamics of rhythmic motor networks under these conditions, undoubtedly because of the experimental difficulties of making long-term electrophysiological recordings from freely moulting animals.

Here, we address this problem in a combined *in vivo* and *in vitro* study of one of the best understood models of rhythmic motor behaviour, the stomatogastric nervous system (STNS) of decapod Crustacea. The STNS of the European rock lobster *Homarus gammarus*, as in other large crustaceans, generates rhythmic food-processing movements of the foregut and consists of four ganglia: the two commissural ganglia (CoG), the oesophageal ganglion (OG) and the stomatogastric ganglion (STG). The STG itself lies on the dorsal wall of the stomach, where it is situated in the ophthalmic artery and is therefore

continuously bathed in arterial blood. The STG contains approximately 30 neurones grouped into two motor-rhythm-generating networks, the well-described gastric and pyloric networks (Selverston and Moulins, 1987; Harris-Warrick, et al., 1992). The lobster pyloric network, which consists of 12 identified neurones (Cazalets et al., 1990; Massabuau and Meyrand, 1996), is continuously active *in vivo* (Clemens et al., 1998a) controlling peristaltic movements of the pyloric chamber. Since the pyloric chamber is at the posterior end of the foregut, which undergoes replacement during ecdysis, we were interested in how the pyloric circuit might participate in the moulting process and especially in the mechanical separation of the foregut from the non-ectodermal midgut. At this time, levels of blood-borne factors change dramatically (Greenaway, 1985; Durliat et al., 1988) and, significantly, the operation of the pyloric network is known to be extremely sensitive to a variety of exogenous hormonal and other modulatory influences (Harris-Warrick et al., 1992). Recently, such influences have been found to include behaviour-related changes in blood oxygenation levels (Massabuau and Meyrand, 1996; Clemens et al., 1998b).

By using electromyographic (EMG) recording techniques that do not hinder the lobster's normal behaviour (Clemens et al., 1998a), the aim of the present study was to monitor the motor output of the pyloric network prior to and after moulting. Importantly, since this neural network is composed mainly of motor neurones, activity patterns recorded from target muscles provide a direct insight into the operation of the central circuit from which these patterns arise. Our data show that, despite a long-lasting physiological preparation of the animal as it enters the late premoult stage, the pyloric network continues to express regular and rhythmic patterns of activity until approximately the last 1–2 days prior to the moult. We show that at ecdysis some of the ensuing changes in network expression can be correlated with a transient hypoxic status and a dramatic decline in arterial P_{O_2} to near-anoxia. Since experimental manipulation of arterial P_{O_2} *in vivo* in intermoult animals and *in vitro* in isolated STNS preparations mimicked these changes in network operation, we suggest that this oxygen deficit may play a direct role in modifying the central nervous activity controlling foregut movements at the time of ecdysis.

Materials and methods

In vivo experiments on moulting animals were attempted on seven adult European rock lobsters, *Homarus gammarus* (400–600 g, carapace length approximately 120 mm), of either sex and in the late premoult stage (stage D3; Aiken, 1973) purchased from commercial suppliers. Animals of this size have a moult cycle of approximately 1.5–2 years. EMG recordings were successfully obtained from three different animals before and after the moult, and blood O_2 status at these times was determined on two further lobsters. Control experiments were performed *in vivo* on a total of six intermoult animals and *in vitro* on nine STNS preparations. Before and during *in vivo* experiments, all lobsters were maintained in large (50 l) tanks of filtered and aerated sea water at 15–16 °C, renewed at a rate of 0.5 l min^{-1}. The inspired P_{O_2} was 20–21 kPa (for reference, 1 kPa=7.5 mmHg or 7.5 Torr; in water, a P_{O_2} of 1 kPa corresponds to an O_2 fraction of approximately 1 %).

Electromyographic recording of animals before and after moulting

The general methods have been described elsewhere in detail (Clemens et al., 1998a). Briefly, Teflon-insulated silver wire electrodes with a core diameter of 125 μm (AM Systems Inc., USA) were implanted into the appropriate pyloric muscles *via* small holes (diameter 0.5 mm) drilled through the cephalothorax using a fine syringe needle (Rezer and Moulins, 1983). To record the activity of the muscles innervated by the pyloric motor neurones LP (Lateral Pyloric), PY (PYloric) and PD (Pyloric Dilator), electrodes were implanted near to the midline between the cervical and subcervical grooves (Fig. 1A). Since pyloric activity is always expressed spontaneously, it is possible to locate the tip of an electrode close to a muscle by using an audio monitor as a steering device. In some cases, the electrode could be placed so close to the neuromuscular junction that presynaptic potentials in the motor neurone terminal could be clearly distinguished in the electromyographic recording. Electrodes were then glued to the carapace using tissue glue (Histoacryl; Braun Melsungen, Germany) and connected to highly flexible wires (diameter 1 mm) that maintained mechanical stability without restricting the animal's movements. The connections at the free end of the electrodes were waterproof-sealed using a commercial silicone glue and fixed to the carapace using dental cement (Durelon, ESPE, Germany). After electrode implantation, animals were kept isolated in darkened tanks (light intensity above the tanks 50–100 lx at noon) placed on vibration-isolated tables and with a 'natural-like' aquatic environment in which the animal could move freely and hide. Water supply was provided by an external 50 l reservoir, maintained at 16±1 °C under closed-circuit conditions. The free ends of the electrodes were connected to amplifiers (Grass P5 AC preamplifier), and recorded data were displayed on a Tektronix 5113 oscilloscope, stored on a Schlumberger tape recorder (Enertec S.A., France) and transposed simultaneously onto a Gould ES 1000 electrostatic chart recorder.

Arterial P_{O_2} *measurements at moulting*

Because of the extreme difficulty in predicting the precise timing of ecdysis, successful blood P_{O_2} analysis at moulting could be performed on only two lobsters. As moulting usually takes place at night and generally lasts no longer than 20–30 min, animals suspected of impending exuviation were also equipped with a laboratory-constructed moult detector (Fig. 1A, inset). This consisted of a fine Teflon-insulated silver wire (core diameter 75 μm, A-M Systems Inc., USA) which was glued across the cleft between the thorax and first abdominal segment. It was then connected to an alarm bell that was triggered at moult onset when the cleft opened and the wire ruptured. At this time, the thoracic carapace is lifted by approximately 3–5 cm, which also permits blood sampling by direct puncturing of the heart beneath the new and very soft

