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IS THE RESTING RATE OF OXYGEN CONSUMPTION OF LOCOMOTOR MUSCLES IN CRUSTACEANS LIMITED BY THE LOW BLOOD OXYGENATION STRATEGY?

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Summary

Numerous water-breathers exhibit a gas-exchange regulation strategy that maintains O_2 partial pressure, P_{O_2}, in the arterial blood within the range 1–3 kPa at rest during the daytime. In a night-active crustacean, we examined whether this could limit the rate of O_2 consumption ($\dot{M}_{O_2}$) of locomotor muscles and/or the whole body as part of a coordinated response to energy conservation. In the crayfish *Astacus leptodactylus*, we compared the *in vitro* relationship between the $\dot{M}_{O_2}$ of locomotor muscles as a function of the extracellular P_{O_2} and P_{CO_2} and *in vivo* circadian changes in blood gas tensions at various values of water P_{O_2}. *In vitro*, the $\dot{M}_{O_2}$ of locomotor muscle, either at rest or when stimulated with CCCP, was O_2-dependent up to an extracellular P_{O_2} of 8–10 kPa. *In vivo*, the existence of a night-time increase in arterial P_{O_2} of up to 4 kPa at water P_{O_2} values of 20 and 40 kPa was demonstrated, but an experimental increase in arterial P_{O_2} during the day did not lead to any rise in whole-body $\dot{M}_{O_2}$. This suggested that the low blood P_{O_2} in normoxia has no global limiting effect on daytime whole-body $\dot{M}_{O_2}$. The participation of blood O_2 status in shaping the circadian behaviour of crayfish is discussed.

Key words: crayfish, crustacean, behaviour, oxygen, hypoxia, modulation, muscle, blood O_2 transport, metabolism.

Introduction

In a variety of physiologically different crustaceans, there exists a strategy of gas exchange regulation that consists, in resting and unfed conditions, of maintaining the partial pressure of oxygen (P_{O_2}) in the blood within a low and narrow range irrespective of the oxygenation level of the ambient water. Both in the laboratory (Massabuau and Burtin, 1984; Massabuau et al., 1991; Forgue et al., 1992; Ellis and Morris, 1995; Gannon and Wheatly, 1992, 1995) and in field conditions (Adamczewska and Morris, 1994; Massabuau and Forgue, 1996), measured blood P_{O_2} is usually within the range 1–3 kPa, irrespective of water P_{O_2} between 3 and 40 kPa. This particular adaptive pattern is known as the low blood P_{O_2} strategy (Massabuau and Burtin, 1984; Forgue et al., 1989).

The fact that animals operate with such a low blood P_{O_2} raises numerous questions. Indeed, various O_2-dependent metabolic processes and all O_2-utilizing enzymes, except cytochrome *c* oxidase, exhibit K_{mO_2} values (the oxygenation value at which 50 % of the maximal initial velocity of a reaction occurs) that are much higher than the mean O_2 concentration at the physiological P_{O_2}, suggesting *in vivo* limitations (Jones et al., 1985; Vanderkooi et al., 1991). We have previously studied some consequences of this low blood P_{O_2} strategy on the neural networks of crustaceans that generate the masticating and filtering movements of the foregut (the gastric and pyloric circuits of the stomatogastric nervous system). Using freely behaving animals and *in vitro* preparations of isolated stomatogastric ganglia, we demonstrated that the arterial P_{O_2}, normally 1–2 kPa between meals in the lobster *Homarus gammarus* during the day, limits (in a neuromodulator-like manner) the rapid postprandial expression of the pyloric rhythm and promotes a strong interaction between the gastric and pyloric networks. Interestingly, we also found that, within 1 h of feeding, the arterial P_{O_2} doubles, before returning to resting values some 24 h later, and that this specifically allows an increase in pyloric frequency and the uncoupling of the gastric and pyloric rhythms characteristic of food processing in the foregut. Indeed, the experimental suppression of this transient rise in arterial P_{O_2} prevents these changes (Massabuau and Meyrand, 1996; Clemens et al., 1998). This clearly suggests that regulation of the arterial P_{O_2} to various apparent set points in the low, but physiological, P_{O_2} range makes an active contribution to the generation of behavioural patterns in water-breathers by modulating neural network activity.

The primary aim of the present work was to examine whether similar blood P_{O_2} changes are associated with the

circadian rhythm of locomotor activity in crustaceans (Brown, 1961) and can directly influence the oxidative metabolism of locomotor muscles. In these animals, direct *in vivo* measurements of tissue-specific rates of O_2 consumption raise major technical difficulties, but insights can be gained from cellular studies using the crustacean limb preparation (Fatt and Ginsborg, 1958). This preparation allows direct measurements of rate of O_2 consumption of isolated muscle without the added problems resulting from artificial perfusion. The present work was performed in the freshwater crayfish *Astacus leptodactylus* because a circadian rhythm of ventilatory activity has been reported in this species (Sakakibara et al., 1987) and because of the occurrence of the low daytime blood P_{O_2} strategy (Massabuau and Burtin, 1984). In experiments performed both *in vivo* and *in vitro*, we studied the effects of changes in P_{O_2} and P_{CO_2} that were either freely regulated by the animals or experimentally imposed. Our results show that circadian changes in respiratory blood gas content occurring spontaneously in freely behaving *A. leptodactylus* may participate in shaping the circadian rhythm of activity by direct action on the locomotor muscles themselves in a manner directly related to water O_2 availability, and we also illustrate how intracellular P_{O_2} changes associated with the metabolic activity of the muscle cells can contribute to the rate of O_2 supply.

