

## How a low tissue O<sub>2</sub> strategy could be conserved in early crustaceans: the example of the podocopid ostracods

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### Summary

An adaptation strategy whereby O<sub>2</sub> partial pressure,  $P_{O_2}$ , in the tissues is maintained within a low, narrow range of 1–3 kPa, largely independent of the inspired  $P_{O_2}$ , has been reported in water- and air-breathing poikilotherms and in homeotherms. Based on the postulate that this basic cellular mechanism has been established since the early stages of evolution, it has been hypothesized that it could be the consequence of an early adaptation strategy to maintain cellular oxygenation within the same low and primitive range. To test this hypothesis we studied the basic mechanisms of oxygen regulation in podocopid ostracods, minute crustaceans that have existed on earth for at least 500 million years. Podocopids lack any regulatory mechanism for adapting their ventilation to cope with changes in water

oxygenation, and instead adjust their tissue oxygenation status by migrating through the O<sub>2</sub> gradient to sediment layers where the  $P_{O_2}$  of the water is 3–5 kPa. Experimental manipulation of the O<sub>2</sub> profile induced their vertical migration to follow this precise water  $P_{O_2}$  and demonstrates the existence of a regulation strategy. This strategy must be associated with the lower  $P_{O_2}$  values within the animal's carapace valves, showing that podocopids can actively regulate their tissue  $P_{O_2}$  at constant but even lower values than the water. In conclusion, the low tissue  $P_{O_2}$  strategy could have existed in early crustaceans and, by extension, in early animals.

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

### Introduction

The first eukaryotic cells are thought to have been in existence on our planet for about 2 billion years (Han and Runnegar, 1992) and current evidence suggests that animal life started to evolve in a low oxygen environment. Indeed, the Proterozoic period was characterized by a low O<sub>2</sub> partial pressure ( $P_{O_2}$ ) in the atmosphere (0.2–3 kPa), which was much lower than the normoxic  $P_{O_2}$  of 21 kPa presently found at sea level (Holland, 1994; Berner and Canfield, 1996; Canfield, 1998; Bekker et al., 2004).

There was an explosion of life in the lower Cambrian and, based on both the crustacean fossil record (Vannier and Abe, 1995; Waloszek, 1999; Shu et al., 1999; Siveter et al., 2001; Horne et al., 2002) and molecular phylogeny (Yamaguchi and Endo, 2003), the different species of the Ostracoda, one of the largest groups of crustaceans, appears to have been established since that time. If one compares present and early fauna, very few such primitive animals are still present and so ostracods emerge as an outstanding group covering at least 500 million years of aquatic life. Their carapace fossils are proven values for interpreting the geological age, depth, salinity and other paleoecological parameters of sedimentary rocks.

Remarkably, in water-breathing animals, there is a strategy of gas-exchange regulation that consists of maintaining  $P_{O_2}$  in

the arterial blood within an astonishingly low and narrow range (1–3 kPa, i.e. about 5–10 times lower than in homeotherms; Massabuau, 2001). In mammalian tissues, including the brain, the most frequently measured  $P_{O_2}$  is also at 1–3 kPa (Vanderkooi et al., 1991). Based on the postulate that basic cellular mechanisms have been established since the early stages of evolution, it has been suggested that this similarity in oxygenation status could be the consequence of an early adaptation strategy, which subsequently, throughout the course of evolution, maintained cellular oxygenation in the same low and primitive range, independent of environmental changes (Massabuau, 2001, 2003). The low arterial oxygenation strategy observed in water-breathers could thus be a link in a chain stretching back as far as the Proterozoic ages. The whole story, including the elaboration of sophisticated gas exchange systems that work in either water or air (and the use of respiratory pigments) – plus adaptations to increasing animal sizes, complexity and metabolic levels in earth's changing atmosphere – may represent a remarkable example of homeostasis operating over a vast time scale.

In the present paper we have used podocopid ostracods to test this hypothesis by gaining more insight into the O<sub>2</sub>-supply control mechanisms in early crustaceans. Podocopid ostracods

are minute crustaceans (0.3–3 mm) with a laterally compressed body within a calcified bivalved carapace that encloses a domiciliary cavity. They inhabit diverse benthic environments both in fresh- and seawater ecosystems. The podocopids are the most diverse and widespread ostracods today. It is important to note that they lack gills and heart, which suggests a clearly primitive morphofunctional structure. Interestingly, however, they all possess a pair of ventilatory appendages, physiologically analogous to the scaphognathites present in present Crustacea (Hughes et al., 1969). These appendages beat rhythmically and bring water currents into the domiciliary cavity where gas exchanges ( $O_2$ – $CO_2$ ) occur.

### Materials and methods

Experiments were performed all the year round on a total of 347 ostracods (body size ranging from 300 to 700  $\mu\text{m}$ ) belonging to six genera. Three species (*Leptocythere castanea*,  $N=180$ ; *Cyprideis torosa*,  $N=125$ ; *Loxococoncha elliptica*,  $N=14$ ) were collected locally in the Bay of Arcachon, SW France, by hand-sampling sediment cores at low tide on muddy sand sediments. Two species (*Cytheropteron alatum*,  $N=10$ ; *Argilloecia conoidea*,  $N=2$ ) were sampled in muddy sediment from the Bay of Biscay during the Oxybent program cruises (1998–2000) at depths ranging from 300–800 m using a suprabenthic sledge (Macer-Giroq sledge; Dauvin et al., 1995). Three main types of analysis were performed on a mixed population of ostracod species: (1) ventilatory responses to various water oxygenation levels, (2) behavioural positioning in an oxygen gradient, and (3) morphofunctional anatomy to determine maximum  $O_2$  diffusion distances.

Animals were acclimated in the laboratory for at least 1 month before experiments began (Massabuau, 2001) and then remained in the experimental set-ups for 3–8 weeks. As no significant mortality or statistical difference was observed as a function of experimental duration, all the data are presented together. Note that this illustrates the stability of our procedure and observations in our experimental conditions. Altogether, a total of about 750 h of observation was performed. For reference, 1 kPa=7.5 Torr or mmHg. In seawater (salinity=30‰) equilibrated with air, the partial pressure is 21 kPa, and the oxygen concentration is 9.33 mg  $\text{l}^{-1}$  (280  $\mu\text{mol l}^{-1}$ ) at 10°C and 7.90 mg  $\text{l}^{-1}$  (242  $\mu\text{mol l}^{-1}$ ) at 18°C.

### Maintenance conditions

The animals, together with their natural sediment, were placed in open-flow PVC tanks (40 cm×15 cm×15 cm) in a dark room thermostated at 10 or 18°C. They were all supplied with seawater from the Bay of Arcachon (water  $P_{O_2}\approx 20$ –21 kPa; water pH $\approx 7.8$ ; salinity $\approx 30$ ‰). Given 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 under a binocular microscope before experiments. To minimise external disturbances, the experimental tanks were isolated from laboratory vibrations on anti-vibrating benches.