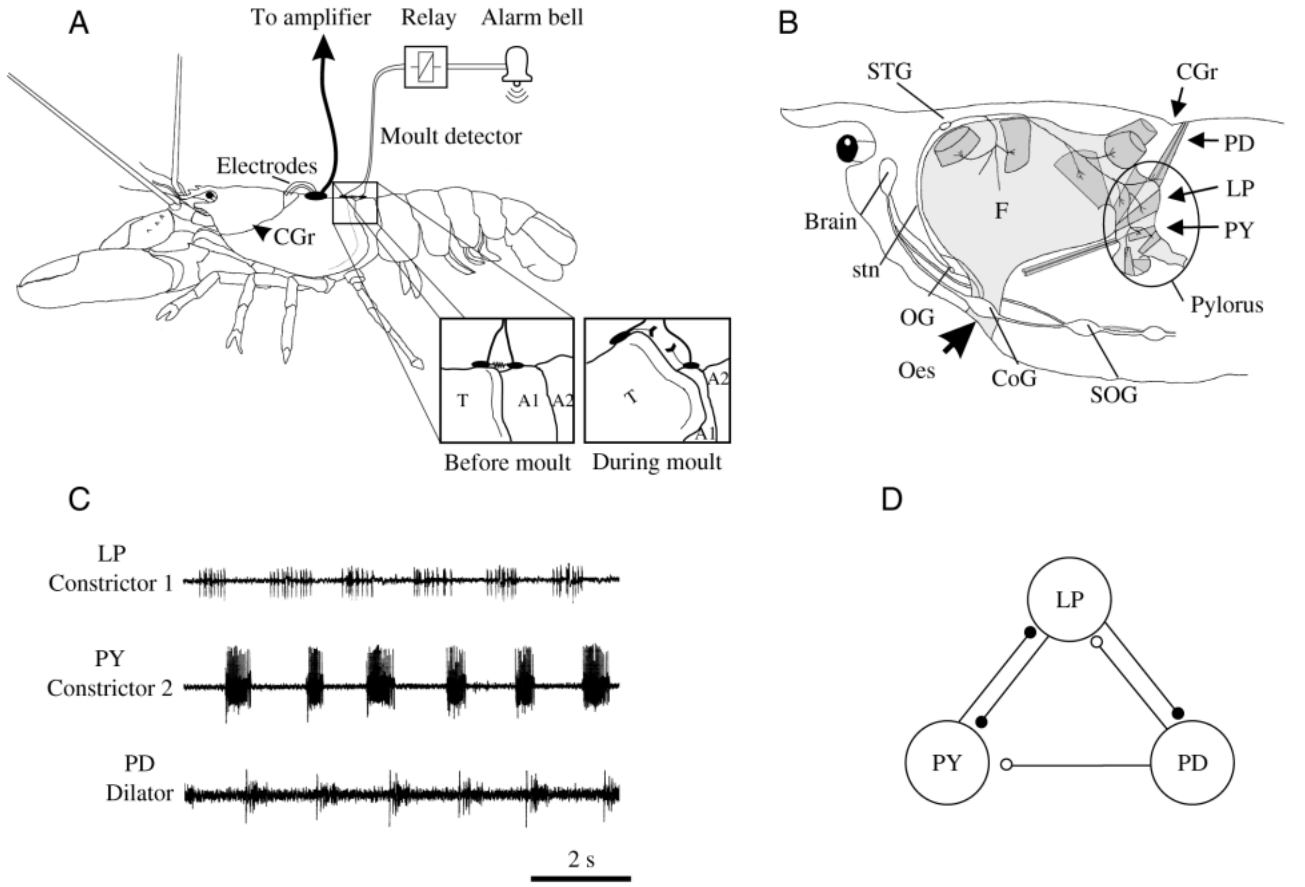

Fig. 1. *In vivo* experimental set-up and pyloric system of the lobster *Homarus gammarus.* (A) Electromyographic (EMG) recordings were made with wire electrodes inserted through the dorsal carapace anterior to the cervical groove and fixed with dental cement above the heart. A moult-detecting device was also installed by glueing a fine Teflon-insulated silver wire between the posterior rim of the cephalothorax and the first abdominal segment (A1) (see insets). The ends of the wire were connected *via* a relay to an alarm bell triggered by the rupture of the wire when the thoracic carapace lifted at the onset of ecdysis. This raising of the carapace also facilitated access to the heart for blood sampling. (B) Location of the foregut and muscles of the pyloric chamber in the intact animal. CoG, commissural ganglion; CGr, cervical groove; F, foregut; Oes, oesophagus; OG, oesophageal ganglion; STG, stomatogastric ganglion; stn, stomatogastric nerve; SOG, suboesophageal ganglion; LP, lateral pyloric neurone; PD, pyloric dilator neurone; PY, pyloric neurone; T, thorax. Pyloric muscles are identified by the motor neurones that innervate them (see text for details and abbreviations). (C) Simultaneous EMG recordings of the three main pyloric muscle types. The highly regular triphasic pattern occurs in the strict order of constrictor muscles 1 before constrictor muscle 2 before dilator muscle (innervated by LP–PY–PD motor neurones, respectively) with a brief pause preceding the start of the next cycle. (D) Simplified synaptic wiring diagram of the pyloric central pattern-generating network. Note the mutually inhibitory synapse between LP and PY neurones. Stick-and-ball connections denote chemical inhibitory synapses: filled ball, glutamatergic synapse; open ball, cholinergic synapse.

carapace. P_{O_2} measurements involved sampling arterial blood (i) from an animal a few hours before moult, (ii) again from the same individual during the moult and (iii) after the moult. For each measurement, arterial blood was obtained in less than 20 s by gently removing the lobster from the water and puncturing its heart with a capillary glass tube equipped with a syringe needle. Using this sampling technique, which was critically assessed by Forgue et al. (1992) and Massabuau and Forgue (1996), the blood sample (volume 100 µl) is driven into the glass tube at each heart beat. Arterial P_{O_2} was determined within 3 min using an E5046 Radiometer polarographic electrode thermostatted at seawater temperature.

Transient hypoxia in vivo

Control experiments on the effects of experimentally induced hypoxia on intermoult animals (N=6) consisted of monitoring pyloric network activity in resting animals exposed to transient hypoxia for 3–5 h. Hypoxic tank water was obtained by bubbling a N_2/air gas mixture *via* mass flow controllers (model FC-260, Tylan General) driven by a laboratory-constructed programmable control unit. Water P_{O_2} was monitored continuously using a YSI oxygen meter (model 57, Yellow Spring Instruments, Ohio, USA). Within 30–60 min, the inspired P_{O_2} in the water was decreased to 1–4 kPa. Recovery towards control oxygenation conditions was achieved within 45 min by bubbling air.

In vitro *extracellular recordings in saline solutions equilibrated at various physiological oxygenation levels*

Experiments were performed on nine preparations. The stomatogastric nervous system was dissected according to the

method of Selverston and Moulins (1987), leaving the anterior paired commissural ganglia, the oesophageal ganglion and their interconnecting nerves attached to the stomatogastric ganglion, and the isolated system was placed in the experimental apparatus previously described by Massabuau and Meyrand (1996). In short, the STG, CoGs and commissural connectives were desheathed, and the preparation was pinned down in a Sylgard-lined Petri dish. The entire preparation was superfused with air-equilibrated saline except the STG, which was pinned onto a separate Sylgard plate and enclosed in a 300 µl glass chamber to allow a separate superfusion with saline at physiological and near-anoxic P_{O_2} levels. The chamber was gravity-fed with saline at a constant flow rate (3–4 ml min^{-1}), and the entire preparation was thermostatted at 15±0.2 °C by means of a laboratory-constructed thermoelectric device. Extracellular nerve recordings were made with monopolar platinum electrodes insulated from the bath with Vaseline and using laboratory-constructed extracellular amplifiers. Data were displayed on a Tektronix 5113 oscilloscope, recorded on a Gould ES1000 electrostatic chart recorder and stored on video tape coupled to a Neurocorder DR890.

