The Journal of Experimental Biology 208, 661-670
Published by The Company of Biologists 2005
doi:10.1242/jeb.01427

The early life history of tissue oxygenation in crustaceans: the strategy of the myodocopid ostracod *Cylindroleberis mariae*

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Accepted 1 December 2004

Summary

We studied basic principles of respiratory physiology in Cylindroleberididae, *Cylindroleberis mariae* Baird 1850, which are millimetre-sized crustaceans (myodocop ostracod) having a fossil record dating back to about 425 millions years ago. Facing experimental changes of O_2 partial pressures in the range 2–40 kPa (normoxia is 21 kPa), *C. mariae* lack any regulatory mechanism to adapt their ventilatory and circulatory activity. Thus, the oxygenation status of their internal milieu must follow, as a dependant variable, the ambient oxygenation. Freely behaving *C. mariae* exhibit a marked diurnal activity rhythm. They are actively swimming in the water column during night, where they inspire in normoxic–normocapnic water. They are resting in self-made nests during daytime, where they are rebreathing in a confined and hypoxic environment. By analogy to extensive previous literature data, we suggest that these changes of respiratory gas content, and the associated tissue gas status, participate to the shaping of their metabolic activity and behaviour. To conclude, as Cylindroleberididae are early crustaceans exhibiting a remarkable stasis since the Palaeozoic, present data illustrates how principles of tissue oxygenation strategy can cover an impressive time scale.

Key words: respiration, evolution, crustacea, control of breathing, oxygen regulation, hypoxia, hyperoxia, circadian rhythm.

Introduction

Myodocopid ostracods are small bivalved crustaceans that have a fossil record dating back to the lower Silurian, about 425 million years ago. A well-preserved fossil of Cylindroleberididae has been recently described, which demonstrates that there has been a remarkable evolutionary stasis at the morphological level within this group because it has striking similarity to the extant myodocops (Siveter et al., 2003). Consequently, the study of present Cylindroleberididae offers an invaluable and, obviously, rare opportunity to analyse how the respiratory process and tissue-oxygenation strategy has evolved since early times.

Water-breathers are usually tolerant to water oxygenation changes. In hypoxia, the general rule is an increase branchial water flow, and many also increase their blood flow rate. Both adaptations allow the animals to maintain their oxygen consumption independent of water oxygenation. Importantly, it also allows an adaptation strategy whereby O_2 partial pressure, P_{O_2}, in the arterial blood is maintained within a low and narrow range of 1–3 kPa, largely independent of inspired P_{O_2} (Massabuau, 2001). This has been reported in fish, crustacean, mollusc and annelid. Interestingly, in mammalian tissues the most frequently measured P_{O_2} is also in the same low range. Based on the postulate that basic cellular machinery has been established since the early stages of evolution, it has been proposed that this similarity in oxygenation status is the consequence of an early adaptation strategy that, subsequently, throughout the course of evolution, maintained cellular oxygenation in the low and primitive range at which eukaryotic cells appeared two billions years ago (Massabuau, 2001, 2003). Podocopid ostracods, which represent the largest ostracod group, are heart- and gill-less crustaceans although they do possess ventilatory appendages. They have existed on earth for at least 500 million years and they also follow the same regulation strategy. However, and contrary to most water-breathers, podocopids lack any regulatory mechanism of ventilatory adaptation to face changes in water oxygenation. Instead, they adjust their tissue oxygenation status by migrating into sediment O_2-gradients to find low water P_{O_2} niches (Corbari et al., 2004). Thus, the podocopid data set reinforced the ideas that: (1) the level of oxygenation at individual tissue or cellular levels is a fundamental problem of homeostasis irrespective of species difference; and (2) it could have been held constant during the evolution of life to retain the original oxygenation status.

To get more insights into this evolutionary theory we studied myodocopid ostracods. Myodocopid ostracods appear, from a morphofunctional perspective, to be more

evolved by comparison with podocopid ostracods. Indeed, they possess not only a ventilatory system, composed of two scaphognathites ventilating a domiciliar cavity, but also a cardiovascular system composed of a well-differentiated heart and, in the Cylindroleberid family, 6–8 lamellar gills (Vannier et al., 1996; Horne et al., 2002). Based on previous evidence, the homeostasis of their internal milieu, in terms of oxygen, should be maintained by some regulatory mechanisms involving an autonomous or behavioural adaptive process. Consequently, our aim was to study how tissue oxygenation status is regulated in Cylindroleberid myodocops. During evolution, myodocops have acquired a large variety of lifestyles either benthic, nektobenthic or exclusively planktonic. They are confined to seawaters where they colonized the shallowest coastal as well as the deepest bathyal and abyssal environments worldwide (Horne et al., 2002). The species we studied, *Cylindroleberis mariae*, is a nektobenthic representative of Cylindroleberid myodocop. It displays nocturnal upward migrations (Macquart-Moulin, 1999; Fenwick, 1984) and rests during the daytime at the sea bottom where many species inhabit burrows or nests built with sand particles and phytodetritus (Cohen, 1982; Smith and Horne, 2002). Our approach was based on a combination of anatomical, physiological and behavioural analyses to determine the oxygenation strategy of this species.

Materials and methods

Experiments were performed from February to March 2002 and from June to August 2003 on a total of 80 *C. mariae* (myodocopid ostracods Baird 1850; also known as *Asterope mariae* Moore, 1961). All specimens (adults, size ranging from 1.5–2.0 mm) were collected by dredge locally in the Bay of Arcachon, SW France. Animals were acclimated in the laboratory for at least one month before experiments began (Massabuau, 2001) and remained in the experimental set-ups for 1–6 weeks. As no significant mortality and statistical difference was observed as a function of experimental duration and date, all data are presented together. Note that, in our experimental conditions, it illustrates the stability of our procedure and observations. As a whole, a total of about 250 h of observation was performed.