### Ventilatory analysis by video recording

The experiments consisted of studying ostracod ventilatory activity when exposed to various steady water  $P_{O_2}$  conditions at 10°C, by visual inspection after or during video recording (Figs 1 and 2). All video observations were carried out under dim light using infrared light ( $\lambda=880$  nm) to limit any disturbance to the animals. Recordings were made 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 the animals were only motionless on exceptional occasions, no attempt was made to use any automatic frequency counting device.

### Experimental procedure

Analyses were performed on groups of mixed species originating either from the Bays of Arcachon or Biscay. One week before the experiment started, a group of 7–15 ostracods were transferred to a wedge-shaped micro-aquarium (Fig. 1; volume 500  $\mu\text{l}$ ; 2 cm×2 cm, thickness, 0–3 mm; water renewal rate 10–20  $\mu\text{l min}^{-1}$ ). This was hand-made using a microscopic slide fixed using SYLGARD (Down Corning; Michigan, USA) on a thermostated glass plate (10 cm×6 cm×0.5 cm). It was provided with *in situ* muddy sand and vegetal remains to mimic a ‘natural-like’ environment, in which the animal could move freely, dig and hide between sand particles. This aquarium was part of a 1 l closed recirculatory system with constant entry and exit levels, set at  $10\pm 0.1^\circ\text{C}$  for all experiments using a

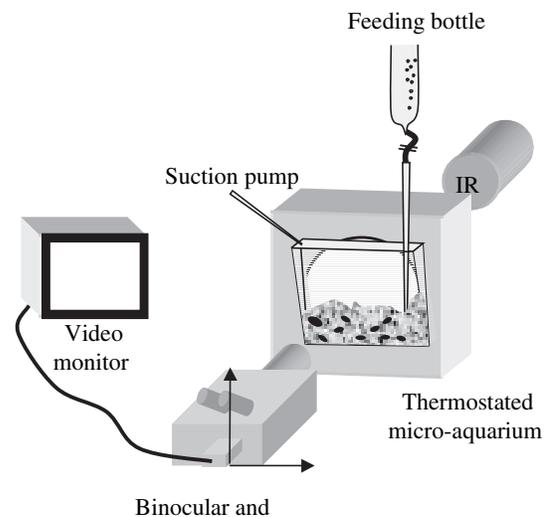


Fig. 1. Experimental set-up for ventilatory analysis by video recording. Animals were free ranging in a vertical layer of natural sediment and ventilatory activity was measured by visual inspection through the animals. Analysis was performed under dim light by means of infra-red (IR) camera and micro-spotlight (see text for details).

laboratory-constructed thermoelectric device. During the experiments  $P_{O_2}$  varied from 2–21 kPa (27–282  $\mu\text{mol l}^{-1}$ ). The partial pressure of CO<sub>2</sub> ( $P_{CO_2}$ ) was maintained at 0.1 kPa, a value typical of water  $P_{CO_2}$  in air-equilibrated environments. The gas mixtures were bubbled through the reservoir of seawater feeding bottles. The N<sub>2</sub>/O<sub>2</sub>/CO<sub>2</sub> gas mixture was obtained *via* mass flow controllers (Tylan General, model FC-260; San Diego, USA) driven by a laboratory-constructed programmable control unit.

Two sub-types of experiments were performed in this set-up: (1) analysis of reference ventilatory pattern in normoxia and response to 3-day exposure periods at 3 kPa, and (2) ventilatory responses to 2 h exposure periods by decreasing the range from 21 to 2 kPa.

#### Long-term exposure at water $P_{O_2}=21$ and 3 kPa

Analysis of the reference ventilatory pattern to long-term exposure in normoxia (21 kPa, 282  $\mu\text{mol l}^{-1}$ ) and 3 days in hypoxia (3 kPa, 40  $\mu\text{mol l}^{-1}$ ) was performed from April to June on two podocopid species from the Bay of Arcachon and two species from the Bay of Biscay. Animals were first studied for 3 days in reference normoxic conditions (21 kPa), where the animals had adapted to the set-up for 1 week, then in hypoxia for 3 days ( $P_{O_2}=3$  kPa, hypoxic test) and finally in normoxia after 3 days of recovery (recovery). When the analysis started (day 1), the experiment consisted of focussing on individuals (recognizable by their species, location in the aquarium, size and shell marks) and studying them for 1 h periods in normoxia, hypoxia and, finally, normoxia. Thus, a total of 3 h (3×1 h) of analysis was performed on each individual studied. Their ventilatory pattern was described during reference days 1, 2 or 3, test days 4, 5 or 6 and recovery days 7, 8 or 9. For each animal, the time spent actively ventilating during the studied hour ( $\text{min h}^{-1}$ ), the mean ventilatory burst duration (min), the burst number ( $\text{h}^{-1}$ ) and the mean ventilatory frequency within bursts ( $\text{min}^{-1}$ ) were determined. There were no statistically significant differences as a function of time at each water  $P_{O_2}$  level, so all data were pooled. Comparisons

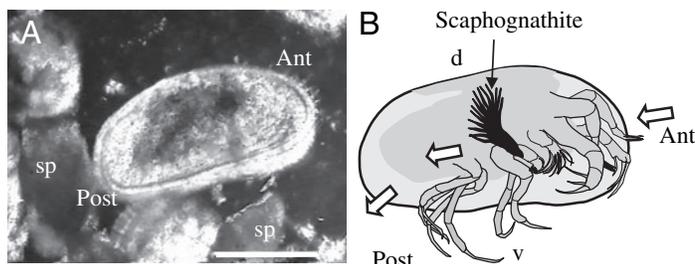


Fig. 2. Morphofunctional anatomy of *Cyprideis* sp., a typical podocopid ostracod from the Bay of Arcachon. (A) *In situ* picture in the experimental micro-aquarium. Note the size of the animal in comparison to the sand particles (sp). (B) Schematic drawing (right valve not shown) illustrating the ventilatory flow pattern (white arrows) through the animal. The inspired water enters from the anterior aspect and superfuses the soft body. Ant, anterior; post, posterior; d, dorsal; v, ventral; scaphognathites, ventilatory plates. Scale bar, 250  $\mu\text{m}$ .

were performed using a paired *t*-test, as an individual was its own reference.

#### Resistance to 24 h-anoxia

To obtain some insight into the potential ability of podocopids to survive anoxic exposure, a group of ten specimens from the Bay of Arcachon (*Leptocythere* and *Cyprideis*) were exposed to  $P_{O_2}=0$  kPa and  $P_{CO_2}=0.1$  kPa during a 24 h exposure period. After recovery at  $P_{O_2}=21$  kPa, ventilatory activity and behaviour were studied during the following 72 h period.

#### Short-term exposure at various oxygenation levels

These experiments were done on three species from the Bay of Arcachon and two from the Bay of Biscay. Each group was exposed to seven plateau levels of different water  $P_{O_2}$  presented in the following order (21, 10, 6, 4, 3, 2 and 21 kPa). The duration of exposure was 2 h per oxygen level, and ventilatory frequencies within ventilatory bursts ( $\text{min}^{-1}$ ) were measured during the last 30 min of exposure. Again, each specimen was identified. Under each experimental condition, measurements were performed twice per individual and both determinations were pooled.