The physiological saline used for superfusion consisted of 479 mmol l^{-1} NaCl, 13.2 mmol l^{-1} KCl, 13.7 mmol l^{-1} $CaCl_2$, 10 mmol l^{-1} $MgSO_4$, 3.9 mmol l^{-1} Na_2SO_4 and 5 mmol l^{-1} Hepes. The pH was adjusted to 7.45 using HCl. Low-P_{O_2} gas mixtures were obtained by mixing $N_2/O_2/CO_2$ as described above and by Massabuau and Meyrand (1996). During experiments, P_{O_2} was varied between 0 and 20 kPa, and the CO_2 partial pressure (P_{CO_2}) was maintained at 0.4 kPa, a typical value in water-breathing animals (Rahn, 1966). Between experiments, the composition of the gas phase was analysed using a paramagnetic O_2 analyser (Servomex 1100A) and an infrared CO_2 analyser (Servomex 1410B) calibrated with high-grade N_2 and precision gas mixtures (F_{O_2}=3.99±0.04 %; F_{CO_2}=1.01±0.01 %). The O_2 concentration (C_{O_2}) in the saline was calculated according to Henry's law ($C_{O_2}=\alpha_{O_2}\times P_{O_2}$) with α_{O_2}=12.4 µmol l^{-1} kPa^{-1} at 15 °C.

Values are reported as means ±1 S.E.M. except where stated otherwise.

Results

Background and general findings

The pyloric network of *H. gammarus* drives rhythmic contractions of striated muscles that produce peristaltic-like movements of the pyloric chamber at the posterior end of the stomach (see Fig. 1B). The pyloric muscles consist of the dorsal dilator muscles innervated by two PD motor neurones, the constrictor 1 muscles innervated by the single LP motor neurone, and a set of constrictor 2 muscles innervated by eight PY motor neurones. Together, these three muscle groups

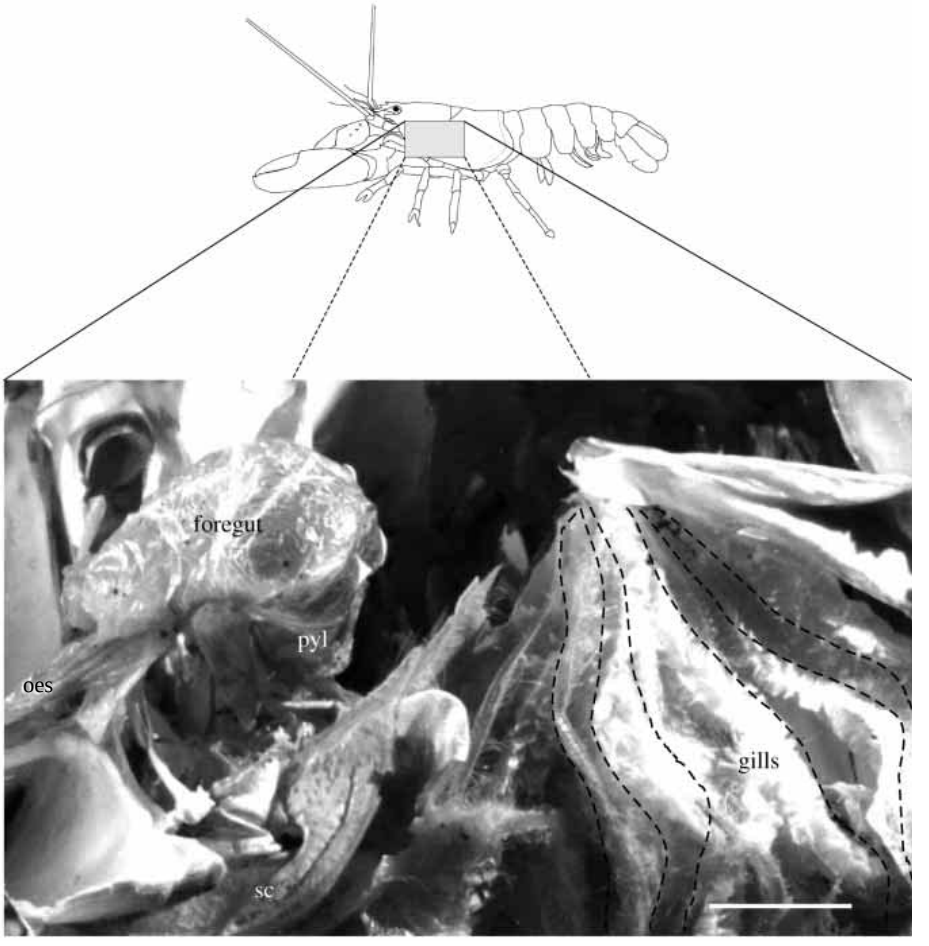

Fig. 2. Photograph of a discarded thoracic cuticle after moulting. All internal ectodermal structures of the foregut including the oesophagus (oes) and pyloric chamber (pyl) are shed during exuviation and remain within the carapace. The scaphognathites (sc) and gills also undergo moulting. Scale bar, 1 cm.