Morphofunctional anatomy

The study was performed on three *C. mariae* measuring 1.7, 1.8 and 2.0 mm. Whole animals were immersed in a fixative for electron microscopy (6% glutaraldehyde buffered with 0.4 mol l^{-1} sodium cacodylate, pH 7.4, osmotic pressure 1100 mosmol l^{-1}) for 12 h at 4°C and subsequently rinsed in cacodylate buffer (0.4 mol l^{-1}, NaCl 4%). They were embedded separately in Araldite. Serial sections were performed with a Reichert automatic ultra-microtome (Depew, NY, USA). The observations were measured on enlarged pictures (semi-thin preparations) after visual inspection using a microscope LEICA TCS 4D.

Maintenance conditions

The animals, together with their natural sediment and phytodetritus, were placed in an aquarium with a running flow system in a dark thermostated room set either at 10 or 18°C (Aquarium size: L, 50 cm; W, 50 cm; H, 50 cm). The aquaria were all supplied with seawater from the bay of Arcachon (water P_{O_2}≈20–21 kPa; water pH≈7.8; salinity ≈28–32‰). Considering the animal size and the amount of organic material and microfauna naturally present in the sediment, no external food was added. When required, specimens were isolated on binocular microscope before experiments. To minimise external disturbances, experimental tanks were isolated from laboratory vibrations with anti-vibrating benches.

Physiological analysis of ventilatory and circulatory activity by video recording

We analysed the myodocopid ostracod ventilatory system of animals exposed to various steady water P_{O_2} conditions at 10°C by visual inspection after or during video recording activity. All video observations were achieved during daytime (i.e. between 9 am and 5 pm) under dim light by using infra-red light (λ=880 nm) to limit animal disturbance. Recordings were performed by using an X-Y driven Leitz MZ12 binocular microscope (Oberkochen, Germany) equipped with a B/W Ikegami camera (CDD Camera, ICD42B; Maywood, USA). Data were displayed on a Sony TV monitor (HR Trinitron PVM 1453MD; Tokyo, Japan). They could be either analysed on line and/or, stored on a JVC tape recorder (S-VHS, HRS75000MS; Tokyo, Japan) or a Panasonic tape recorder (VHS, NV/SD45; Osaka, Japan). As animals were mostly moving, no attempt was made to use any automatic frequency counting device.

Experimental procedure

One week before experimentation started (the systematic acclimation period before any experiment), myodocops were transferred to an experimental micro-aquarium (Fig. 1A,B; volume 1.2 ml; L, 20 mm; W, 3 mm; H, 20 mm; water renewal rate 60–100 µl min^{-1}). It was hand-made with a microscopic slide fixed by using SYLGARD (Dow Corning, Michigan, USA) on a laboratory made thermostated glass plate (10×6×0.5 cm). It was equipped with muddy sand and phytodetritus from the Bay of Arcachon to mimic a 'natural-like' environment in which animals could move freely, dig and hide. This aquarium was part of a 1 l closed re-circulatory system with constant entry and exit levels. It was set at 10±0.1°C for all experiments by means of a laboratory-constructed thermoelectric device. During experiments, P_{O_2} varied from 2–40 kPa (27–540 µmol l^{-1} or 0.9–17.3 mg l^{-1}). The CO_2 partial pressure (P_{CO_2}) was maintained at 0.1 kPa, a value typical of water P_{CO_2} in air-equilibrated environments. The gas mixtures bubbled through the reservoir of seawater feeding bottles, which was connected to the aquarium by means of glass tubes to avoid gas leaks. The $N_2/O_2/CO_2$ gas mixture was obtained *via* mass flow controllers (Tylan General, model FC-260; San Diego,

CA, USA) driven by a laboratory-constructed programmable control unit.

Different subtypes of experiments were performed at 10°C in this set-up: analysis of reference ventilatory pattern in normoxia; ventilatory and cardiac responses to 2–15 h exposure periods at various O_2 partial pressures ranging from 40 to 2 kPa; respiratory adaptation to 3 day exposure periods at 4 kPa.

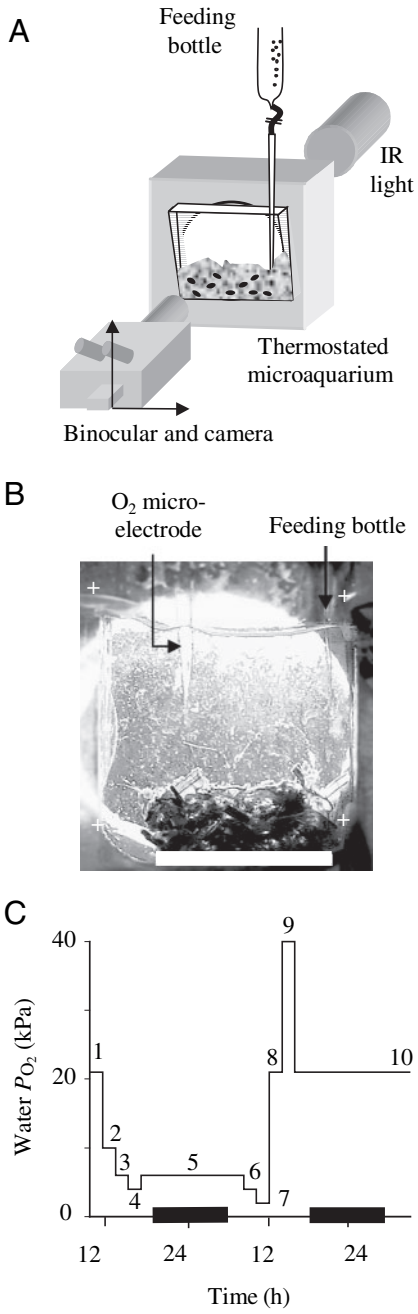

Fig. 1. (A) Experimental set-up for ventilatory analysis by video recording. Animals were free ranging in a vertical layer of natural sediment, cardiac and ventilatory activities were measured by visual inspection through the animals. Analyses were performed under dim light by means of infra-red (IR) camera and micro-spotlight (see text for details). (B) Mini-aquaria with myodocop nest. Scale bar, 1 cm. (C) Experimental procedure of short-term exposures at various oxygenations numbered from 1 to 10.