#### Behavioural regulation of organism oxygenation status

This experiment, performed in August, consisted of studying the ostracod positioning into natural and experimentally manipulated O<sub>2</sub> gradients at 18°C. Thirty five cores of muddy sandy sediment (sediment depth, 15 cm; water column, 5 cm;  $N=35$ ) were hand-collected with glass tubes (diameter 5 cm, length 20 cm) in the Bay of Arcachon at Malprat Island (day 0; Carbonel, 1978, 1980). In an attempt to minimize heterogeneity between cores, the size of the sampled area was 1 m<sup>2</sup>. They were transferred within 1–1.5 h to a laboratory thermostated at 18°C and placed in experimental tanks fed with running seawater (water  $P_{O_2}\approx 21$  kPa, water pH $\approx 7.8$ , salinity=30‰). Great care was taken not to disturb the sediment at the interface. Five cores were randomly chosen 1–2 h later to determine reference normoxic O<sub>2</sub> profiles and ostracod location. Following O<sub>2</sub>-profile determination, the cores were immediately frozen in liquid N<sub>2</sub> to fix the ostracod positions in the sediment. They were kept at –20°C prior to analysis. One day after field sampling (day 1), 15 cores were exposed to hyperoxia (water column  $P_{O_2}=40$  kPa) to manipulate the anoxic zone. The remaining 15 cores were kept in parallel under normoxic conditions. O<sub>2</sub> profiles and ostracod location were determined on five normoxic and five hyperoxic cores at days 4, 7 and 11.

#### Oxygen profile determinations

O<sub>2</sub> profiles were measured with O<sub>2</sub> polarographic microelectrodes (UNISENSE Microsensors; Aarhus, Denmark) driven by a PRIOR micromanipulator (Cambridge, UK; steps, 0.2 mm). The microelectrode was advanced through the central region of the core, recording the O<sub>2</sub> changes with depth. Preliminary experiments, during

which five profiles were measured per core (1 central, 4 peripheral), demonstrated that in our experimental conditions a single central measurement was representative of the entire vertical  $O_2$ -distribution, except when a burrow was present. Following this preliminary observation, two cores were eliminated from the full analytical process during the experimental run, as a burrow was detected. Thus, the total number of analyzed cores for ostracod positioning was 33 instead of 35.

#### *Ostracod position in the oxygen gradients*

The analysis was performed on melting cores. Each core was sliced (thickness, 400  $\mu\text{m}$ ) using razor blades and a precision micromanipulator. The slices were obtained from +1 cm above the sediment–water interface to –1 cm. Ostracod number (4–10 per core) and species analysis were determined for each slice after animal sorting under binocular.

#### *Oxygen diffusion distances*

The study was performed on a total of five *Argilloecia* and five *Cyprideis*. Whole animals were immersed in a fixative for electron microscopy (6% glutaraldehyde buffered with 0.4 mol  $\text{l}^{-1}$  sodium cacodylate, pH 7.4, osmotic pressure 1100 mOsmol  $\text{l}^{-1}$ ) for 12 h at 4°C and subsequently rinsed in cacodylate buffer (0.4 mol  $\text{l}^{-1}$ , NaCl 4%). They were then embedded separately in Araldite. Serial sections were cut using a Reichert (Depew, NY, USA) automatic ultra-microtome. Ultra-thin sections were taken from randomly distributed areas of the Araldite block. Maximum diffusion distances were measured on enlarged pictures (semi-thin preparations) after visual inspection using a Leica TCS 4D microscope (Solms, Germany).

#### *Statistical analysis*

Values are reported as mean values  $\pm 1$  standard error (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

Throughout all the experiments, animals were systematically observed at the bottom of the aquarium, crowding between sand particles and organic remains (Fig. 2A). They were only exceptionally present at the water–sediment interface and their swimming velocity ranged from 1–2  $\text{mm min}^{-1}$ . Water currents were drawn into the domiciliary of the animals by the rhythmic action of both scaphognathites (Fig. 2B), as could be seen by particle displacements, which passed over the soft body surface and were then forced backwards. Interestingly, the ventilatory movements were very similar to what is observed in green crabs *Carcinus maenas* (Hughes et al., 1969) or crayfish *Astacus leptodactylus* (Massabuau, 1983), for example. Although never strictly identical, these movements were always very similar on both sides. No reversals of flow direction, or any pause by a single scaphognathite were ever observed. However, ventilatory activity was not continuous. Indeed, a typical pattern was characterized by a spontaneous switch from active beating to pauses or apnoea. Fig. 3 is an illustration of this variability for *Cyprideis torosa*, which can be taken as representative of all studied species, independent of the original biotope (deep sea or shallow water) and period studied. This variability was considerable, both within and between animals (Fig. 3A,B). Fig. 3C,D and Table 1 illustrate this as a function of time and water  $P_{O_2}$  (21 and 3 kPa, respectively), in a group of 10 *Cyprideis*. During the 3-day period of analysis (Fig. 3C,D), the total burst duration per hour ( $\text{min h}^{-1}$ ; Fig. 3C) and the number of bursts per hour ( $\text{h}^{-1}$ ;

Table 1. Characterisation of ventilatory pattern in four species of podocopids during 3-day exposure periods in water of  $P_{O_2}=21$  and 3 kPa

Genus	<i>N</i>	$P_{O_2}=21$ kPa					$P_{O_2}=3$ kPa				
		Burst				Apnea %	Burst				Apnea %
		Number ( $\text{h}^{-1}$ )	Mean duration ( $\text{min h}^{-1}$ )	Total duration ( $\text{min h}^{-1}$ )	<i>f</i> <sub>R</sub> ( $\text{min}^{-1}$ )		Number ( $\text{h}^{-1}$ )	Mean duration ( $\text{min h}^{-1}$ )	Total duration ( $\text{min h}^{-1}$ )	<i>f</i> <sub>R</sub> ( $\text{min}^{-1}$ )	
<i>Cyprideis</i>	10	18 $\pm$ 6	3 $\pm$ 2	24 $\pm$ 8	53 $\pm$ 7	40	22 $\pm$ 9	5 $\pm$ 4	19 $\pm$ 6	58 $\pm$ 4	50
<i>Leptocythere</i>	10	11 $\pm$ 5	14 $\pm$ 8	38 $\pm$ 8	84 $\pm$ 12	30	11 $\pm$ 4	4 $\pm$ 2	33 $\pm$ 11	53 $\pm$ 4	60
<i>Cytheropteron</i>	5	4 $\pm$ 2	31 $\pm$ 17	42 $\pm$ 14	52 $\pm$ 3	20	3 $\pm$ 2	22 $\pm$ 18	38 $\pm$ 18	53 $\pm$ 4	40
<i>Argilloecia</i>	2	7 $\pm$ 6	31 $\pm$ 28	38 $\pm$ 21	63 $\pm$ 11	0	1 $\pm$ 0	60 $\pm$ 0	60 $\pm$ 0	77 $\pm$ 1	0

*Cyprideis* and *Leptocythere* were from the Bay of Arcachon; *Cytheropteron* and *Argilloecia* from the Bay of Biscay.