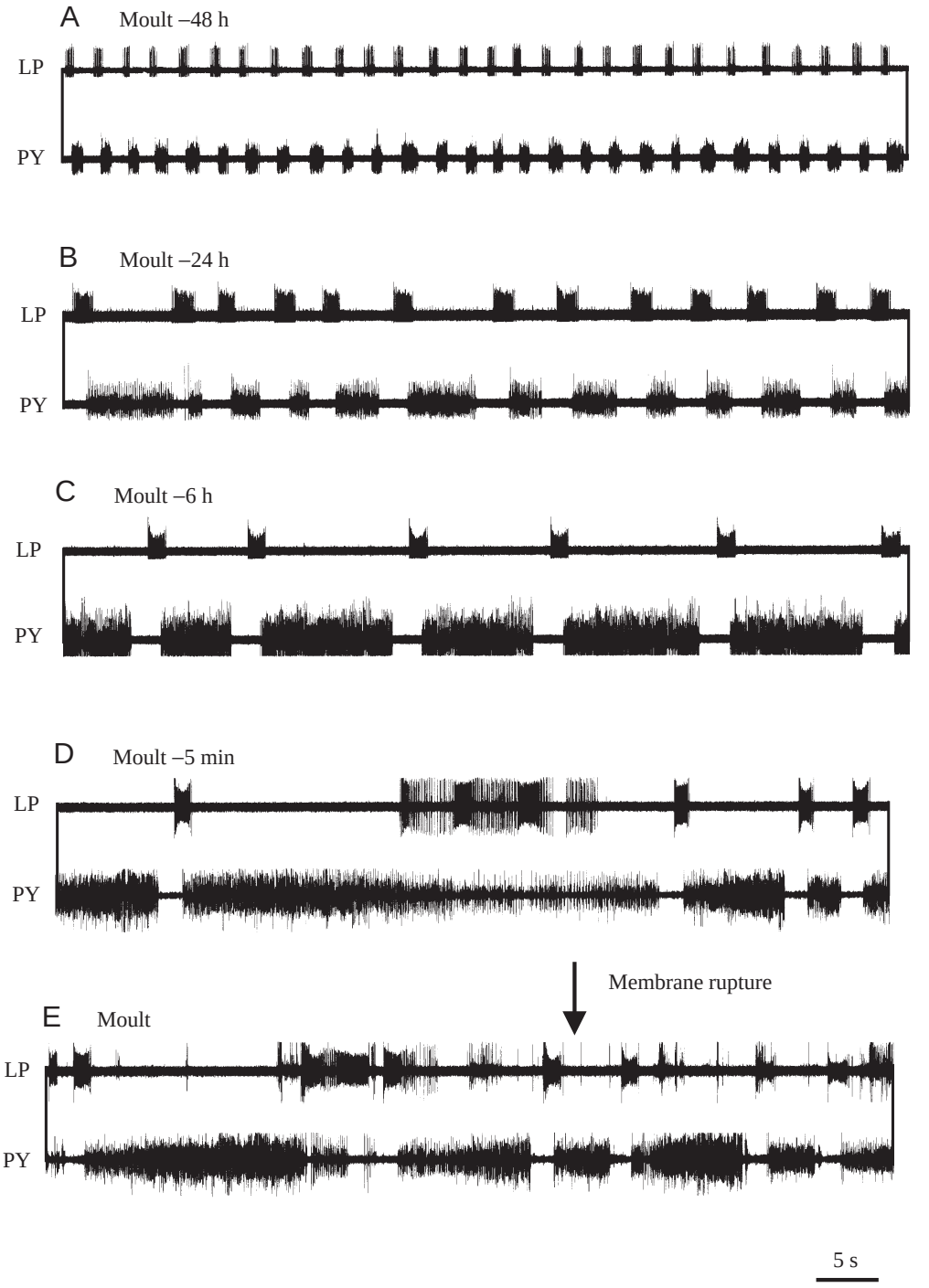

Fig. 3. Electromyographic (EMG) recordings of a lobster from late premoult until the onset of ecdysis. Recordings are from pyloric LP and PY neurone-innervated muscles. (A) Typical intermoult pattern of pyloric motor activity 2 days before moulting. (B) One day before ecdysis, pyloric periods have become considerably longer, and mean PY neurone burst durations may attain 5–8 s. (C) Six hours before the moult, a very slow but regular pyloric pattern is expressed with LP and PY neurone bursts of 2–3 s and 10–20 s, respectively. (D) A few minutes before moulting, a disrupted pattern occurs: PY neurone bursts may occasionally last 60 s, at which time LP and PY neurones may be co-active. (E) When moulting starts (at arrow), pyloric activity is still present although erratic. LP, lateral pyloric neurone; PY, pyloric neurone.

express a triphasic pattern (Fig. 1C) that is organised by inhibitory connections between the innervating motor neurones that themselves constitute the central rhythm-generating circuit (Fig. 1D). As reported initially for *Jasus lalandii* (Rezer and Moulins, 1983) and more recently for *H. gammarus* (Clemens et al., 1998a), pyloric rhythmicity is expressed spontaneously and continuously *in vivo*.

When exuviation takes place, the thoracic part of the carapace lifts up progressively (see inset to Fig. 1A) and thereby opens a wide cleft through which the animal will leave its old shell. All ectodermal tissue is shed with the moult, including the respiratory organs (gills and scaphognathites) and the anterior part of the digestive tract (oesophagus, stomach and pylorus). This was confirmed in the present study, as illustrated in Fig. 2 which shows a lateral view inside a shed carapace that had been dehydrated immediately after exuviation and partially opened to visualise internal moulted structures. Ectodermal remnants of the stomach, oesophagus and pylorus, as well as a scaphognathite and gills, are clearly visible.

Activity of the pyloric network before moulting

In resting and unfed intermoult lobsters, the pyloric network operates with a cycle period of approximately 2.5 s (Clemens et al., 1998a). It is only in a 12–48 h period just prior to ecdysis that the pyloric rhythm begins to show clear premoult influences. Fig. 3A shows one such example (*N*=3 animals) where the pyloric pattern at −48 h was still comparable to the intermoult rhythm in unfed and resting lobster. The cycle period is extremely regular (2.3±0.1 s) and gives no indication of internal physiological changes associated with the approaching moulting process. Thus, LP and PY neurone bursts last 0.7±0.1 s and 0.9±0.1 s, respectively, while the PY–LP burst interval (during which the dilator PD neurones burst; see Fig. 1C) is 1.0±0.1 s. During this time, the animal moves normally around its aquarium, but refuses food. It is not until approximately −24 h that the pyloric pattern begins to change noticeably in that cycle periods become progressively longer (6.3±1.0 s in Fig. 3B). Constrictor neurone activity also becomes substantially longer in each cycle, with LP neurone bursts now lasting 1.6±0.1 s (+130 %) and PY neurone bursts lasting as long as 10 s (3.1±0.5 s, +250 %). In contrast, the PY–LP interval increased by only 20 % to 1.2±0.1 s. A few hours later, pyloric periods reach 12–15 s (Fig. 3C), and individual constrictor burst durations begin to vary substantially from one cycle to another. However, the strictly alternating sequence of constrictor LP–PY neurone bursting remains unchanged and the PY–LP interburst interval increases slightly to 1.4±0.1 s.

Several minutes before the rupture of the thoracico-abdominal membrane and the onset of the actual moulting process, pyloric periods become even more variable and may attain as much as 60 s (see PY nerve trace; Fig. 3D). Significantly, moreover, the functional inhibitory relationship between the PY and LP neurones (see Fig. 1D) appears occasionally to weaken, since in some of these very long pyloric cycles the alternating LP–PY firing sequence becomes disrupted and the two neurone types fire concomitantly (Fig. 3D). Despite this occasional conjoint bursting in the final minutes before moult, overall rhythmic activity of the pyloric network continues to be expressed even at the moment when the thoracico-abdominal membrane ruptures and the physical process of extrusion from the old shell begins (Fig. 3E; see arrow). Such a premoult modification in pyloric activity, notably with a progressive increase in period length, occurred in all three animals observed (see Fig. 5A). Although the time course of the increase in the pyloric period displayed variability between individuals, in all cases it did not become evident until between 12 and 48 h premoult. This is further indicated by the inset of Fig. 5A, which plots mean cycle period measurements from an animal recorded for more than 3 weeks prior to moulting.