Short-term adaptation ability at various oxygenation levels

These experiments were done on a group of 12 animals from the Bay of Arcachon, which was exposed to 10 plateau levels of different water P_{O_2} presented in the following order: ≈21 (Reference), 10, 6, 4, 6, 4, 2, 21 (Recovery 1), 40 kPa and 21 kPa (Recovery 2; Fig. 1C). The duration of exposure for each oxygen level was ranging from 2 to 15 h, with the exception of the reference normoxic condition that lasted 7 days. Ventilatory frequencies within ventilatory bouts (min^{-1}) and cardiac frequencies (min^{-1}) were measured during the last 30 min of exposure time. Each animal was identified based on location in the aquarium, size and shell marks to avoid replicate analysis on the same individuals.

Three-day exposure periods at water P_{O_2}*=21 and 4 kPa*

The analysis of reference ventilatory pattern in normoxia (21 kPa, 282 μmol l^{-1}) and 3 day exposure under hypoxia, 4 kPa (53 μmol l^{-1}), was performed in March 2002 on one group of seven animals. After acclimation, myodocops were first studied in reference normoxic conditions (21 kPa) during 3 days in the mini-aquarium, then under hypoxia during 3 days (P_{O_2}=4 kPa, hypoxic test) and finally in normoxia after 2 days of recovery. When the analysis started, the experiment consisted of focussing on an individualized specimen and to study it during a 1 h period. Thus, for each animal, its ventilatory pattern was described during the reference days 1, 2 or 3 and the test days 4, 5 or 6. For each animal, the percentage of active ventilation during the studied hour (hourly duration, %), the mean ventilatory bout duration (min^{-1}), the bout number (h^{-1}), the ventilatory frequency within bouts (min^{-1}) and the cardiac frequency (min^{-1}) were determined. As no significant difference was observed between animals (paired *t*-test), all data were pooled together for each water P_{O_2}. Consequently, comparisons were performed on paired analysis.

Behavioural regulation of organism oxygenation status during the diurnal rhythm

Many myodocops exhibit a clear diurnal activity rhythm (they are active at night and resting in nests during daytime, Macquart-Moulin, 1999; Smith and Horne, 2002) whereas the above analyses were essentially performed during daytime. To get more insights into the organism's oxygenation strategy at various activity levels, we thus turned to an analysis of (1) activity pattern and (2) oxygenation status in nests.

Diurnal rhythm of activity in C. mariae

The analysis was performed on 50 specimens of *C. mariae* (measuring 1.9±0.1 mm) in August 2003, following a 15 day acclimation period in the laboratory. The temperature in the room was set at 18°C to enhance oxidative metabolism and oxygen dependency by comparison to the above experiment that were performed at 10°C. After being collected, animals were placed in a glass aquarium (10×5×30 cm, L×l×h)

whose bottom was covered by a natural substrate (sand, mud and phytodetritus; thickness, 1 cm). The acclimation period was 7 days. The aquarium was exposed to natural day light cycles and, to permit nocturnal camera recording, an infrared floodlamp (λ=870 nm) was added. The floodlamp was facing the camera (camera Watec WAT-902H equipped with a macro zoom lens, Computar MLH-10X) to allow animal counting at night and it was continuously switched on. A total of 10 diurnal activity rhythms were recorded. One picture was caught per second by driving the videocamera with a PC (software, PVR Perception player; Enfield, UK). On each picture, the total number of animals present in the water column was then determined. The activity index we derived (expressed as arbitrary unit, a.u.) was the number of animals present in the water column during a 30 min observation period.

Oxygenation status in myodocops nests

To characterize the partial pressure of oxygen into a myodocop nest, 15 *C. mariae* were placed in a mini-aquarium (Fig. 1B) during one week. The aquarium was perfused with normoxic–normocapnic water and O_2-profiles (N=5) were measured with an O_2 polarographic microelectrode (UNISENSE Microsensors) driven with a PRIOR micromanipulator (steps, 0.2 mm). The microelectrode was impaled in the central part of the nest, close to the animals.

Statistical analysis

Values are reported as mean values ±1 standard error of the mean (S.E.M.) or 1 standard deviation (S.D.). 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). P<0.05 was taken as the fiducial limit of significance.

Results

Morphofunctional background and general findings

Fig. 2A,B presents the general morphology of *C. mariae*. Fig. 2B (left valve not shown) shows the position of well-developed scaphognathites and a large heart (Fig. 2C), located in dorsal position. The scaphognathites, or ventilatory plates, are paired appendages, which are beating rhythmically to bring water currents between valves, in the domiciliar cavity. Water circulates from the anterior to the posterior part of the animal, along the animal soft body and at gill level. Interestingly, the observed ventilatory movements were closely analogous to what is recorded in green crabs (*Carcinus maenas*, Hughes et al., 1969) or crayfish (*Astacus leptodactylus*, Massabuau, 1983). In *C. mariae*, scaphognathites could work independently with a main tendency to operate in synchrony. Interestingly, opposition phase and unilateral ventilatory arrest were occasionally observed. As shown in Fig. 2D and E, large gills are present and their total number is seven. These gills consist in a set of integumental lamellae overlapping each other as in a wide-open book. Gill cross-section reveals that, at mid level in the gill basket, the mean lamellae thickness was 17.3±0.2 µm (mean ± 1 S.E.M.; N=6–7 measurements per individual; three individuals). As shown in Fig. 2D and E, numerous lacunae were present. The mean diffusion distance from water to hemolymph was 3.8±0.7 µm (N=12–14 measurements per individual). Note that in the same specimen, the maximum diffusion distance between water and soft-body core was ranging from 150–300 µm.