Water temperature = 10°C.

Number, number of ventilatory burst per hour; mean duration, mean burst duration per hour; total duration, total duration of active ventilation per hour; *f*<sub>R</sub>, respiratory frequency within bursts.

All values are means  $\pm 1$  S.E.M.

Apnea, percentage of animals presenting a total absence of ventilatory activity during the 1 h period studied.

\*Significantly different from reference values at 21 kPa; *N* = number of animals studied. Mean values do not include apnoeic animals.

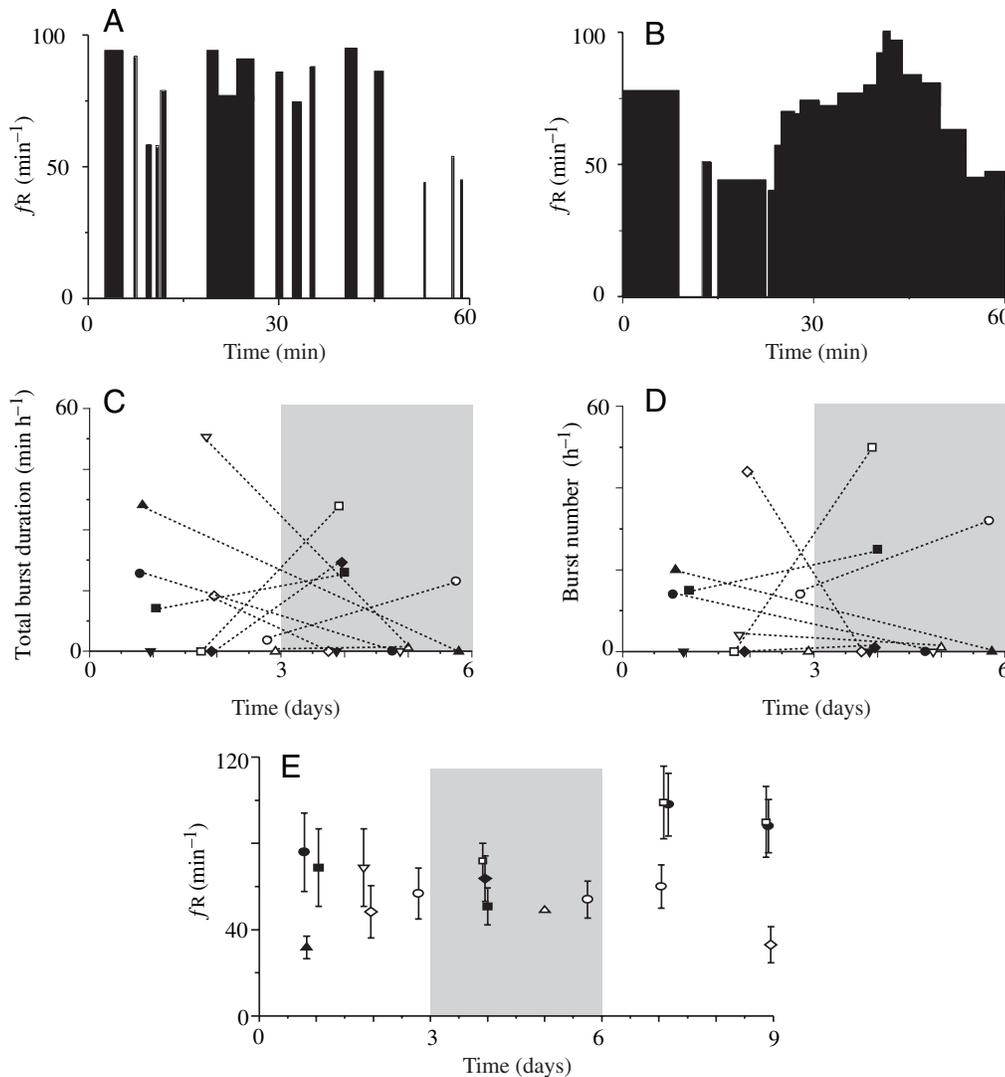


Fig. 3. Ventilatory response of *Cyprideis torosa* to consecutive 3-day exposure periods at reference water  $P_{O_2}=21$  kPa, hypoxia,  $P_{O_2}=3$  kPa, and recovery  $P_{O_2}=21$  kPa. See text for details. (A,B) Typical ventilatory pattern ( $f_R$ , ventilatory frequency) in two specimens of *Cyprideis* during 1 h observation periods at  $P_{O_2}=21$  kPa. Both activities are contrasted and characterized by alternations of ventilatory bursts and pauses. (C–E) Ventilatory activity per hour (C;  $\text{min h}^{-1}$ ), burst number per hour (D;  $\text{h}^{-1}$ ), mean ventilatory frequency within burst per minute (E;  $f_R$ ,  $\text{min}^{-1}$ ) at  $P_{O_2}=21$  and 3 kPa (grey-shaded). Each data point (mean  $\pm$  1 S.D.) was obtained from a single podocopids, randomly chosen and analysed during the 1 h period. The ventilatory frequency within burst was also analysed during a 3-day recovery period (days 7 and 9) at  $P_{O_2}=21$  kPa (E). One symbol per individual,  $N=10$  animals. No significant change was observed as a function of water  $P_{O_2}$  and time.

Fig. 3D) were studied for each animal. Clearly, both parameters were highly variable at each  $P_{O_2}$  (total burst duration= $14.3\pm 6.1$   $\text{min h}^{-1}$  at 21 kPa and  $9.5\pm 4.1$   $\text{min h}^{-1}$  at 3 kPa, paired  $t$ -tests,  $t=0.47$ , d.f.=20,  $P=0.64$ ; burst number= $11.1\pm 4.4$   $\text{h}^{-1}$  at 21 kPa and  $10.9\pm 5.7$   $\text{h}^{-1}$  at 3 kPa, paired  $t$ -tests,  $t=-0.13$ , d.f.=20,  $P=0.89$ ; means  $\pm$  S.E.M.,  $N=10$ ; no different values; zero data included to take into account apnoeic animals). For further insight into this first set of observations, we then analysed the respiratory frequency,  $f_R$ , within bursts before, during and after exposure at 3 kPa (Fig. 3E). Clearly,  $f_R$  did not significantly change as a function of time and  $P_{O_2}$  ( $f_R=53.1\pm 7.1$   $\text{min}^{-1}$  at 21 kPa during the reference period (ref),  $57.9\pm 4.3$   $\text{min}^{-1}$  during the test period at  $P_{O_2}=3$  kPa, and  $78.0\pm 10.7$   $\text{min}^{-1}$  following recovery (rec) at  $P_{O_2}=21$  kPa; means  $\pm$  S.E.M., no different values, paired  $t$ -tests: ref 21 kPa vs 3 kPa,  $t=-0.54$ , d.f.=10,  $P=0.59$ ; 3 kPa vs rec 21 kPa,  $t=-1.6$ , d.f.=10,  $P=0.14$ ; ref 21 kPa vs rec 21 kPa,  $t=1.9$ , d.f.=10,  $P=0.08$ ). Specifically, no significant hypo- or hyperventilations were observed. Table 1 shows that this ventilatory pattern and absence of ventilatory response to hypoxia was not only typical of *Cyprideis* but appeared as a

general observation in most studied podocopid genus. Finally, it must be added that all animals survived the 3-day hypoxic exposure, without any sign of behavioural and/or physiological change. However, no individual survived a 24 h anoxic exposure in our experimental conditions.