Materials and methods

Animals and ambient conditions

Experiments were performed on 62 male crayfish *Astacus leptodactylus* in the intermoult stage and weighing 30–60 g. They were acclimated in the laboratory for at least 1 month before the experiments and fed weekly on carrots and fish. To minimise external disturbance during *in vivo* studies (blood sampling and O_2 consumption analysis), experimental tanks were isolated from vibrations by antivibration benches, and the animals could not see the experimenters. They were maintained under natural light conditions at 13 °C (P_{O_2} 2.5, 6, 10, 21 or 40 kPa; P_{CO_2} 0.1 kPa; pH 8.30–8.40). Prior to experiments, the animals were not fed for 5–7 days. For reference, 1 kPa=7.5 mmHg (=7.5 Torr) and, in a saline solution or in water, 1 kPa corresponds to an O_2 fraction of approximately 1 %. Three different types of experiment were performed.

Oxygen consumption of isolated muscle

Carpopodite extensor muscles from locomotor limbs were prepared as described by Fatt and Ginsborg (1958). The limbs were obtained by autotomy, and the carpopodite (attached to a piece of meropodite) was isolated. A window was cut on its convex side, and the flexor muscles, the gross innervation and the arteries were removed to expose the muscle from its apodeme to the cuticle. The preparation was then enclosed in a 2 ml open-flow respirometer thermostatted at 13 °C with the mero-carpopodite joint slightly bent to keep the extensor muscles under tension. The respirometer was supplied with saline at a constant flow rate by a Braun–Melsungen perfusion pump placed in series after the respirometer and a reservoir of saline. The saline was stirred magnetically (rotation frequency, 1 Hz) to ensure a homogeneous composition in the chamber so that the gas composition of the saline at the level of the muscle was representative of the saline exiting the chamber. The extracellular P_{O_2} value was measured directly in the chamber using a YSI electrode whose output was displayed continuously on a Sefram pen recorder. At steady state, the rate of oxygen consumption, $\dot{M}_{O_2}$, can be described according to the Fick principle:

$$\dot{M}_{O_2} = \dot{V}_{sal} \times \alpha_{salO_2} \times (P\mathrm{in}_{O_2} - P\mathrm{e}_{O_2}),$$

where $\dot{V}_{sal}$ is the rate of flow of saline through the respirometer; α_{salO_2} is the solubility of oxygen in the saline (12.4 μmol l^{-1} kPa^{-1}), $P\mathrm{in}_{O_2}$ is the O_2 partial pressure of the saline entering the respirometer and $P\mathrm{e}_{O_2}$ is the O_2 partial pressure of the saline exiting from the respirometer and is representative of the extracellular P_{O_2}.

The $N_2/O_2/CO_2$ gas mixture was obtained using Wösthoff pumps that bubbled gas through the reservoir of saline feeding the preparation. Two subsets of experiments were performed: (i) variable saline P_{O_2} (2–30 kPa) and constant P_{CO_2} (0.4 kPa) and (ii) variable saline P_{CO_2} (0.1–0.5 kPa) and constant P_{O_2} (5 kPa). The duration of exposure was 60–90 min at each P_{O_2} or P_{CO_2}. In experiment i, to detect possible effects of cellular damage resulting from hypoxia on the measurements, the order of presentation of P_{O_2} was 12–15, 8–9, 6–8, 4–5, 3–4 and 25–30 kPa with 12–15 kPa taken as the reference $\dot{M}_{O_2}$ and 25–30 kPa as the control $\dot{M}_{O_2}$. Note that all the CO_2 partial pressures we used are close to normal *in vivo* blood P_{CO_2} in *Astacus leptodactylus* (Sakakibara et al., 1987). In all conditions, O_2 consumption by the isolated limb preparations was stimulated by the addition of 4 μmol l^{-1} carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, Sigma), an uncoupler of H^+ transport. The composition of the physiological saline used for superfusion was: NaCl, 195 mmol l^{-1}; KCl, 5 mmol l^{-1}; $CaCl_2$, 10 mmol l^{-1}; $MgCl_2$, 10 mmol l^{-1}; $NaHCO_3$, 6 mmol l^{-1}. The pH was adjusted to a physiological value of approximately 7.8 with CO_2.

At the end of each experiment, the mass of muscle was estimated by measuring differences in wet mass before and after separation of the muscle cells from the exoskeleton.