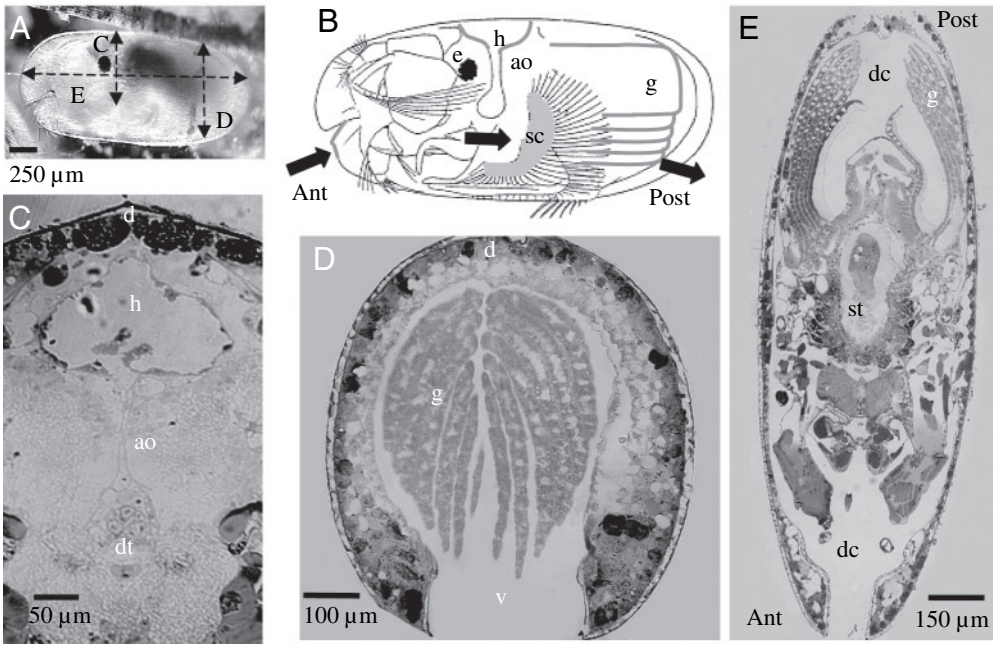

Fig. 2. Morphofunctional anatomy of *C. mariae*, myodocopid ostracod. (A) *In situ* picture in the experimental micro-aquarium. Dashed lines C, D and E indicate section planes for Fig. 2C–E. (B) Schematic drawing, left valve not shown, illustrating the ventilatory flow pattern (arrows) through the animal. The inspired water enters from the anterior aspect and superfusates the soft body (inspired from Cannon, 1933). (C) Cross section through the heart. (D) Cross section through the gills. (E) Longitudinal section showing the seven pairs of gills. ant, anterior; ao, aorta; d, dorsal; dc, domiciliar cavity; dt, digestive tract; e, eyes; g, gills; h, heart; post, posterior; sc, scaphognathite; st, stomach; v, ventral.

Characterisation of the ventilatory pattern at various P_{O_2} levels

In resting *C. mariae*, a typical ventilatory pattern in air-equilibrated water (P_{O_2}=21 kPa) was characterized by

spontaneous switch from active ventilation to transient pauses (Fig. 3). Interestingly, a continuous ventilatory activity was occasionally observed during at least 1 h periods (2/7 studied animals) and the longer pause we recorded was 8 min. Note finally, that either scaphognathite could work alone although this was rarely observed. The mean ventilatory bout number was 3 h^{-1} (minimum–maximum, 1–8 h^{-1}) and the total ventilatory duration per hour varied from 31–60 min. During active ventilation periods, called ventilatory bouts, the mean-recorded ventilatory frequency was ≈90 min^{-1} (see Table 1; minimum 30 min^{-1}; maximum 168 min^{-1}). Cardiac pauses were never observed and the mean cardiac frequency was ≈40 min^{-1} (minimum 38 min^{-1}; maximum 54 min^{-1}, Table 1).

The existence of ventilatory and/or circulatory regulatory mechanisms was tested by exposing animals to various water oxygenation levels during exposure periods ranging from 2–15 h (Fig. 1C). The result of our experiments is presented in Fig. 4. The striking observation was that, when the frequencies under hypoxic (2<P_{O_2}<10 kPa) and hyperoxic (P_{O_2}=40 kPa) conditions were compared with normoxia during reference and recovery conditions (P_{O_2}=20.5–21.5 kPa,), no change of ventilatory and cardiac frequency could be noticed (Fig. 4 upper panels). Specifically, in hypoxia, no hyperventilatory response was recorded. The relationship between the ventilatory frequency, f_R and water P_{O_2} was: f_R=0.028 water P_{O_2}+96.08 (r^2=0.00121, P<0. 76). The relationship between the cardiac frequency, f_H, and water P_{O_2} was: f_H=0.092 water P_{O_2}+54.75 (r^2=0.0072, P<0. 89; Fig. 4 lower panels). Note finally that despite a 23 h exposure at P_{O_2}<6 kPa, no recovery impairment was discernible. Indeed, this mid-term hypoxic exposure did not lead to any statistical difference between recovery and reference frequencies for both ventilatory (ANOVA, $F_{7,116}$=0.99, P=0.44) and cardiac (ANOVA, $F_{7,57}$=1.58, P=0.16) aspects. To reinforce this observation, especially facing hypoxic challenge, we then exposed the *Cylindroleberis* to water P_{O_2}=4 kPa during 3 days and we analysed all characteristics of the corresponding respiratory activity. The results are presented in Table 1. Clearly, the mean number of bouts, bout duration, hourly duration of ventilation and ventilatory frequency within bout per hour did not significantly change as a function P_{O_2} (no different values, paired *t*-tests). Fig. 5 extends on this theme by comparing the distribution frequencies of these parameters. Without a doubt, both ventilatory patterns were similar. Thus, even during long-term exposure to hypoxia, no significant ventilatory and circulatory adaptability could be observed in *C. mariae*. Consequently, it strongly suggested that at constant temperature and metabolic level, the P_{O_2} value in their milieu intérieur should vary passively – as a dependent variable – following changes of P_{O_2} in the inspired water.