In a next step towards determining the existence or absence of ventilatory adaptation mechanisms to face water  $P_{O_2}$  changes, we then examined the effects of exposure in 2 h stages in the same five species (plus *Loxoconcha elliptica*, an extra genus from the Bay of Arcachon). These observations are presented in Table 2. When the hypoxic values are compared to the normoxic reference and recovery status, no hyperventilation could be detected in response to hypoxia. The relationship between  $f_R$  and water  $P_{O_2}$  was:  $f_R=0.6(\text{water } P_{O_2})+72.9$  ( $r^2=0.49$ ,  $P<0.079$ ). Note in addition that the percentage of apnoeic animals remains independent of water  $P_{O_2}$  in the range 21–2 kPa [number of apnoeic animals= $0.09(\text{water } P_{O_2})+46.2$ ;  $r^2=0.015$ ,  $P<0.79$ ]. Fig. 4A,B, presents all pooled data to illustrate this absence of ventilatory adaptation ability.

What could be then the adaptive solution, if any,

Table 2. Respiratory frequency within bursts in podocopids exposed to various oxygenation levels and percentage of apneic animals

Genus	N	$P_{O_2}$ (kPa)														$r^2$	
		21 (Ref.)		10		6		4		3		2		21 (Rec.)			
		fR (min <sup>-1</sup> )	Apn. (%)														
<i>Leptocythere</i>	19–48	91±6	40	84±5	52	85±8	59	87±8	52	85±12	37	102±9	52	93±8	47	0.01	0.10
<i>Cyprideis</i>	18–30	107±9	61	79±11	64	73±22	84	103±14	71	62±4*	60	71±18	56	86±5	72	0.30	0.00
<i>Loxococoncha</i>	6–14	74±11	29	73±13	46	74±17	54	86±11	43	46±18	0	41±15	0	90±18	50	0.35	0.18
<i>Cytheropteron</i>	5	62±6	20	63±12	40	50±2	40	51±13	20	41±11	20	53±4	0	61±5	60	0.59	0.32
<i>Argilloecia</i>	2	60±20	0	71±44	0	46±17	0	45±10	0	62±3	0	42±2	33	34±15	33	0.00	–

Water temperature = 10°C.

Apn., percentage of animals presenting a total absence of ventilatory activity during the studied period; Ref., respiratory characteristics during reference period; Rec., respiratory characteristics during recovery period.

All values are means ± 1 S.E.M.

\*Significantly different from reference value at water  $P_{O_2}$  = 21 kPa. N = number of animal;  $r^2$  = correlation coefficient.

developed by these animals? Are they using an alternative strategy to maintain their cellular oxygenation status, or are they exhibiting a total absence of tissue  $P_{O_2}$  regulation? They naturally inhabit oxygen gradients in the upper layers of the sediment, so we studied their positioning in naturally occurring and experimentally manipulated  $O_2$  gradients. In addition, experiments were performed at 18°C to stimulate the animals'  $O_2$  requirements and  $O_2$  dependency. Fig. 5 presents the results of these experiments performed in natural cores from the Bay of Arcachon. As  $O_2$  penetration varied between cores, and was independent of exposure time, all data were grouped by core  $O_2$ -profile characteristics (redox fronts were in the range 2–3, 3–4, 4–5, 5–6 and 7–8 mm, indicated by dotted lines in Fig. 5). Two types of podocopid species were found in the cores, *Leptocythere castanea* (N=120) and *Cyprideis torosa* (N=82). Interestingly, 2 h after field sampling, most animals were naturally found in the 3–5 kPa layer at 1–2 mm below the surface. They then stayed there during the 10-day experimental period in all cores supplied with normoxic water, independent of the acclimatory adaptation period to laboratory conditions. In cores where the  $O_2$  profile had been experimentally manipulated, the distribution also remained clearly linked to the same low oxygenation layers, and this was independent of depth and sampling time ranging from 4–11 days. Fig. 6A is a frequency distribution diagram summarizing these observations. It is clear that the  $P_{O_2}$  in water where animals

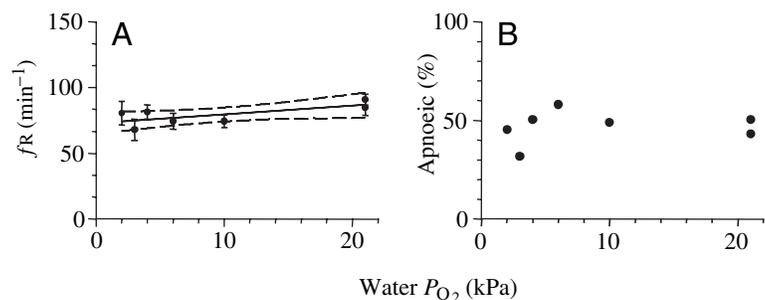
were most frequently found (N=202) ranged from 3 to 5 kPa, although data were not normally distributed and higher values of up to 16–18 kPa were occasionally observed. The above data set consequently demonstrates the existence of a behavioural regulation mechanism of body oxygenation in ostracods.

We next addressed the issue of  $O_2$  diffusion problems in these animals, which lack any blood circulatory system. Specifically, we measured maximum diffusion distances from ventilated water in the domiciliary cavity to the more central tissues. Fig. 7 presents a typical transverse section from a large 500  $\mu$ m *Argilloecia* (Fig. 7A) and a 600  $\mu$ m *Cyprideis* specimen (Fig. 7B), showing that the maximum diffusion distance for oxygen between ventilated water located between the valves and the body core ranged from 50–100  $\mu$ m. The cuticle thickness at soft body level was  $2.4 \pm 0.2$   $\mu$ m (N=10 measurements).

## Discussion

In this work we have studied the basic principles of respiratory physiology in one of the most primitive animal groups living on earth, the ostracod podocopids. We report that these crustaceans, which lack any blood circulatory system, (1) possess a pair of ventilatory appendages that beat in a similar fashion to modern malacostraceans, (2) lack any regulatory

Fig. 4. Ventilatory response to 2 h exposure periods at various oxygenation levels for all studied podocopids (N=56–106 animals; same data as in Table 2). (A) Respiratory frequencies within bursts, fR, are given as means ± 1 S.E.M. (B) Number of apnoeic animals during each exposure period expressed as percentages of the studied animals. No significant difference was observed as a function of  $P_{O_2}$ .