Pyloric network activity after moulting

Because of the difficulty in re-attaching EMG electrodes onto the very soft carapace immediately after ecdysis, successful recordings of pyloric activity following the moult were obtained from three animals but from only one animal in the minutes following ecdysis. Figs 4A and 5A (right side) show that pyloric rhythmicity can be recorded as little as 10 min post-moult and that, at this time, cycle periods are in the region of 4–5 s. Some 2 h later (Figs 4B, 5A), the rhythm has accelerated to period lengths of 1.1±0.1 s, and an alternating relationship can be clearly seen between the

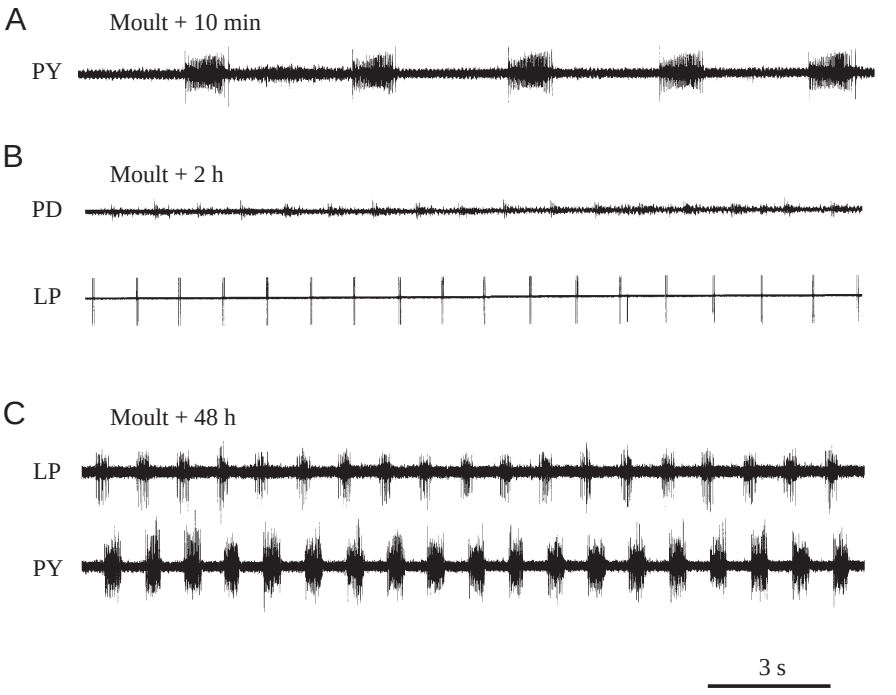

Fig. 4. Electromyographic (EMG) recordings of pyloric motor activity after the moult. (A) Rhythmic bursting in a PY neurone-innervated muscle as soon as the EMG electrode could be replaced (approximately 10 min after ecdysis). The pattern is already regular and has a mean cycle period of 4–5 s. (B) The same animal 2 h after the moult; pyloric periods have declined to 1–1.2 s. (C) Recovery of typical, albeit faster, pyloric rhythmicity at 48 h postmoult in a different animal. LP, lateral pyloric neurone; PD, pyloric dilator neurone; PY, pyloric neurone.

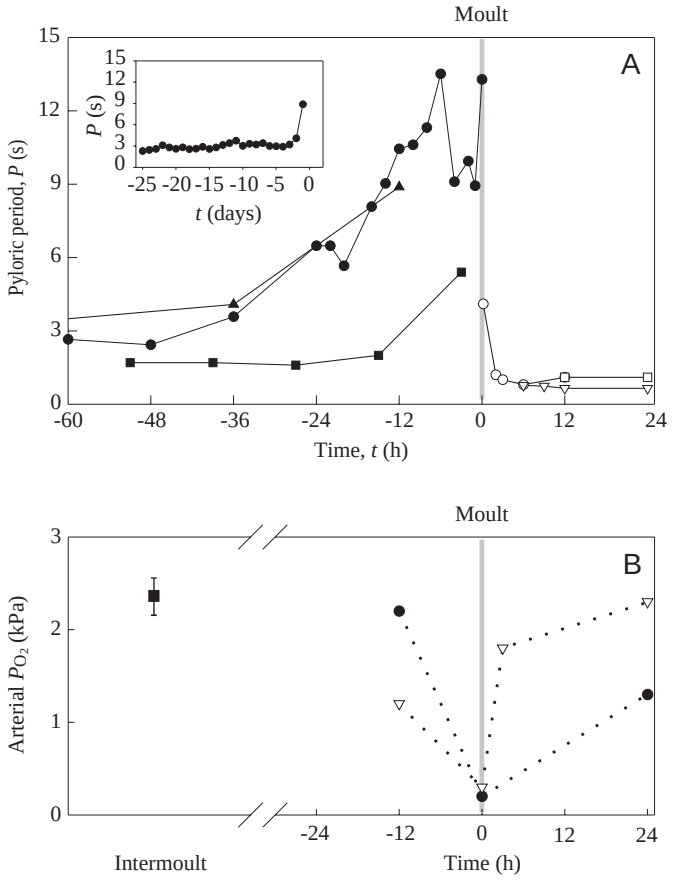

Fig. 5. Changes in the pyloric cycle period before and after moulting (A) and relationship with changes in arterial P_{O_2} (B). (A) Changes in cycle period (N=3 animals) do not appear earlier than 12–48 h premoult: the inset shows the pyloric periods of an animal recorded throughout 26 days before moulting. After the moult, typical robust pyloric rhythmicity is rapidly re-established. Each point represents the mean cycle period in a 5 min recording sample. (B) Changes in arterial P_{O_2} as a function of moult stage. Late premoult and early postmoult measurements were made in two animals; a mean intermoult value (± S.E.M.) was obtained from 22 animals. At ecdysis, P_{O_2} falls from resting values of 1–2 kPa, comparable with that during intermoult, to approximately 0.2 kPa. Postmoult recovery to control levels can occur within 2 h.

activities of the different pyloric muscles. At moult +48 h, as recorded from another animal (Fig. 4C), the activity pattern is again similar to that seen in intermoult animals (cf. Fig. 3A) albeit with a still diminished cycle period (1.0±0.1 s). Finally, it is noteworthy that, in our experimental conditions, lobsters (N=5) refused food for 1–2 weeks before and after ecdysis.

Moulting is accompanied by a transient deep hypoxia in arterial blood

Since moulting involves renewal of all ectodermal tissue, the gills and scaphognathites are replaced as well. Thus, new functional gills must already be in place beneath the old cuticle prior to the moult. This in turn necessitates a substantial thickening of the branchial tissues (Passano, 1960; Andrews and Dillaman, 1993), which causes an enhanced diffusion barrier for oxygen as well as a deterioration in pumping efficiency of the scaphognathites. Moreover, water uptake prior to ecdysis dilutes the blood and consequently decreases the O_2-transport capacity of the blood. Since the ensemble of these changes leads towards an asphyxic state characterised by a significant rise in blood lactate levels after ecdysis (Mangum et al., 1985), we monitored arterial blood oxygen levels in parallel with pyloric network activity during the moulting process to compare the relative timing of these two events. In the two animals that were successfully sampled prior to and during exuviation, arterial P_{O_2} values did not increase during late premoult (although at a time when O_2 consumption has been reported to be strongly enhanced; Passano, 1960; Penkoff and Thurberg, 1982), but fell to near-anoxic values (0.2–0.3 kPa) during ecdysis (see Fig. 5B). At this stage, the lobster is completely immobile, the cephalothorax has lifted 3–5 cm and the animal is approximately half-way through the extrusion process. Two hours after the moult, arterial P_{O_2} has returned towards the normal operational range found in the intermoult animal. This fall in arterial P_{O_2} at a time when the pyloric network activity changes so dramatically was therefore the first indication that an oxygen limitation may be modulating the pyloric circuit during ecdysis.