Numerous myodocops are reported to emerge at dust from the sediment and swim in the water column, i.e. in air-equilibrated water. By contrast, during daytime Cylindroleberids rest in burrows or nests on the sea bottom. To analyse if this particular diurnal behaviour applies to *C. mariae* and whether it participates to a rhythm of tissue oxygenation, we then turned to a behavioural study of animals free to move in a water column

Table 1. *Characterisation of ventilatory and cardiac activity*

		Bout						Bout				
	N	Number (h^{-1})	Mean duration (min)	Hourly duration (%)	f_R (min^{-1})	f_H (min^{-1})	Apnea (%)	Number (h^{-1})	Mean duration (min)	Hourly duration (%)	f_R (min^{-1})	f_H (min^{-1})	Apnea (%)
Myodocopids		21 kPa						4 kPa					
C. mariae	7	3±1	31±8	88±7	93±3	42±4	0	3±1	36±9	90±7	72±10	46±2	0
*P**		–	–	–	–	–	–	0.73*	0.88*	0.88*	0.94*	–	–
Podocopids		21 kPa						3 kPa					
Five species	31	9±2	18±6	61±5	55±10	–	26	8±4	30±12	69±13	48±11	–	42
P		0.03	0.01	0.03	0.02	–	–	0.01	0.67	0.10	0.03	–	–

Characterisation of ventilatory and cardiac activity during a 3 day exposure period at water P_{O_2}=21 and 3 or 4 kPa in the myodocopid ostracod *Cylindroleberis mariae* (upper) and comparison with podocopid ostracods (lower; from Corbari et al., 2004). Note the absence of significant respiratory adaptation under hypoxia in both ostracod groups (see text for other details). Number, number of ventilatory bouts per hour; mean duration, mean bout duration per hour; hourly duration, total duration of active ventilation per hour; f_R, respiratory frequency within bouts; f_H, cardiac frequency. Apnea, percentage of animals presenting a total absence of ventilatory activity during the 1 h studied period. All data expressed as mean ± 1 S.E.M., *N*=number of studied animals. *P**, *P* values for comparisons between hypoxia and normoxia in *C. mariae*; *P*, *P* values for comparisons between normoxic- and hypoxic-respiratory activities in podocopid ostracods and *C. mariae* (myodocopid ostracod).

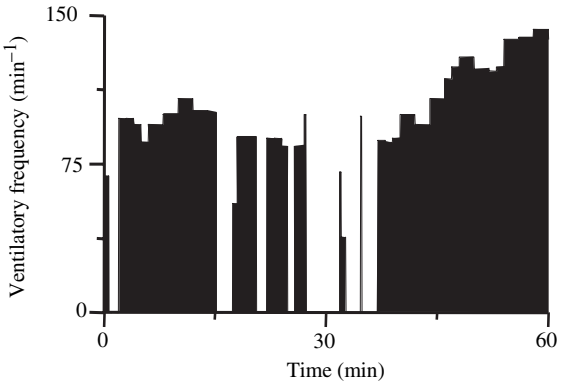

Fig. 3. Typical ventilatory pattern (f_R, ventilatory frequency, min^{-1}) in one *C. mariae* specimen during 1 h observation period at P_{O_2}=21 kPa. Ventilatory activity is characterized by alternations of ventilatory bouts and pauses.

and build nests. Fig. 6 demonstrates first the existence of a very marked daily rhythm of activity in this species. It summarizes the result obtained during 10 daily cycles through a summer season. Clearly, no specimen (0/50) was recorded in the water column during daytime while it was only at night that animals were active. At night, maximum swimming velocities of 20 mm s^{-1} were recorded, while during daytime, in the sediment, it was only ≈0.7 mm s^{-1} as animals were mostly inactive in the nests. Fig. 7A1–A4 illustrate the different phases of a nest building. On Fig. 7A2, a specimen is shown surrounded by filaments of mucus-like slime and Fig. 7A3 shows two animals gliding into a nest. Finally, Fig. 7A4 illustrates the density that can be reached within a nest in which 15 individuals were observed. Fig. 7B presents an oxygen profile performed during daytime in this nest. Clearly, the nest water was confined as illustrated by the measurements of hypoxic P_{O_2} values ranging from 8–10 kPa. Thus depending on their activity level, *Cylindroleberis* are either rebreathing a hypoxic and hypercapnic water in nests when there are resting during daytime, or breathing a normoxic–normocapnic water, when they are actively swimming in the water column at night.

Discussion

C. mariae are cylindrolerid ostracods already existing 425 million years ago and equipped with scaphognathites, gills and cardiovascular system. Present data demonstrate that they are unable to adapt their ventilatory and circulatory activity to face water oxygenation changes. Thus, the oxygenation status of their internal milieu is closely dependent on the oxygenation of their external environment. During the diurnal rhythm, the animals positioned themselves in hypoxic water when they are resting, and in normoxic water when their are active. This is the result of a behavioural and social strategy as groups of *C. mariae* build nests where they are buried during daytime, rebreathing in a confined environment.

Comparison with previous data

To date very little data are available concerning respiratory properties and evolution in early crustaceans and arthropods although the evolution of their cardiovascular system has been reviewed by Wilkens (1999). Horseshoe crabs, *Limulus polyphemus*, are certainly an exception (Watson, 1980; Mangum and Ricci, 1989). They probably existed since the Silurian period (410–440 million years ago) and, interestingly, as reported here for myodocops, their ventilatory pattern has also been reported as highly variable. Moreover, an absence of ventilatory rate change in response to oxygenation changes has been reported (Mangum and Ricci, 1989) which fits quite well with present observation in *Cylindroleberis*. In podocopid ostracods (Corbari et al., 2004), the ventilatory pattern is also highly variable, but numerous statistically significant differences exist when bout characteristics – number, mean, hourly duration and ventilatory frequencies – are directly