Blood P_{O_2} *measurements as a function of circadian rhythm*

Five groups of 14 crayfish were each exposed to one level of oxygenation (P_{O_2} 2.5, 6, 10, 21 or 40 kPa) to which they were acclimated for 2–3 days prior to sampling. The $N_2/O_2/CO_2$ gas mixture was obtained *via* laboratory-made mixing systems using mass flow controllers (Tylan General, model FC-260). The crayfish were kept in a 7 l tank, and the water was renewed at a rate of 1 l h^{-1}. During experiments, the pH and the P_{CO_2} of the water were controlled using a pH/CO_2-stat (Dejours and Armand, 1980). On day 1, between 09:30 and 10:00 h, the crayfish were placed in the experimental tanks (time t_0) in which the water P_{O_2} had been adjusted to the

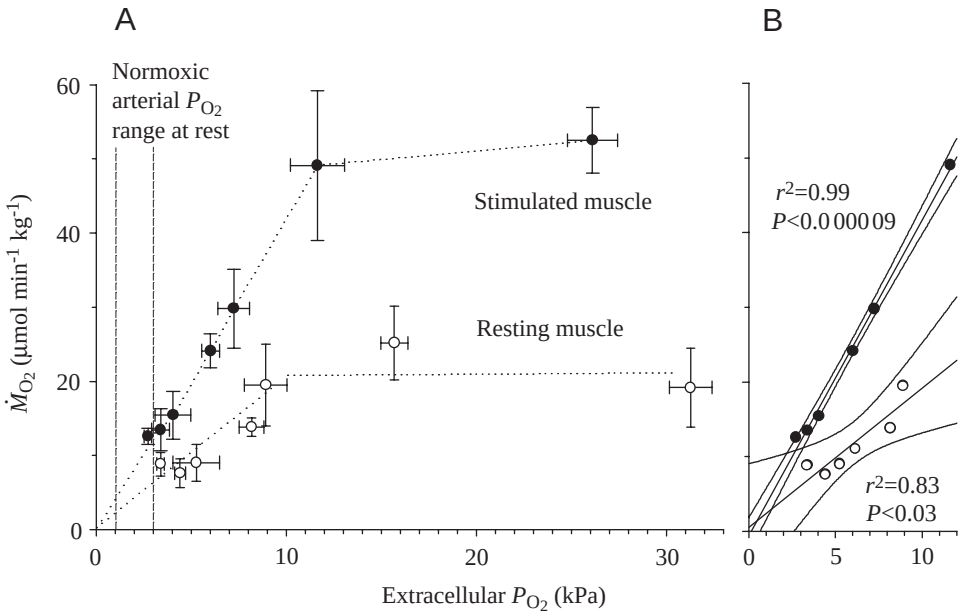

Fig. 1. *In vitro* relationship between the rate of O_2 consumption ($\dot{M}_{O_2}$) of carpopodite extensor muscles from the locomotor limbs of crayfish *Astacus leptodactylus* and extracellular P_{O_2} in resting and CCCP-stimulated preparations. (A) In resting and CCCP-stimulated muscle, $\dot{M}_{O_2}$ was independent of P_{O_2} only at extracellular P_{O_2} values above 8–10 kPa. At all saline P_{O_2} values, $\dot{M}_{O_2}$ was significantly (P<0.005) enhanced by the addition of CCCP. Characteristically, the arterial P_{O_2} range in resting normoxic crayfish (vertical broken lines) was in the low range of the O_2-dependent part of the relationships, suggesting the existence of *in vivo* O_2-limitation. Values are means ± S.E.M., N=7. (B) Statistical analysis of the O_2-dependent relationships over the range 0–12 kPa; linear regression lines and 95 % limits. Extracellular P_{CO_2}=0.4 kPa.

desired value. Animals from one group were sampled twice: first 1 day later in the morning, between 10:00 and 12:00 h (t_0+23–24 h) and then in the evening of the following day between 22:00 and 24:00 h (t_0+60–62 h). To sample arterial blood, a hole was drilled in the shell at the level of the heart, leaving a thin layer of cuticle in place. A piece of rubber was glued over this hole. This procedure was carried out at least 5 days before measurements were taken. Blood samples were collected by removing the crayfish from the water and puncturing the rubber membrane with glass capillary tubes equipped with a needle. The blood sample (100 μl) was obtained within the first 15–30 s of emersion. Using this technique, arterial blood enters the tube spontaneously and heart beats are visible. This sampling technique was assessed critically by Massabuau and Forgue (1996) and considered to provide true *in vivo* resting values not significantly different from values measured using indwelling chronic catheters (Massabuau and Burtin, 1984). After sampling, the blood was stored on ice and analysed within 1–3 min.