To examine this hypothesis more directly, we imposed hypoxic conditions on intermoult animals by specifically decreasing their levels of inspired P_{O_2} and consequently their blood P_{O_2}. Although such animals have a much lower rate of oxidative metabolism than at the late premoult stage (Passano, 1960), we wanted to explore the possibility that a forced deficit in O_2 might alone evoke changes in the pyloric motor pattern similar to those seen at ecdysis. One such experiment is shown in Fig. 6 where, under control conditions (Fig. 6A), measured arterial P_{O_2} was approximately 2 kPa and the pyloric cycle period was 2.2±0.2 s. As hypoxic conditions were gradually imposed, changes in the pyloric pattern occurred progressively in a manner similar to those observed during moulting (cf. Fig. 3). Initially, pyloric cycle periods increased up to 10 s (5.1±0.7 s) at an arterial P_{O_2} of 0.5–0.7 kPa (Fig. 6Bi), and the sequence then became disrupted with intense bursts occurring in the PY constrictor motor neurones at an arterial P_{O_2} of 0.3 kPa (Fig. 6Bii). After 3–4 h in these experimentally lowered arterial P_{O_2} conditions (Fig. 6Biii), PY neurone bursting reached 20–30 times its intermoult value, although the strict LP–PY burst alternation was still maintained, as generally seen in moulting animals (cf. Fig. 3E). Control levels of pyloric activity (period, 2.4±0.3 s) were regained after a return to control oxygenation levels in the inspired water (Fig. 6C).

To assess the modulatory effects of these near-anoxic P_{O_2} values and to test the specificity of the action of O_2, we turned to *in vitro* experiments to impose changes in oxygen levels directly and selectively on the pyloric network for 2–3 h at a constant P_{CO_2} (Fig. 7). In spontaneously rhythmic isolated STNS preparations, pyloric activity remains unaltered by P_{O_2} changes between 20 kPa (standard normoxic *in vitro*

conditions) and 3–4 kPa (physiological arterial P_{O_2} levels in postprandial lobster; Massabuau and Meyrand, 1996; Clemens et al., 1998b). Consequently, the recordings of Fig. 7A illustrate control activity (pyloric period 1.3±0.1 s) at a P_{O_2} of 20 kPa that is typical of *in vitro* experiments where O_2 supply is not limiting. The LP and PY neurones recorded expressed their characteristic activity patterns, with PY neurone bursts (0.3±0.1 s) following the LP neurone burst (0.4±0.1 s) in each cycle. When hypoxic conditions similar to those measured during actual ecdysis were imposed (i.e. STG bathing saline at a P_{O_2} of 0.1–0.2 kPa), the pyloric period became significantly longer and more variable (3.4±0.7 s, +160 %). LP and PY burst durations also lengthened and became more variable (1.5±0.4 s, +275 % and 1.2±0.3 s, +300 %, respectively); occasionally, during longer-lasting cycles, these neurones were conjointly active (Fig. 7B). These effects, which were analysed in nine experiments, were also completely reversible, as is evident in Fig. 7C.

Taken together, therefore, the qualitative coherence between these *in vivo* and *in vitro* manipulations suggests that oxygen deprivation occurring naturally in the moulting animal is sufficient to account for at least some of the major changes observed in the expression of the pyloric motor network leading up to, and at the time of, ecdysis.

Discussion

In the present study, we report for the first time on the expression of a rhythmic motor network, the stomatogastric pyloric circuit, during moulting in a crustacean. Despite dramatic and long-lasting physiological changes that occur before and after ecdysis, the pyloric network continues to