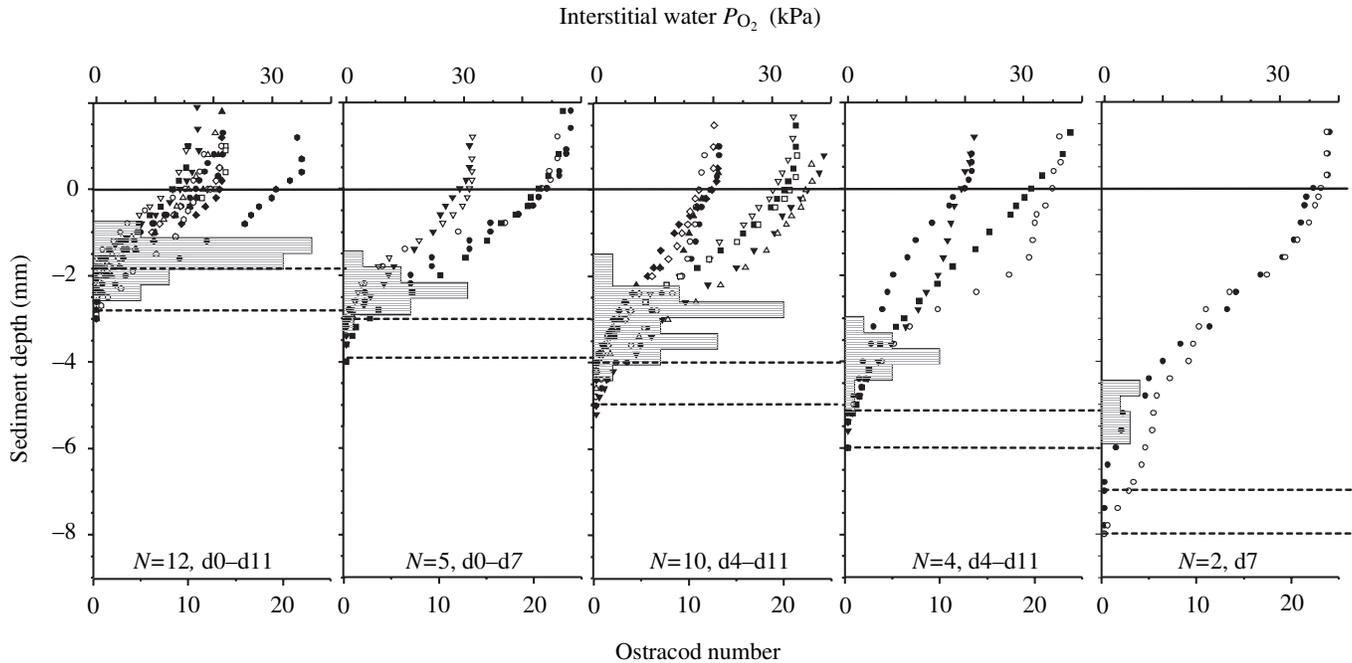


Fig. 5. Relationship between animal positioning (frequency distribution at different depths shown by the grey bars; scale, lower x axis) and  $O_2$  profiles (one symbol type per  $O_2$  profile; scale, upper x axis) as a function of sediment depth in naturally occurring (open and closed triangles, circles and open squares, left panel) and experimentally manipulated  $O_2$ -gradients (water column  $P_{O_2}$ =21 or 40 kPa). Analyses were performed at days (d) 0, 4, 7 and 11. As  $O_2$  penetration velocity varied from core to core for the same water column, values of  $P_{O_2}$ ,  $O_2$  profiles are grouped by similar near anoxic-zone depths (thickness, 1 mm; dotted lines) independent of exposure time. The animal's position followed the  $O_2$  profiles, and were independent of sediment depth and time.  $N$  is the number of analysed cores per  $O_2$  profile penetration depth.

mechanism of ventilatory adaptation to changes in water oxygenation, (3) possess an  $O_2$ -chemosensitivity, and (4) adjust the oxygenation status in their internal environment by migrating into  $O_2$  gradients at water  $P_{O_2}$  values of 3–5 kPa. Importantly, experimental manipulation of oxygen in the gradient induces this migration and demonstrates the existence of a regulation strategy. An inspired  $P_{O_2}$  value of 3–5 kPa must be associated with lower  $P_{O_2}$  values between the animal's valves and evidently at tissue level, showing that podocopids actively regulate their cellular  $P_{O_2}$  to low and constant values. We therefore suggest that the low tissue  $P_{O_2}$  strategy that has been reported in present water-breathers and air-breathers also exists in podocopids. Its aim is to maintain a cellular  $P_{O_2}$  within a low range, possibly 1–3 kPa. This strategy is irrespective of species and phyla, living medium (water or air), animal architecture and size, temperature and resting metabolic level. Podocopid ostracods are early animals and, in blood of modern water-breathers and mammalian tissues,  $P_{O_2}$  levels are within

the same low range (Fig. 6B,C), which reinforces the postulate that evolution has maintained our cellular oxygenation status at a primitive and protective level (Massabuau, 2001, 2003). Ostracods were present from 540–500 million years ago

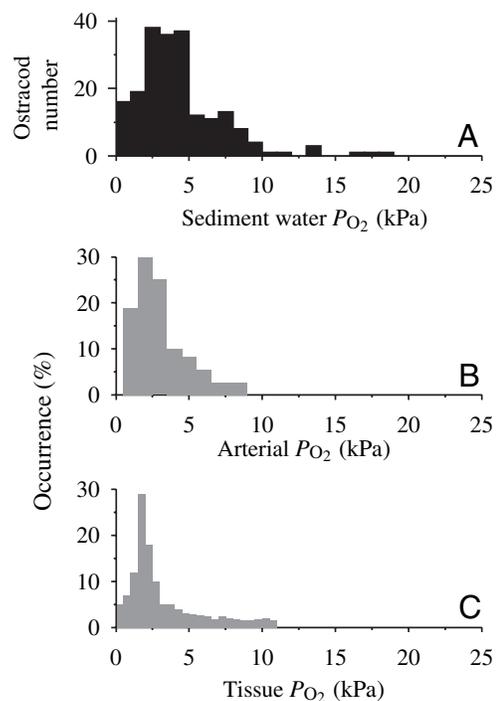


Fig. 6. (A) Frequency distribution of ostracods as a function of water  $P_{O_2}$  in the sediment. Note that the values are not normally distributed and the podocopids were most frequently present in the range 3–5 kPa. (B) Frequency distribution of arterial  $P_{O_2}$  in the Chinese crab *Eriocheir sinensis* in normoxia ( $P_{O_2}$ =21 kPa) from Fogue et al. (1992). (C) Frequency distribution of tissue  $P_{O_2}$  in mammalian brains at 37°C (cerebral cortex of rat from Lübbers; Siesjö, 1978). Note the impressive similarity of oxygen status, despite the large evolutionary gap.

through Phosphatocopids (Müller, 1982; Siveter et al., 2001), and they could be among the first links in a chain originating from the Proterozoic ages.