Whole-body rates of oxygen consumption as a function of circadian rhythm

Rates of oxygen consumption were determined in seven normoxic crayfish, unfed for 3–5 days, as described by Massabuau et al. (1984). The respirometer was equipped with a pH/CO_2-stat and the water was stirred to ensure a homogeneous composition throughout the system so that the composition of the water exiting the chamber closely represented that of the water inspired by the crayfish. A laboratory-made automatic device continuously monitored P_{O_2} in the exit water and adjusted the water flow through the respirometer to clamp the inspired P_{O_2} value of the animal at the desired value. Consequently, the inspired P_{O_2} was maintained within a narrow range (±0.5 kPa) irrespective of the changes in $\dot{M}_{O_2}$ induced by the experimental conditions. The crayfish were placed in a respirometer at approximately 13:00 h, and measurements were performed the following day between 10:00 and 12:00 h and between 22:00 and 24:00 h. Note that steady-state measurements were performed on resting animals that had not been handled for at least 20 h, and P_{O_2} was measured automatically with electrodes manipulated by remote control to ensure unstressful conditions.

Statistical analyses

Values are reported as mean values ± 1 standard error (S.E.M.) unless stated otherwise. Differences were evaluated using a Mann–Whitney U-test, a two-tailed Student's t-test, a Fisher test and/or analysis of variance (ANOVA) as appropriate. P<0.05 was taken as the fiducial limit of significance.

Results

When the resting extensor muscle of the carpopodite were superfused *in vitro* with a control saline equilibrated at a physiological P_{O_2} of approximately 3 kPa (i.e. the upper range of arterial P_{O_2} measured *in vivo*), $\dot{M}_{O_2}$ was O_2-limited (Fig. 1A, open symbols): increasing P_{O_2} in the extracellular medium (Pe_{O_2}) resulted in an increase in $\dot{M}_{O_2}$. Only when the saline P_{O_2} exceeded 8–10 kPa was a plateau value reached. In the O_2-dependent range (3 kPa≤Pe_{O_2}≤9 kPa), the equation describing the relationship between resting $\dot{M}_{O_2}$ and Pe_{O_2} was: $\dot{M}_{O_2}$=1.87Pe_{O_2}+0.5 (r^2=0.83, P=0.03). To gain more insight into this extracellular O_2-limitation problem, muscle $\dot{M}_{O_2}$ was

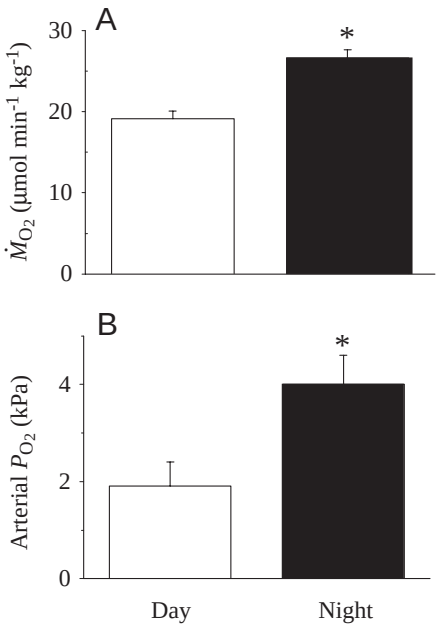

Fig. 2. *In vivo* analysis of the circadian change in the rate of O_2 consumption ($\dot{M}_{O_2}$) and in arterial P_{O_2} occurring spontaneously in freely behaving crayfish *Astacus leptodactylus* in normoxic laboratory conditions. At night, when the animals are spontaneously active and walking in the experimental tanks, whole-body $\dot{M}_{O_2}$ increased (A) and arterial P_{O_2} rose from 2 kPa (resting value) to 4 kPa (B). $\dot{M}_{O_2}$ analysis, N=7 crayfish; blood P_{O_2} analysis, N=14. Water P_{O_2}=20–21 kPa, water P_{CO_2}=0.1 kPa. Values are means + S.E.M.; an asterisk indicates a significant difference between night and day values (P<0.05).

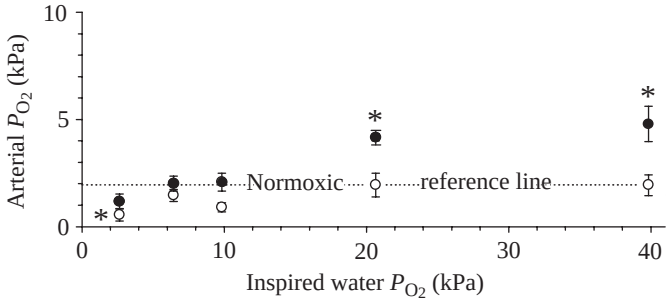

Fig. 3. *In vivo* relationship between arterial P_{O_2} and inspired water P_{O_2} at two different periods (rest and activity) of the circadian rhythm in the crayfish *Astacus leptodactylus*. Daytime values (open symbols) did not differ significantly for water P_{O_2} values ranging from 6 to 40 kPa (horizontal dotted line). They can be attributed to a first apparent set point characteristic of resting behaviour. Nocturnal values (filled symbols) did not differ significantly for water P_{O_2} values of 21 and 40 kPa. They can be attributed to a second apparent set point characteristic of night behaviour. Day and night values are paired measurements determined after a 24 h period at a given water P_{O_2}. Water P_{CO_2}=0.1 kPa. Values are means ± S.E.M.; an asterisk indicates a significant difference in comparison with the day value in normoxia (21 kPa) (P<0.05). N=14 at each water P_{O_2}.