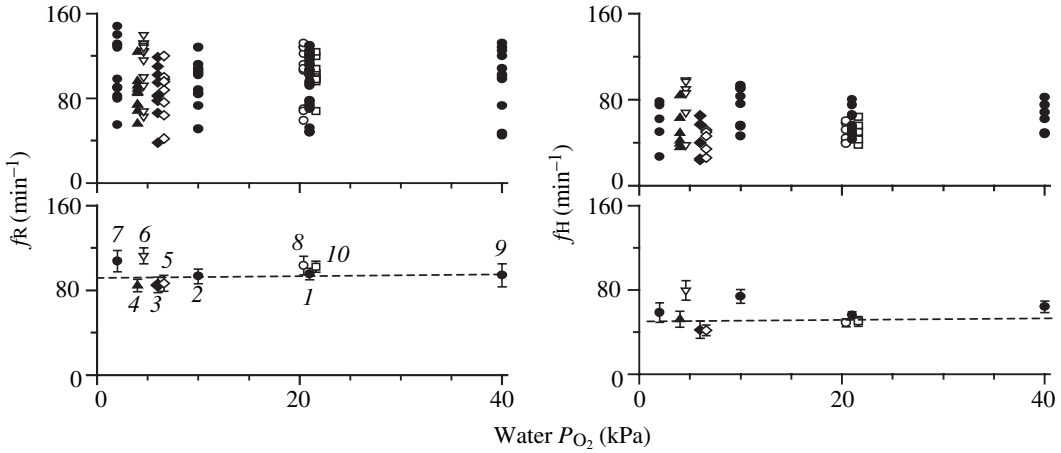

Fig. 4. *C. mariae* ventilatory and cardiac responses to 2–16 h exposure periods at various oxygenation levels. Upper panels: respiratory frequencies within bouts, f_R (min^{-1}) and cardiac frequencies, f_H (min^{-1}) illustrating inter-individual variability. One symbol per oxygenation level and *N*=12 animals per studied level. Lower panels, mean f_R and f_H relationship *versus* water P_{O_2} (±1S.E.M.). No significant trend was observed as a function of P_{O_2}. Italics refer to the protocol shown in Fig. 1C.

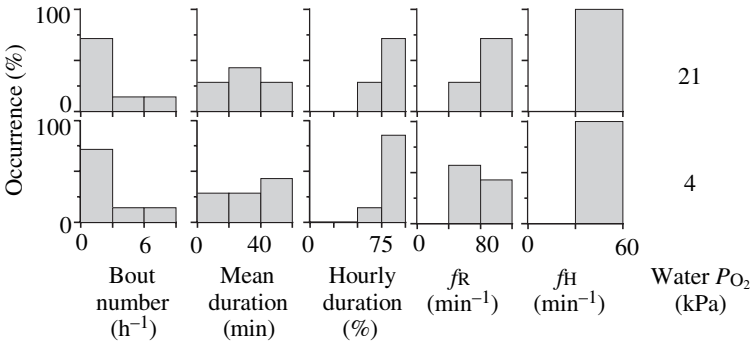

Fig. 5. Frequency distribution of ventilatory bout number, ventilatory mean duration, ventilatory hourly duration, respiratory frequency within bout (f_R) and cardiac frequency (f_H) during 3 day exposure periods at water P_{O_2}=21 and 4 kPa (mean values in Table 1). Note the absence of any ventilatory and cardiac change (N=7 animals).

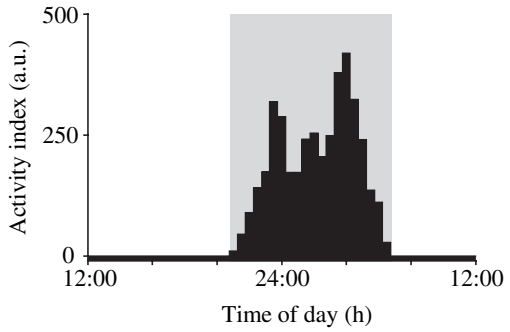

Fig. 6. Diurnal rhythm of activity in *C. mariae* (10 cycles analysed). *C. mariae* was only actively swimming in the water column from 22:00–07:00. Grey shadow, from sunset to sunrise; August 2003; 50 animals in the aquarium.

compared (see Table 1). However, a major difference is certainly that one never observed ventilatory arrest, or apnoea, longer than 8 min in *Cylindroleberis* while it was quite frequent in podocopids as they can stop breathing for periods >1 h. This ability in podocopids could be associated to their activity and metabolic level. Indeed, the velocity of podocopids in the sediment is slower than in *Cylindroleberis* (1–2 mm min^{-1} *vs* 40 mm min^{-1}; podocopid values from Corbari et al., 2004). The ability of *Cylindroleberis* to build nests was already reported by Cannon (1933). He noted that when a specimen is placed in a dish of clean seawater without any mud, in a minute or two, it is found to be surrounded by a mass of mucus like slime. Fage (1933) reported that *Cylindroleberis* can stick together sand particles by using secretory glands and stay in one centimetre long nests for days or weeks under laboratory conditions. Finally, Vannier and Abe (1993) reported that another member of the myodocopid ostracod family, the Cypridinidae *Vargula hilgendorfii* can also stay within the upper layers in the sediment and that they also produce some sticky substance that could be a kind of slime. Thus, in myodocops, nest building appears as a very general behaviour.

Extensive studies on the respiratory physiology in another type of millimetre-sized crustacean, *Daphnia magna*, were already performed. *Daphnia* are equipped with a cardiovascular system but no ventilatory plates. They are planktonic filter feeders and ventilate their filtering chamber with thoracic appendages. In *Daphnia*, the existence of both cardiocirculatory (Paul et al., 1997) and ventilatory responses (Pirow and Buchen, 2004) were reported following changes in water oxygenation levels. In addition, there is haemoglobin in *Daphnia* (Kobayashi and Hoshi, 1984) and, although Fox