#### Comparison with previous data

To our knowledge, very little data relating to respiratory problems in ostracod podocopids have been published. In fact, the study of living ostracods has been dominated by zoologists and micropaleontologists developing geological applications (palaeoenvironment and stratigraphy). Consequently, how the carapace is produced, together with valve morphology in relation to habitat, has been extensively studied. By contrast, there is much less information about the soft anatomy (Okada, 1982; Keyser, 1990) and physiological data is scarce (Van Morkhoven, 1962; Maddocks, 1992). Hagerman (1969) reported on oxygen consumption and anaerobic survival in the brackish water podocopid *Hirschmannia viridis* (Muller) at 20°C. He reported that *Hirschmannia* did not survive anaerobic conditions for 13 h ( $LC_{50}=7$  h) and water  $P_{O_2}\approx 2$  kPa for 160 h ( $LC_{50}=55$  h). Interestingly, he did not observe any mortality at water  $P_{O_2}\approx 4$  kPa. These data are consequently in good agreement with the present report and would suggest that the presence of ostracods at water  $P_{O_2}\approx 2$  kPa, as shown in Figs 5 and 6A, could be only transitory. Variability evidently exists between species, however, as Danielopol et al. (1993) reported that the freshwater *Limnocythere inopinata* survived a 96 h exposure period at 0.2 kPa, but *Metacypris cordata* did not (at 11°C). It is also noteworthy that Geiger (1990) studied the distribution of freshwater podocopids *Cytherissa lacustris* in sediments from Lake Mondsee, Austria. He reported that *C. lacustris* was most abundant 5–10 mm below the sediment–water interface and that the maximum  $O_2$  penetration depth was 5–8 mm, depending on the  $O_2$  concentration in the overlaying water. This evidently reflects the positioning in the sediment  $O_2$  profile as described in the present study. Some information about scaphognathite frequency in *Metacypris cordata* (ostracod podocopid) was also reported by Danielopol et al. (1993). These authors worked on two individuals immobilised upside down at different water  $P_{O_2}$  values. They reported that ventilatory activity was irregular and infrequent at  $P_{O_2}=15$  kPa (11°C), with a ventilatory frequency ranging from 0–10 beats  $min^{-1}$ . Between  $P_{O_2}=0.4$ – $0.8$  kPa, they reported a change in the beating frequency from 0 to 50  $min^{-1}$  but the scarcity of their observations evidently limited any conclusion.

#### $O_2$ diffusion problems in tissues

The present report, together with Geiger's data (Geiger, 1990) demonstrate that ostracod podocopids live at low

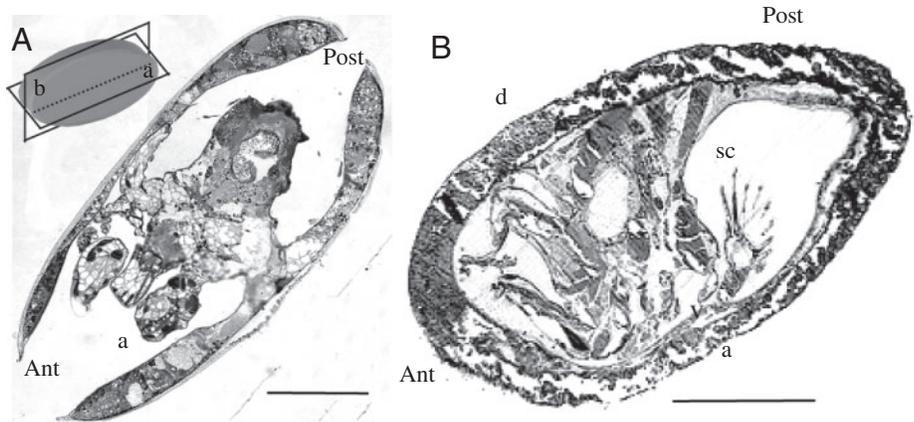


Fig. 7. Ostracod morphology and gas diffusion distance in two typical podocopids. Inset, section positioning view of A and B: a, longitudinal section, view A; b, sagittal section, view B. (A) Longitudinal view in an *Argilloecia* specimen. (B) Sagittal view in a *Cyprideis* specimen. The diffusion distance between water in the domiciliary cavity and the most central tissues never exceeds 60  $\mu m$ . Ant, anterior; post, posterior; d, dorsal; v, ventral; sc, scaphognathite. Scale bars, 100  $\mu m$ .

ambient  $P_{O_2}$ , in the sediment, far from air-equilibrated waters. This evidently raises the question of how oxygen diffuses in the animal's soft body. The problem of the maximal body size of small animals in which  $O_2$  can penetrate by pure diffusion has often been addressed since the pioneer works of Harvey (1928) and Krogh (1941). Assuming a homogeneous spherical body in which oxygen is consumed at a constant rate, and assuming that  $P_{O_2}$  at the centre is 0, the maximum diffusion distance is  $x=\sqrt{6K_{O_2}P_{O_2}/M_{O_2}}$ , where  $K_{O_2}$  is the Krogh's constant of diffusion ( $\mu mol h^{-1} cm^{-1} kPa^{-1}$ ),  $P_{O_2}$  is the  $O_2$  partial pressure in water (i.e. in the domiciliary cavity in podocopids) and  $M_{O_2}$  is the rate of  $O_2$  consumption or flux ( $\mu mol h^{-1} cm^{-3}$ ). Paul et al. (1997), taking as an example *Daphnia magna*, which is also a millimetre sized crustacean (1–4 mm; Kobayashi, 1982), a Krogh's constant of  $0.378 \times 10^{-3} \mu mol h^{-1} cm^{-1} kPa^{-1}$  (Bartels, 1971) and a  $M_{O_2}$  value of  $39 \mu mol h^{-1} cm^{-3}$  (from Kobayashi and Hoshi, 1984), calculated that the maximum diffusion depth is 340  $\mu m$  for an external  $P_{O_2}$  of 21 kPa, 280  $\mu m$  for 13.3 kPa, 200  $\mu m$  for 6.7 kPa and 140  $\mu m$  for 3.3 kPa. Similarly, Vannier and Abe (1995) calculated a critical radius at water  $P_{O_2}=21$  kPa of 1 mm, over which  $O_2$  diffusion is not sufficient to supply a spherical ostracod. In the present work, we studied animals having a maximum diffusion distance of 50–100  $\mu m$  from the domiciliary cavity to the deepest tissues, and report their spontaneous positioning at inspired  $P_{O_2}=3$ – $5$  kPa. Our results are thus consistent with simple  $O_2$ -diffusion capability, especially as *D. magna* is a so-called active species, while ostracods are much more sluggish.