stimulated by adding 4 µmol l^{-1} CCCP to the superfusate. Fig. 1A shows that, at all values of Pe_{O_2}, $\dot{M}_{O_2}$ increased in these stimulated conditions compared with control conditions and that $\dot{M}_{O_2}$ was dependent on Pe_{O_2} up to 10–12 kPa. The relationship between $\dot{M}_{O_2}$ and Pe_{O_2} between 3 and 12 kPa was: $\dot{M}_{O_2}$=4.2Pe_{O_2}–0.5 (r^2=0.99, P<0.000009, Fig. 1B). Importantly, note that the O_2-induced effect was reversible: a 6–7 h exposure period at P_{O_2} values as low as 2–3 kPa did not lead to any statistical difference between the control (Pe_{O_2} 12–15 kPa) and recovery (Pe_{O_2} 25–30 kPa) periods (paired t-tests). This absence of any change in $\dot{M}_{O_2}$ suggests that cellular integrity had mostly been maintained in our experimental preparations irrespective of the P_{O_2}. These observations were the first indication that the low blood P_{O_2} strategy in crustaceans could limit the $\dot{M}_{O_2}$ of locomotor muscles at rest and during activity. It is also important to note that all unstimulated $\dot{M}_{O_2}$ values were doubled by CCCP at all extracellular P_{O_2} values (P<0.005), which suggested, according to the first law of Fick, an unexpected major effect of intracellular P_{O_2} on the supply of O_2 to the muscle cells.

To study what is happening in intact and unrestrained animals when they switch from resting behaviour to exercise in normoxic water, we performed *in vivo* experiments by taking advantage of the spontaneous circadian rhythm of activity in crayfish. Fig. 2A shows the corresponding changes in $\dot{M}_{O_2}$, which increased significantly by 35±5 % at night compared with the daytime value (from 19.2±0.8 to 26.8±1.1 µmol min^{-1} kg^{-1}, means ± S.E.M., N=7). The associated changes in arterial blood P_{O_2} are presented in Fig. 2B. During the day, the resting arterial P_{O_2} was 1.9±0.5 kPa, and this increased to 4.0±0.6 kPa at night (N=14, means ± S.E.M.) when the crayfish were active in the experimental tanks.

These observations suggested that the low arterial P_{O_2} strategy observed *in vivo* in resting animals could contribute to the limitation of O_2 supply to the limb muscles and demonstrate that the nocturnal increase in locomotor activity was associated with an increase in arterial P_{O_2}. During the daytime, arterial P_{O_2} is regulated at an apparent set point over a large range of inspired P_{O_2} values (see Introduction). To determine whether in normoxic water the arterial P_{O_2} measured during the night activity period corresponded to a different apparent set point, we first repeated the analysis under conditions of hyperoxia (water P_{O_2}=40 kPa). Fig. 3 shows that, in these conditions, arterial P_{O_2} measured at night after a 24 h exposure to 40 kPa was 4.7±0.8 kPa (N=14, mean ± S.E.M.), which was not significantly different from the night-time value in normoxia (21 kPa, P<0.05). This was a strong indication that the higher arterial P_{O_2} reached in these non-limiting water P_{O_2} conditions during the nocturnal period of activity may indeed be a second apparent set point. To test whether this level of arterial P_{O_2} was also maintained in hypoxic conditions, we then exposed another group of animals to a series of 24 h exposures at various selected water P_{O_2} values ranging from 2.5 to 10 kPa. The results presented in Fig. 3 show that arterial P_{O_2} was significantly reduced. Although such an observation was expected for water P_{O_2} values of 2.5 and 6 kPa, this result was more surprising for a water P_{O_2} of 10 kPa, at which the night-time arterial P_{O_2} was

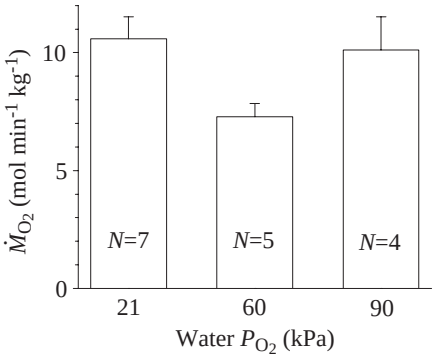

Fig. 4. Whole-body rates of O_2 consumption ($\dot{M}_{O_2}$) in crayfish *Astacus leptodactylus* at water P_{O_2} values of 21, 60 and 90 kPa. All animals studied at 21 kPa were exposed at 60 and/or 90 kPa (paired analysis). Measurements were made in resting animals during the day after a 24 h exposure period. $\dot{M}_{O_2}$ in normoxic water (21 kPa) was taken as the reference value. Hyperoxic exposure was not associated with an increase in $\dot{M}_{O_2}$ despite an associated increase in arterial P_{O_2}. Values are means + S.E.M.; the number of crayfish studied at each water P_{O_2}, is given.