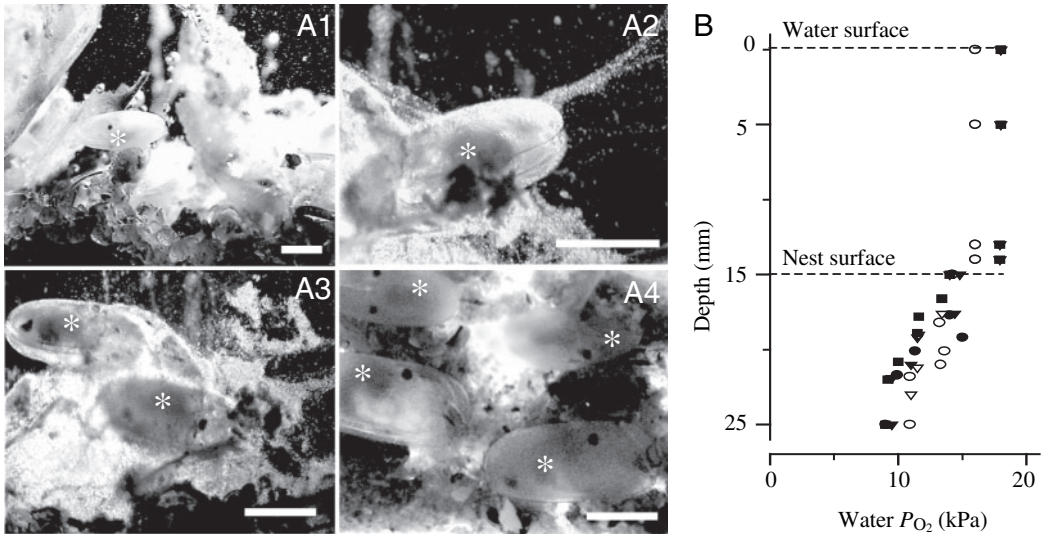

Fig. 7. *In situ* pictures of nesting behaviour in *C. mariae* and nest oxygenation status. (A1) First steps of nest building. (A2) Production of mucus-like slime. (A3) Two individuals entering in a nest. (A4) Illustration of animal density in a nest during daytime. (B) Water O_2-profile in the nest shown in A1–A4. Asterisks indicate individuals. Scale bars, 1 mm.

(1957) reported that it also occurs in *Cypria* and *Pseudocypris* (freshwater ostracods), the question certainly requires further investigation (Hourdez et al., 2000; Weber and Vinogradov, 2001). Again, any explanation about these differences remains highly speculative but it is worth noting that *Daphnia* were only reported from the Permian (250–300 million years ago; Schram, 1982). Respiratory control mechanisms could have logically evolved from Cambrian to Permian.

The respiratory control system in ostracods

In ostracods, the rhythmic movement of the scaphognathites is controlled by four muscles innervated by nerves originating from the circumoesophageal ganglia (Hartmann, 1967). Although of primitive aspect by comparison to decapod crustaceans, the central nervous system is already well-differentiated as a cerebrum, a circumoesophageal ring, a chain of ventral ganglia and a network of motor nerves connecting to various muscles were described (Rome, 1947; Hartmann, 1967). Remarkably, in present decapods, the scaphognathite beating movement is fairly similar to what is observed in myodocops (present data) and podocops (Corbari et al., 2004). In crabs like *Carcinus maenas*, these movements are driven by a set of five levator and five depressor muscles (Young, 1975), innervated by motor neurones arising from a central pattern generator (CPG; Simmers and Bush, 1980). By analogy, this strongly suggests that a respiratory CPG also exists in myodocops (Harris-Warrick et al., 1992; Marder and Bucher, 2001). Indeed, there is now considerable evidence from a variety of different invertebrates that the motor patterns underlying rhythmic behaviour are essentially determined by CPG within the central nervous system (Harris-Warrick et al., 1992; Marder and Bucher, 2001). This would be coherent with the demonstration that the heartbeat in *Vargula hilgendorfii* is neurogenic and driven by a CPG located in a cardiac ganglion. Interestingly, the *Vargula* cardiac CPG is composed of a single neuron (Ando et al., 2001; Ishii and Yamagishi, 2002) when in many decapods, it is composed of nine neurons. It illustrates the level of complexity that could be expected for an early ventilatory CPG.

Ventilatory pattern and evolution of central nervous mechanisms controlling ventilatory activity in crustaceans

In ostracods, the ventilated water flows backwards, i.e. from the anterior to the posterior aspects of the animals. Remarkably, in present decapod Crustaceans, the predominant mode is opposite: the scaphognathites draw water forwards *via* openings located at the base of the walking limbs and chelae and expel it through the hydrostomes, that are excurrent openings located on the anterior part, below antennae. Occasionally, the system reversed the direction of ventilatory currents and water is inhaled *via* the anterior aspect (Arudpragasam and Naylor, 1964; Hughes et al., 1969). Backward pumping (alternatively called, reversals) were only reported to be a predominant mode (1), in the crabs *Corystes cassivelaunus*, which normally live buried in sand (Arudpragasam and Naylor, 1966) and (2) in the shore crabs *C. maenas*, which, when exposed to progressive hypoxia in shallow water, partially emerge into air and aerate their branchial cavities by reversing the direction of their irrigation (Taylor et al., 1973). Simmers and Bush (1983) studied the neuronal basis of bimodal beating in *Carcinus*. They reported that a single pattern-generating network produces the motor programmes appropriate for both forward and backward beating. Switching between beating modes originates from selective inputs, which either inhibit one or the other pattern.

Thus, in decapods, a single ventilatory CPG produces two different motor patterns (forward and backward) when in ostracods, the backward motor pattern is the only observed ventilatory mode. What could be the origin of such an apparent divergence? Is it the result of evolution? Decapods have a fossil record dating back to the Permian (286–245 million years ago; Benton, 1993) when ostracods are dated from the early Paleozoic (400–500 million years ago). We propose then that the backward ventilatory pattern could have preceded the forward pattern. In *Carcinus*, Arudpragasam and Naylor (1964) shows that during backward flow, water only irrigates the upper surface of the posterior gills, which is evidently of limited efficiency. On the contrary, during forward flow, water irrigates most of the gill lamellae. Finally and importantly, when crustaceans are facing hypoxic challenges, an increase of forward flow is the major adaptation to maintain the oxygen consumption and blood oxygenation status (Taylor, 1982; McMahon, 2001). The above observations could then explain why to our knowledge no large crustacean is currently relying on backward ventilatory activity: in large crustaceans facing an hypoxic stress or an increased O_2-demand, forward flow is of higher adaptative value as it allows a better gill ventilation efficiency than the backward flow. Regarding *C. cassivelaunus* (Arudpragasam and Naylor, 1966), which appears as an exception in this scheme, one must keep in mind that it is normally living in sand. The causal explanation for their extensive use of a backward mode is likely that a forward flowing current could carry sand into their gill chambers.