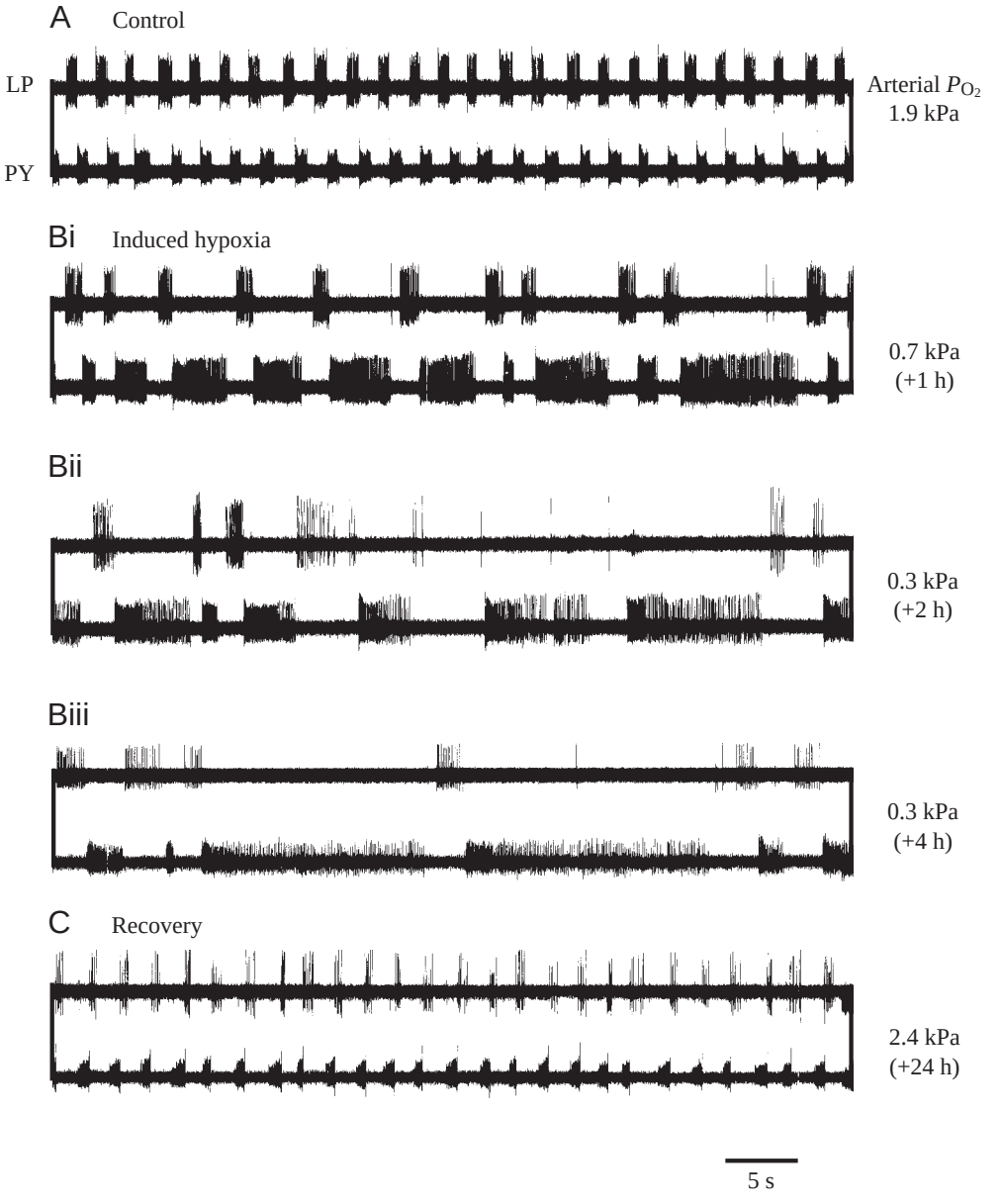

Fig. 6. The influence of an imposed deep hypoxia on pyloric motor output *in vivo*. (A) Control conditions in an intermoult animal; with arterial P_{O_2} measured at 1.9 kPa, the pyloric network operates with a cycle period of 2.5–3 s, which is typical of the intact animal. LP and PY neurone burst durations are 0.8–1 s. (B) Forcing the animal to inspire hypoxic water prolongs pyloric cycling and neurone bursting when arterial P_{O_2} reaches 0.7 kPa (Bi), and the rhythm then becomes disrupted with very long PY bursts and irregular LP neurone bursts when arterial P_{O_2} is reduced further to 0.3 kPa for 2 h (Bii) and 4 h (Biii). (C) Recovery of the regular fast pyloric rhythm recorded 24 h after a return to normoxic water.

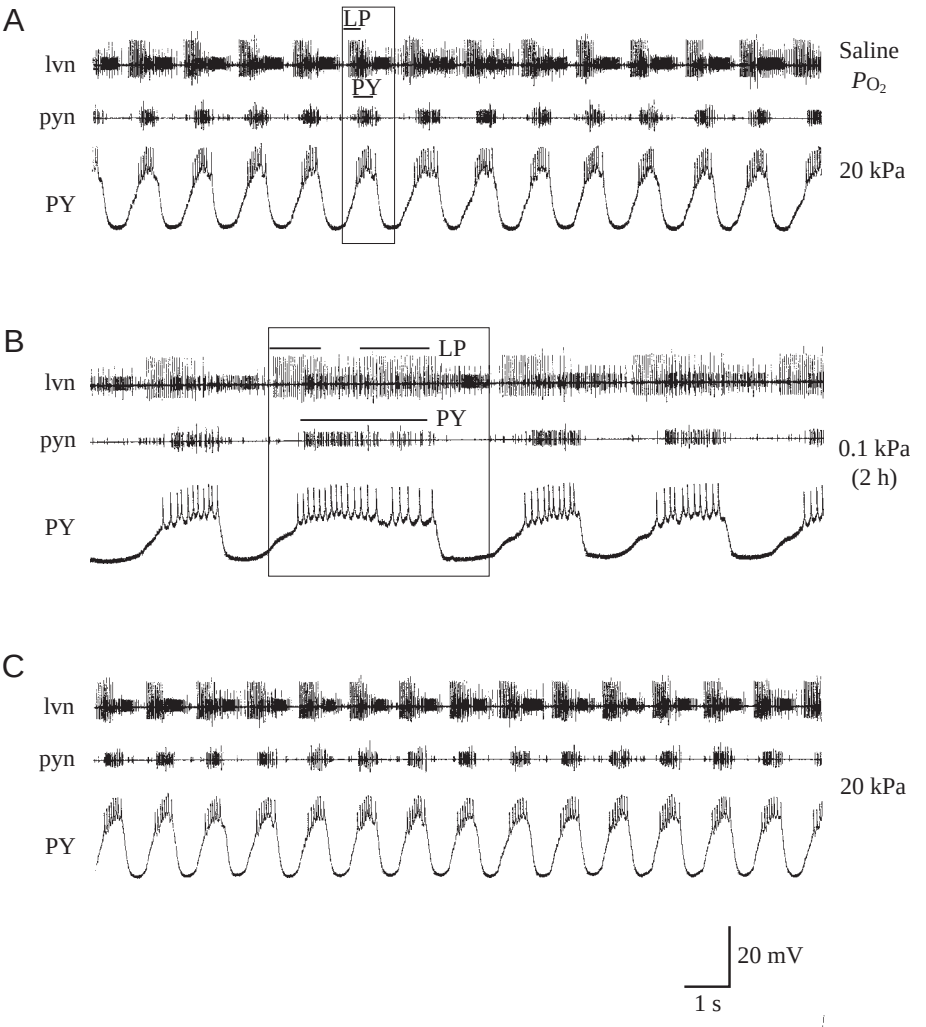

Fig. 7. The influence of a temporary deep hypoxia on pyloric network activity *in vitro*. (A) Under control conditions in saline at a P_{O_2} of 20 kPa, LP and PY neurones express typical activity patterns with LP bursts preceding PY neurone bursts (see boxed area). Extracellular recordings are from LP and PY axons in the lateral ventricular nerve (lvn) and PY in the pyloric neurone nerve (pyn), while an individual PY neurone was also recorded intrasomatically. (B) Imposing severe hypoxic conditions (P_{O_2}=0.1 kPa, similar to values measured in the moulting animal) on the stomatogastric ganglion for 2 h leads to a slowing of pyloric cycling and a prolongation of LP and PY neurone bursts. During longer-lasting PY neurone bursts (see boxed area), LP and PY may fire simultaneously. (C) Recovery of control activity after a return to normoxic saline conditions. LP, lateral pyloric neurone; PY, pyloric neurone.

elaborate a fairly typical triphasic pattern (i.e. a triphasic sequence of LP–PY–PD bursting) throughout most of this time. Our data demonstrate that only when entering the final hours prior to ecdysis, and in association with a short-lasting near-anoxia, does the functional integrity of the pyloric network become profoundly affected. Our *in vivo* data from intermoult animals and *in vitro* experiments suggest that a transient O_2 deficit is at least partly responsible for this modification, a finding that is consistent with an earlier proposal of a neuromodulator-like role for oxygen in influencing rhythmic network activity *in vivo* (Massabuau and Meyrand, 1996; Clemens et al., 1998b). It is important to note here that, since moulting is associated with an asphyxic process, the decline in available oxygen is coupled to an increase in blood P_{CO_2} (Mangum et al., 1985). However, that CO_2 itself is not contributing significantly to pyloric network modulation is strongly suggested by our intermoult control experiments in which oxygen levels were manipulated either selectively *in vitro* or without a P_{CO_2} increase *in vivo*.