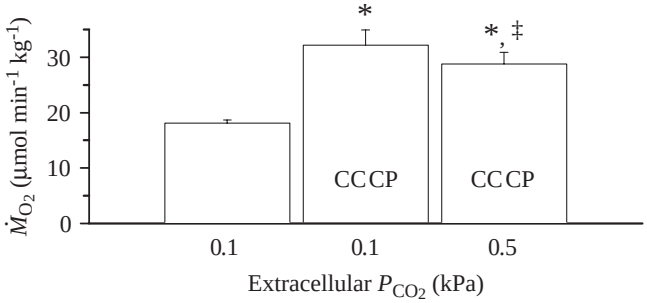

Fig. 5. *In vitro* influence of extracellular P_{CO_2} on the CCCP-stimulated rate of O_2 consumption ($\dot{M}_{O_2}$) of carpopodite extensor muscles from the locomotor limbs of crayfish *Astacus leptodactylus* (extracellular P_{O_2}=5 kPa). The CCCP-stimulated $\dot{M}_{O_2}$ at an extracellular P_{CO_2} of 0.1 kPa was taken as the reference value. In CCCP-stimulated preparations, an increase in P_{CO_2} from 0.1 to 0.5 kPa reduced $\dot{M}_{O_2}$ significantly (paired analysis). Values are means + S.E.M.; N=8 crayfish. An asterisk indicates a significant difference compared with the extracellular P_{CO_2} of 0.1 kPa (P<0.05); a double dagger indicates a significant difference compared with the CCCP-stimulated $\dot{M}_{O_2}$ at an extracellular P_{CO_2} of 0.1 kPa (P<0.05).

only 2.1±0.4 kPa (N=14, mean ± S.E.M.). To test whether this was the result of a true gas-exchange limitation or was a new set point, we stressed a group of crayfish by handling (during the day) them for 5 min in a tank in which the water P_{O_2} was 10 kPa. Under these stressful conditions, the arterial P_{O_2} was 7.3±1.4 kPa (N=5), demonstrating that, at a water P_{O_2} of 10 kPa, the arterial P_{O_2} measured at night in unstressed animals was not O_2-limited. Finally, it is noteworthy that, at water P_{O_2} values of 2.5–10 kPa, most animals remained remarkably inactive and their $\dot{M}_{O_2}$ was not significantly different from daytime values at the same water P_{O_2}.

This new set of data shows that, at water P_{O_2} values of 21 and 40 kPa, resting behaviour during the day is associated with low arterial P_{O_2} and that activity at night is associated with higher P_{O_2} values. What happens if the arterial P_{O_2} is experimentally increased during the daytime resting period? To study this question, we experimentally manipulated the blood P_{O_2} by exposing two groups of animals for 24 h to water P_{O_2} values of 60 or 90 kPa (at a water P_{O_2} of 60 kPa, the mean arterial P_{O_2} was 8.2±1.9 kPa, N=5). The aim was to determine whether a daytime arterial P_{O_2} of approximately 1–3 kPa actively contributes to the lowering of $\dot{M}_{O_2}$ in a manner similar to hypoxia-induced metabolic rate depression. Fig. 4 clearly shows that this was not the case: whole-body $\dot{M}_{O_2}$ was not increased by this manipulation. To clarify the clear-cut difference in the data obtained with the isolated limb muscle (see Fig. 1), we weighed the total limb muscle (eight limbs) and compared this with the whole-body mass. The muscle represented only 0.4±0.1 % of the total body mass, explaining the lack of any possible influence of O_2-dependent limb muscle metabolic activity (and of any other comparable muscles, both in terms of fibre type and tissue quantity) on whole-body $\dot{M}_{O_2}$. Note also that hyperoxia did not stimulate the level of activity in the experimental animals.

Finally, it must be borne in mind that the low blood P_{O_2} strategy observed in animals at rest during the day is the result of a low ventilatory activity, reported to be associated with a hypercapnic acidosis (Sakakibara et al., 1987). This change in the blood acid–base balance associated with changes in metabolic rate is reminiscent of previously described relationships in various cellular studies and during aestivation and hibernation (Busa and Nucitelli, 1984; Malan, 1993; Guppy and Withers, 1999). Fig. 5 shows that the $\dot{M}_{O_2}$ of CCCP-stimulated muscles could also be pH-dependent. In this experiment, the $\dot{M}_{O_2}$ of eight preparations was again CCCP-stimulated, and P_{CO_2} was changed from 0.1 to 0.5 kPa. It is clear that the addition of CO_2 was associated with a significant decrease in $\dot{M}_{O_2}$ in the CCCP-stimulated muscle (P<0.05). Thus, the ventilatory-induced hypocapnic alkalization associated with the night-time increase in arterial P_{O_2} could favour night-time metabolic activation.