To summarize, following the above hypothesis regarding the genesis of ventilatory motor pattern generation in crustaceans, we propose that the backward ventilatory mode should be considered as the early ventilatory mode and the forward mode, as a more recent acquisition, appearing later during evolution. It certainly allowed an increased O_2-uptake ability and, thus, possibly facilitated the evolution of larger animals. In this view, the backward mode should be considered as a vestigial motor pattern in decapods, having an accessory role in gill chamber cleaning.

The strategy of tissue oxygenation in Cylindroleberids

In the present report we show an absence of ventilatory and circulatory adaptation ability facing water oxygenation changes (Fig. 4). Nevertheless, the demonstration of a different positioning, depending on the level of activity strongly suggests that Cylindroleberids adjust their tissue oxygenation level at set values. Indeed, this observation recalls previously described behaviour in the crayfish *Astacus leptodactylus*

during the circadian rhythm of activity (Sakakibara et al., 1987; Forgue et al., 2001) as well as numerous data on metabolic modulation by O_2 and CO_2 (Busa and Nucitelli, 1984; Hochachka and Somero, 1984; Malan, 1993; Guppy and Withers, 1999; St-Pierre et al., 2000). In *A. leptodactylus*, it has been shown that changes of activity at night compared with daytime are associated with changes in arterial blood P_{O_2} and P_{CO_2}. These changes are performed by ventilatory adjustments that ensure the autonomous homeostasis of the internal milieu, in terms of O_2 (Massabuau et al., 1984) as well as to the blood and tissue acid–base balance regulation. In Cylindroleberids, the ability of ventilatory adjustments obviously did not exist, but a social behaviour could play this role on the blood and tissue gas composition as the water P_{O_2} in a nest is hypoxic and the water P_{CO_2} must be hypercapnic, due to rebreathing in a confined space (Fig. 7). Interestingly, when the crayfish *A. leptodactylus* (Forgue et al., 2001) is experimentally exposed to a water P_{O_2} of 10 kPa (remember that the measured value in the Cylindroleberid nest was very close, 8 kPa) during a 24 h exposure period, it remains inactive and stops exhibiting a circadian rhythm of activity. By analogy, this depression effect in the crayfish, is thus a first indication that the oxygenation status found in the Cylindroleberid nests could participate to the shaping of a resting behaviour and that, in this way, these animals do possess an O_2-chemosensitivity. In Crustacea, the existence of three types of hypoxia-induced metabolic rate depressions were proposed (Forgue et al., 2001). The first one is an environmentally induced 'deep' hypoxia during which the water P_{O_2} is so low ($\leqslant$3–4 kPa), that the gas-exchange processes are limited and the resting O_2-consumption cannot be maintained. It imposes a strict limit to the oxidative metabolism and forces its depression. Under these conditions, which we suggest are extreme, any increase of activity relies on anaerobiosis. The second type is observed in 'mild' hypoxic environments (water $P_{O_2}\approx$6–10 kPa). Under these conditions, the animals spontaneously limit their activity in a medium in which excessive exercise could become O_2-limited. Finally, the third type is a behaviourally self-imposed blood hypoxia by hypoventilation, which allows a limited O_2-metabolism even in normoxic or hyperoxic environments. In the crayfish, it limits the aerobic scope at rest during daytime and forms part of the normal physiological repertoire of the animal comportment. Forgue et al. (2001) demonstrated that it did not limit the global animal's oxidative metabolism but typically the locomotor muscle O_2-consumption. It has been proposed to participate to the shaping of the resting behaviour of crayfish *via* direct action on the locomotor muscles themselves. We demonstrate in this report that, contrary to decapods, the ostracods are unable to control directly the oxygenation status of their internal milieu as they cannot adjust their ventilatory activity according to P_{O_2} (see Fig. 4). We suggest that the behaviour of Cylindroleberids breathing in the confined environment of a nest (as reported here) corresponds to a strategy of metabolic depression that underpins the daytime Cylindroleberid resting behaviour.

As stated above, in *Astacus leptodactylus*, a circadian rhythm of acid–base balance in the internal environment was reported (Sakakibara et al., 1987). The animal's blood is hypercapnic during daytime when animals are resting, and hypocapnic at night when animals are active. Arguments in favour of a role of pH in changing the activity of metabolic pathways were largely developed and it is agreed that acidification is associated with a lowering of metabolic rate while alkalinisation is linked to its enhancement (Busa and Nucitelli, 1984; Bickler, 1986; Malan, 1993). In addition, Malan (1985) proposed that changing blood P_{CO_2} is a fast and economical means to change cellular acid–base balance and Forgue et al. (2001) reported that increasing P_{CO_2} favours metabolic depression in the locomotor muscle of the crayfish. Thus, the modulation of metabolic activity by O_2 and CO_2 has been extensively studied. The mechanisms described above offer a guideline strongly suggesting that in Cylindroleberids, and possibly in other myodocopid ostracods, self-imposed changes of water gas composition could contribute to the shaping of the diurnal behaviour rhythm. These physiological mechanisms should depress their metabolic activity during daytime and help them to reach a kind of torpor.

In conclusion, Cylindroleberids are unable to regulate the oxygenation status in their internal environment autonomously by respiratory adjustments. They build nests in which they are resting during daytime, certainly to protect themselves against predators, but an additional consequence is that they are breathing under hypoxic and hypercapnic conditions. By contrast, when they are active in the water column, they inspire in a normoxic and normocapnic environment. The net result is that they experience changes in respiratory conditions, which are similar to what have been extensively described in the literature on metabolic modulation by O_2 and CO_2. Consequently, Cylindroleberids are early crustaceans illustrating a remarkable stasis since the Paleozoic, both in morphological (Siveter et al., 2003) and physiological terms (present data). Indeed, we illustrate here how a single basic set of principles of respiratory physiology could apply to the behaviour and life history of an animal that has existed over an impressive time scale.

We thank N. Mesmer-Dudons and P. Ciret for their invaluable technical support. Thanks to J. Vannier and J. Simmers for helpful discussions or comments.

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