The first notable change in the ongoing activity of the pyloric network during late premoult, and not earlier than approximately 48 h before actual exuviation begins, is a slowing of the pyloric rhythm (Fig. 3). At this time, arterial P_{O_2} is in the normal physiological range of 1–2 kPa (Fig. 5B) for resting, unfed animals (Clemens et al., 1998b). Subsequently, cycle periods continue to become progressively longer and more irregular, with significant cycle-to-cycle changes in phasing of neuronal activity but maintaining the basic alternating motor sequence of PD–LP–PY. Interestingly, this slowing of pyloric activity is mimicked in intermoult animals only when imposed arterial P_{O_2} reaches values as low as 0.5–0.7 kPa. Presumably, these differences in oxygen levels at which the pyloric network eventually expresses a hypoxic activity phenotype are due to differences in oxygen demand, which is known to increase considerably in premoult lobsters (Penkoff and Thurberg, 1982) and other crustaceans (Passano, 1960).

Several minutes before breakage of the thoracico-cervical membrane, the basic alternating pyloric sequence finally breaks down: burst firing becomes irregular, with variable cycle periods, and occasionally the reciprocally inhibitory LP and PY motor neurones may even fire conjointly (Fig. 3D). At this time, blood gas analysis of such animals reveals that arterial P_{O_2} values have dropped to 0.2–0.3 kPa (Fig. 5B). The conclusion that this transient near-anoxia and moult-related perturbation of the pyloric pattern may be causally linked

derived from further experiments in which intermoult animals and *in vitro* STG preparations were exposed to equivalent severe hypoxic conditions. In both cases, major alterations in LP and PY neurone bursting similar to those occurring in very late premoult animals were observed (Figs 6, 7), although *in vivo* a clear conjoint activity of LP and PY neurones was not found. Finally, it is important to note that the effects of low P_{O_2} *in vitro* were observed in experiments where O_2 partial pressure was modified selectively at the level of the STG itself. This further demonstrates the specificity of the modulatory influence of O_2 on the pyloric network, especially since, in the premoult lobster, O_2 is necessarily exerting its effects on the entire nervous system.

In arthropods, the construction of a new exoskeleton requires substantial thickening of ectodermal structures. In a recent ultrastructural study on crayfish gill epithelia (Andrews and Dillaman, 1993), it was reported that the gill is the last ectodermal tissue to be so prepared for ecdysis. These authors showed that the respiratory and ion-transporting gill filaments started deposition of pre-exuvial layers as late as premoult stages D_3 and D_4, respectively. In our experimental conditions using adult *H. gammarus* with a moult cycle of 1.5–2 years, stage D_4 corresponds to the final 10 h or so before the moult (Passano, 1960; Aiken, 1973). Andrews and Dillaman (1993) proposed that the retarded preparation of the gill for moulting was a protection against premature respiratory distress. It is also noteworthy that, at a time when the animal's O_2 needs are strongly enhanced, the ventilatory apparatus (the paired scaphognathites) itself undergoes ectodermal replacement and therefore contributes to the late premoult asphyxic status. Thus, our findings are consistent with the interpretation that these progressive alterations to the cuticle on the gill and scaphognathite structures finally impair respiratory function to the extent that P_{O_2} levels in the arterial blood decrease dramatically to 0.2–0.3 kPa (see Fig. 5). Although the pyloric network has previously been shown to be sensitive to changes in P_{O_2} at low physiological levels of 1–4 kPa (Massabuau and Meyrand, 1996; Clemens et al., 1998b), to our knowledge the effects of near-anoxic conditions on an identified and intact motor network *in vivo* have not been explored. The present study, in which perturbations to pyloric network activity both *in vivo* and *in vitro* were correlated with severe hypoxic levels during ecdysis, is the first reported example of how such extreme transient asphyxic conditions may participate in the normal physiological repertoire of the crustacean life cycle.

In the early postmoult period, the pyloric cycle frequency remained higher than during intermoult, although blood oxygenation did not increase above a P_{O_2} of 1–2 kPa. This finding is in apparent contradiction to earlier observations from *in vitro* experiments in which O_2 level was manipulated exclusively (Massabuau and Meyrand, 1996). Presumably, therefore, other as yet unknown modulatory factor(s) are acting on the pyloric pattern at this time. Also consistent with the absence of a direct relationship between O_2 level and pyloric activity is the occurrence of anaerobic metabolism during the immediate postmoult period (Mangum et al., 1985).

What could be the functional consequences, if any, of these O_2-induced changes in STNS activity? A feature of moulting in Crustacea is that considerable water ingestion provides the main source of swelling of the cephalothorax throughout the late premoult stages (Dall and Smith, 1978), and this process is thought to occur mainly *via* the lining of the digestive tract (Passano, 1960). More specifically, Drach (1939) suggested that water absorption takes place principally at the level of the foregut and, in addition to requiring an appropriate osmotic gradient, necessitates mechanical pressure generated by active muscle contraction. Although data on digestive tract motility of *H. gammarus* are lacking, an attractive possibility is that, during the late premoult phase, the progressive increase in pyloric cycle period associated with long intense bursting in the pyloric constrictor muscles (but not in the dilator muscles; see Fig. 3) participates in occluding the posterior foregut and forcing ingested water into the internal milieu. Two further roles served by the even stronger, prolonged and sometimes conjoint pyloric muscle constrictions during the very last minutes before exuviation could be (i) to compress the old pyloric cuticle (see Fig. 2) into the lumen of the pyloric chamber and thereby facilitate its eventual extrusion and (ii) to sever contact with the more posterior, non-moulting midgut. In support of these proposals, De Fur et al. (1985) reported the occurrence of large pressure pulses in the infrabranchial sinus in the blue crab *Callinectes sapidus* at ecdysis. This *de novo* activity is related to neither cardiac nor ventilatory activity but, interestingly, occurs at a frequency of $5\,min^{-1}$, which corresponds to pyloric cycle rates we find in late premoult *H. gammarus* (Fig. 3D).

It was somewhat surprising that the ongoing activity in the pyloric network is not more profoundly and long-lastingly modified until within 48 h prior to ecdysis, since circulating levels of moulting hormone (Greenaway, 1985), as well as ecdysteroid titres (Durliat et al., 1988), are enhanced early (weeks to months) in the premoult stages and do not decline until the early postmoult period. Evidently, therefore, despite undoubted major differences in the internal metabolic environments of premoult and intermoult animals, the strict pyloric motor burst sequence remains fundamentally stable until just prior to ecdysis. We have already reported that, *in vivo* under resting conditions, the pyloric network performs with a high degree of regularity (Clemens et al., 1998a) even after behavioural stimulation by feeding (Clemens et al., 1998b). The results of the present work extend this theme, indicating the extent to which the pyloric network and its ensemble of modulatory and other extrinsic influences are able to sustain network expression in the freely behaving animal faced with such a dramatic physiological event as moulting.

S.C. was the recipient of European Union Ph.D. grants ERBCHBICT 930509 and ERBFMBICT 960693 under the third and fourth Framework programme. This work was also supported by grants from the Région d'Aquitaine and the Human Frontier Science Program (HFSP). The authors wish to thank Pierre Ciret for technical assistance.

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