Discussion

In this study, we present evidence to show (i) that the low blood P_{O_2} measured in resting crayfish *Astacus leptodactylus* during the day can limit the resting $\dot{M}_{O_2}$ of locomotor muscles, (ii) that, in freely behaving normoxic or hyperoxic crayfish, there is a circadian rhythm in arterial P_{O_2} that occurs concomitantly with the circadian activity rhythm, (iii) that the experimental increase in blood P_{O_2} above normoxic daytime values does not increase whole-body $\dot{M}_{O_2}$ and (iv) that both intracellular P_{O_2} and the P_{O_2} difference across the cell membrane may deeply affect the flow of O_2 to working muscle. More specifically, we show that, for water P_{O_2} values ranging from 21 to 40 kPa (i.e. when the water oxygen supply is not limiting), daytime resting behaviour is associated with an arterial P_{O_2} of approximately 2 kPa and nocturnal activity with

an arterial P_{O_2} of approximately 4 kPa. This is in good agreement with the circadian rhythm of ventilatory activity previously reported by Sakakibara et al. (1987) in the same species. When the crayfish are at rest during the day, an experimental increase in arterial P_{O_2} from 2 to approximately 8 kPa does not increase whole-body $\dot{M}_{O_2}$, suggesting that, rather than having a global limiting effect on daytime $\dot{M}_{O_2}$ (*via* a type of hypoxia-induced depression of the metabolic rate), the circadian changes in crayfish blood O_2 levels could selectively influence the oxidative metabolism of locomotor muscle by limiting its resting rate of O_2 uptake. We propose that the low blood P_{O_2} value regulated during the daytime in unfed animals may contribute to shaping the resting behaviour of crayfish *via* direct action on the locomotor muscles themselves.

In normoxic and hyperoxic water, the low blood P_{O_2} strategy does not appear to be part of a coordinated response to improve global energy conservation during daytime resting periods. However, at night and for water P_{O_2} values in the range 2.5–10 kPa, the situation may be quite different, and we suggest that arterial P_{O_2} values at night could contribute to this global depression. We propose the existence of two types of hypoxia-induced metabolic rate depression: (1) the accepted environmentally induced hypoxia during which $\dot{M}_{O_2}$ drops below a minimal water P_{O_2}, and (2) a behaviourally self-imposed blood hypoxia that limits activity in mild hypoxic media, thereby creating a safety margin for blood oxygenation level so that the animal does not have to resort to anaerobic metabolism.

The first type is the classical phenomenon reported in the literature (Hochachka and Somero, 1984; Guppy and Withers, 1999; St-Pierre et al., 2000) and was unequivocally observed in the present work at a water P_{O_2} of 2.5 kPa. For *A. leptodactylus*, this P_{O_2} corresponds to the critical value below which resting $\dot{M}_{O_2}$ measured during the day cannot be maintained at 13–15 °C (Massabuau and Burtin, 1984). At this water P_{O_2}, both day and night arterial P_{O_2} values were significantly lower than the corresponding day and night values at water P_{O_2} values of 21 and 40 kPa (Fig. 3). It is obvious that the blood P_{O_2} values at 2.5 kPa were gas-exchange-limited and that they imposed a strict limit on any daytime rise in $\dot{M}_{O_2}$, forcing a depression of night-time $\dot{M}_{O_2}$. The second type of hypoxia-induced metabolic rate depression was observed at a water P_{O_2} of 10 kPa, i.e. during mild hypoxia. Indeed, at rest during the day, the arterial P_{O_2} (Fig. 3) and whole-body $\dot{M}_{O_2}$ were not significantly different from normoxic values (Fig. 3; Massabuau and Burtin, 1984) and the oxygenation level in the interior milieu was far from being gas-exchange-limited, as illustrated by the experiment with stressed animals. However, at night, the arterial P_{O_2} values were at daytime levels, the $\dot{M}_{O_2}$ did not increase and the crayfish remained inactive. We suggest that, at a water P_{O_2} of 10 kPa, the crayfish freely imposed on themselves this low night arterial P_{O_2} value, which limits their aerobic scope and can be attributed to a particular type of hypoxia-induced metabolic rate depression. This observation is, to our knowledge, the first reported example of how such a behavioural adaptation may form part of the normal physiological repertoire of the crustacean life cycle. Note also that, in an ecology-based context, this strategy provides two safety factors. First, it limits the switching on of activity in a hypoxic medium in which excessive exercise could become O_2-limited. Second, in the case of unavoidable stress, it provides a safety margin for increasing the blood oxygenation level and thus fuelling the oxidative locomotor muscles without the animal being on the verge of anaerobic metabolism.