#### Oxygen control mechanism in ostracods

The present results show that the ostracod podocopids we studied lack the ventilatory regulation mechanism present in crustaceans (Childress, 1971; Massabuau and Burtin, 1984;

McMahon, 2001; Pirow and Buchen, 2004), fish (Eclancher, 1972; Shelton et al., 1986), molluscs (Tran et al., 2000), amphibians and reptiles (Shelton et al., 1986), birds and mammals (Dejours, 1981; Bouverot, 1985). Note that the six species we studied were randomly chosen among podocopids inhabiting deep-sea and shallow waters and are thus representative of many, if not all, endobenthic podocopids. Paul et al. (1997) and Pirow and Buchen (2004) also studied the principles of respiratory physiology in the minute crustacean *D. magna*. In brief, they reported the existence of cardio-circulatory adaptations to hypoxia, and recently Pirow and Buchen (2004) demonstrated an O<sub>2</sub>-ventilatory drive. Thus, in contrast to our findings, in *Daphnia* a high ventilatory activity copes with a decrease in ambient oxygen availability. There are, however, numerous fundamental differences between cladocera and podocopids. First, *Daphnia* are planktonic filter feeders, which lack scaphognathites but do possess thoracic appendages. The beating of these thoracic appendages causes efficient ventilation within the animal's filtering chamber because this region is well irrigated and P<sub>O<sub>2</sub></sub> is lowered in the exiting water (Pirow et al., 1999). In addition *Daphnia* possess a heart and a simple circulatory system containing haemoglobin, whose concentration and affinity varies according to ambient oxygenation status. Podocopids, on the other hand, possess a pair of scaphognathites, as present in malacostraceans, but no circulatory system. Finally, ostracod podocopids have been present since the lower Cambrian, whereas *Daphnia* probably appeared more recently as they have only been reported from the Permian (250–300 million years old; Schram, 1982).

Importantly, we found that podocopids regulate their tissue O<sub>2</sub> status by behavioural adaptation. Indeed, they escape both the more oxygenated and anoxic pore waters by moving into the sediment and following O<sub>2</sub> profile displacements, independent of time and sediment depth. The maximum velocity reported was 1 cm min<sup>-1</sup> in *Metacypris cordata* (Danielopol et al., 1993) which is consistent with the O<sub>2</sub> kinetics that we imposed in sediments. To our knowledge, this is the first time that such a chemotropism has been demonstrated in ostracods. It demonstrates in these early crustaceans the existence of an O<sub>2</sub> chemosensitivity that is either of peripheral or central origin. In crayfish (Massabuau et al., 1980; Ishii et al., 1989) and fish (Shelton et al., 1986), the presence of peripheral O<sub>2</sub> chemoreceptors has been reported at gill level. Importantly also, the ventilatory control loop appears incomplete in podocopids, as the ventilatory frequency was definitively independent of any change in water P<sub>O<sub>2</sub></sub>. In their biotopes, ostracod positioning is evidently not only driven by oxygenation problems but also by feeding. Ostracods sweep bacteria, algae, protozoa and small particles of detritus into their mouths with the fine, feather-like hairs attached to their appendages (Elofson, 1941; Horne, 2003). In the Bay of Arcachon, organic material is homogeneously present in the first centimetres of sediment, partly due to bioturbation processes (Relexans et al., 1992). Thus, it is likely that in the present experimental conditions, food availability in

the layers where the podocopids are living was not a limiting step that significantly interfered with ostracod displacement.

*Physiology of the crustacean respiratory system viewed from an evolutionary perspective*

In Macrura and Brachyura, which are modern malacostraceans, the rhythmic movement of each scaphognathite is controlled by five levator and five depressor muscles, innervated by two motoneurons arising from a central pattern generator (CPG; Simmers and Bush, 1983) located either in the suboesophageal or thoracic ganglion (Pasztor, 1968; Young, 1975). In ostracods, despite the fact that they are early crustaceans, there is already a well-developed cerebrum, a circumoesophageal ring of ganglia, a chain of ventral ganglia and a network of motor nerves connected to the various muscles of the oral and posterior regions (Rome, 1947; Hartmann, 1967). As we have reported, the activity pattern of the scaphognathites is also perfectly well organised (Vannier and Abe, 1995) and similar to that observed in modern crustaceans (Young, 1975). Their rhythmic movement is controlled by four muscles innervated by nerves originating from the circumoesophageal ganglia (Hartmann, 1967). Because there is now considerable evidence from a variety of different invertebrates that the major features of 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), it is thus very likely that such a respiratory CPG does exist in podocopids. The idea of a central unique CPG is indeed reinforced by the observation of a strong bilateral coordination, which suggests a unique central neuronal connectivity. This strongly suggests that the existence of central neuronal circuits (here respiratory centres) capable of producing a rhythmic movement, possibly appeared very early in the course of evolution.

Horseshoe crabs *Limulus polyphemus* are more closely related to chelicerates than they are to true crustaceans, but they have also evolved little in the past 250 million years and have probably existed since the Silurian period (440–410 million years ago). Interestingly, their ventilatory pattern has also been reported as highly variable (Watson, 1980; Mangum and Ricci, 1989), a CPG displaying pattern motor outputs characteristic of rhythmic gill ventilation has been described (Wyse et al., 1980), and an absence of ventilatory change from normoxia to hypoxia has been reported (Mangum and Ricci, 1989). All taken together, this set of observations appears to be similar to our findings in the podocopids.

Finally, it is worth noting that diverse features can be taken as signs of immaturity and/or primitive status of the respiratory centres in podocopids. First, in resting crustacean decapods such as the green crab *Carcinus maenas*, the gill chambers are irrigated by regular rhythmic beating of the bilateral pair of scaphognathites with short pauses (mean duration, 13 s; frequency, 70 h<sup>-1</sup>; Jouve-Duhamel and Truchot, 1983), while in podocopids longer pauses (up to at least 60 min), exhibiting an apparently erratic frequency are observed (Fig. 3A,B). In *Carcinus*, their frequency and duration were largely decreased

by exposure to hypoxia (Taylor, 1982; Jouve-Duhamel and Truchot, 1983), which contrasts markedly with the situation reported here. Second, most decapods are capable of periodically reversing the direction of ventilatory current flow (Arudpragasam and Naylor, 1964; Hughes et al., 1969). One motor programme, driven by a specific set of motoneurons and underlying reversed beating of the scaphognathites, is responsible for this pattern (Simmers and Bush, 1983). Its functional significance is not clearly understood, but it appears to be important in cleaning detritus in the branchial cavities. In podocopids, it could also be important to clean the domiciliary cavity, as the animals are living between detritus in muddy sandy sediments. However, despite a total of ~750 h analysis of breathing patterns, we never once observed any reversal, which strongly suggests their absence or, at least low occurrence. Finally, but importantly, the present data demonstrate that changes in water  $P_{O_2}$  had no regulatory effect on the breathing rhythm, which is most certainly a major primitive characteristic. Indeed, the ability of an organism to maintain the homeostasis of its internal environment, independently of its external environment, has been one of the fundamental keys of evolution. Based on the present work, podocopids appear, by contrast, firmly subordinated to sediment layers containing low oxygen levels.

In conclusion, the present data obtained in podocopids strongly suggest that the strategy of low tissue  $P_{O_2}$  could have existed in early animals, even if they exhibited immature physiological ventilatory regulation mechanisms. This reinforces the hypothesis of an appropriate regulation of the cellular  $O_2$  status, strongly conserved throughout the evolutionary process. In this view, one can suggest that, once the oxygen concentration on the earth started to increase, podocopids used the hypoxic layers in the sediment as an ecological refuge while their ancestors could live in an open but hypoxic ocean. This is of course speculation, but it is obvious that, whatever evolutionary solution they developed, it has been exceptionally efficient as they are today among the oldest living animals present on our planet and one of the largest crustacean groups.

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