A very significant observation from Fig. 1, besides the fact that the $\dot{M}_{O_2}$ of locomotor muscle was limited to 8–10 kPa, was that at all extracellular P_{O_2} values the resting $\dot{M}_{O_2}$ of the isolated limb preparation was significantly enhanced when mitochondrial oxidation was stimulated by the addition of CCCP. Importantly, this demonstrated that when the O_2 demand increased, the extracellular P_{O_2} (and consequently the arterial P_{O_2}) did not dictate an absolute limit to the $\dot{M}_{O_2}$ of the carpopodite extensor muscle. How can $\dot{M}_{O_2}$ double under such conditions? It must first be recalled that, once the external O_2 concentration is sufficient, i.e. once there is a large enough O_2 reservoir in the vicinity of the muscle, as in our open-flow respirometer, the rate of O_2 transfer from extra- to intracellular medium depends on diffusive conductance and the P_{O_2} difference (ΔP_{O_2}) across the cell membrane (first law of Fick, see Dejours, 1981). In the present *in vitro* experimental design, the diffusive conductance remained constant (constant temperature and homogenisation rate in the extracellular space), so that, at constant extracellular P_{O_2}, the intracellular P_{O_2} decreased in response to the mitochondrial O_2 demand. Hence, the doubling of $\dot{M}_{O_2}$ seen in Fig. 1 in response to treatment with CCCP indicates a doubling of ΔP_{O_2}, which in turns implies that, prior to stimulation, the intracellular P_{O_2} was no less than half the extracellular P_{O_2}. Taken together, these observations suggest that, in the carpopodite extensor muscle, the diffusive conductance should be reasonably low (in comparison with P_{O_2}), and this is consistent with the fact that crustaceans can operate at extremely low blood P_{O_2} values (McMahon, 1985; Forgue et al., 1992; Truchot, 1992; Ellis and Morris, 1995). Obviously, this phenomenon may play a significant role in any increase of O_2 influx into crustacean cells. It can be associated with an increase in arterial P_{O_2} (present data) or, at constant and low arterial P_{O_2}, it can explain the postprandial doubling of $\dot{M}_{O_2}$ 24 h after a meal in the green crab *Carcinus maenas* (Legeay and Massabuau, 1999).

In *Astacus leptodactylus*, blood P_{O_2} changes occurring during the circadian rhythm are associated with a statistically significant daytime hypercapnic acidosis and a night-time hypocapnic alkalosis (the blood P_{CO_2} change is approximately 0.1 kPa and the blood pH change is 0.07–0.1 irrespective of the water P_{O_2} between 10 and 30 kPa; Sakakibara et al., 1987). From cellular studies and analyses performed at the level of the whole organism, arguments in favour of a role of pH in changing the activity of metabolic pathways have been developed, and it is generally agreed that acidification appears to be linked with the lowering of metabolic rate and *vice versa*

(Busa and Nucitelli, 1984; Bickler, 1986; Malan, 1993). Moreover, Malan (1985) proposed that changing extracellular P_{CO_2} provides a fast and economical means of changing intracellular pH. The present results obtained with the isolated limb preparation suggest that the daytime hypercapnic acidosis observed *in vivo* could indeed favour metabolic depression because the experimental addition of CO_2 reduced the stimulation induced by CCCP.

Our observation that at, resting metabolic rates, the O_2 supply could limit the oxidative metabolism of crustacean locomotor muscles is novel for crustaceans and water-breathers, but the idea that the $\dot{M}_{O_2}$ of tissues *in situ* could be O_2-limited has been extensively analysed for mammals (Lubbers, 1968; Jobsis, 1972; Jones et al., 1985; Connett et al., 1990; Vanderkooi et al., 1991). As regards muscle physiology, Chinet and Mejsnar (1989) have suggested that, although it is almost axiomatic that an increased metabolic rate in an organ triggers an increased O_2 supply, there are strong arguments to support the hypothesis that, in mammalian skeletal muscle, this relationship may sometimes be reversed. It is generally agreed that muscle cell respiration in resting mammalian tissue is determined physiologically by the intra-organ control of O_2 availability in the cells (Chapler et al., 1984; Richmond et al., 1997). Interestingly, in the present work, we show that such control exists in crustaceans but is implemented by a global control of blood P_{O_2} carried out at the level of the whole body by ventilatory adjustments. Note, however, that the redistribution of blood flow between organs, based on cardiac valve control, has been documented (De Wachter and McMahon, 1996).

The modulatory mechanisms described above do not mean that O_2 and blood pH are acting only at the effector level, contributing to the shaping of the circadian behaviour rhythm. Oxygen and pH could also be acting at a more central level, i.e. directly in the central nervous system. Such a neuromodulatory-like action has been demonstrated for O_2 in the lobster pyloric and gastric networks (Massabuau and Meyrand, 1996; Clemens et al., 1998, 1999) and possibly for pH in the crab pyloric network (Golowasch and Deitmer, 1993). Regarding the *in vivo* modulation of the pyloric and gastric work before and after feeding, the data available suggest that O_2 may act not only at the level of the central pattern generator but also directly on the pyloric and gastric muscles. Another interesting comparison with the stomatogastric work is the scale of the arterial P_{O_2} changes involved. Indeed, before feeding, the most frequently measured arterial P_{O_2} in the lobster was 1–2 kPa, and in the crayfish at rest during the day it was also 2 kPa. After feeding in the lobster it was 2–4 kPa, and during activity in *Astacus leptodactylus* the mean value was approximately 4 kPa. In exercising *Callinectes sapidus*, Gannon and Wheatly (1995) reported changes in blood P_{O_2} of the same order of magnitude.

It is becoming clear that crustaceans can and do operate complex behaviour patterns at low blood P_{O_2}.

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