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A COMPARISON OF THE RESPIRATORY FUNCTION OF THE HAEMOCYANINS OF VERTICALLY MIGRATING AND NON-MIGRATING PELAGIC, DEEP-SEA OPLOPHORID SHRIMPS

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Summary

The effects of temperature and pH on haemocyanin oxygen-binding were compared for three species of diurnally vertically migrating and two species of non-migrating, pelagic oplophorid shrimps from the deep sea off the Hawaiian island of Oahu. The effects of L-lactate were also measured for three of these species. Haemocyanin concentrations were higher in the haemolymphs of oplophorids that migrate vertically (39.4, 46.8 and 57.6 mg ml^{-1}) than in those of non-migrators (26.0 and 36.4 mg ml^{-1}). Moderately high Bohr effects were found for vertically migrating and non-migrating oplophorids at all temperatures examined (5–25°C, ϕ=−0.46 to −0.80, and −0.55 to −0.88, respectively). The vertically migrating species had temperature-sensitive haemocyanins (ΔH=−23.1 to −41.2 kJ mol^{-1}) across the normal temperature range (5–25°C) encountered during diurnal vertical migration. This results in haemocyanins that have relatively high affinities (P_{50}=0.80–1.06 kPa at pH 7.8, 5°C) at the low temperatures and low O_2 partial pressures (approximately 2.66 kPa O_2 at 5°C) found at depth, and low affinities (P_{50}=4.00–4.66 kPa at pH 7.5, 25°C) at the higher temperatures and higher O_2 partial pressures (approximately 13.33–17.50 kPa at 25°C) found in the near-surface waters. In contrast, the non-migrating species, which live within a narrower temperature range (3–6°C) and at a constant, low partial pressure of O_2 (2.66–4.00 kPa), have haemocyanins with a high affinity for oxygen (P_{50}=0.67–0.93 kPa at pH 7.8, 5°C) and lower sensitivity to temperature (ΔH=−4.2 to −21.6 kJ mol^{-1}). The effects of temperature on the haemocyanin oxygen-affinities of the vertical migrators appear to be highly adaptive, enabling these haemocyanins to be functional across the entire depth (and thus, temperature and oxygen partial pressure) range encountered.

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Key words: haemocyanin, deep-sea, oplophorid, *Acanthephyra*, *Oplophorus*, *Systellaspis*, haemolymph, pelagic, oxygen binding, lactate, vertical migration.

Introduction

The vast pelagic realm of the world ocean is inhabited by a wide variety of animals that are different in lifestyles and physiological adaptations from those that live on the bottom in shallow environments. These animals must be adapted to maintain their vertical position in the water column either by actively swimming or by neutral buoyancy. In addition, a large fraction [off Oahu 47% of total micronekton and 60% of crustaceans between 400 and 1200 m (Maynard *et al.* 1975)] of the pelagic animal biomass in the ocean undertakes extensive diurnal vertical migrations. These animals spend the daytime in the cold and often low-O_2 conditions that prevail at depths of several hundred metres and then swim up to the warmer high-O_2 environment near the surface at night to feed, only to return to depth at dawn (Marshall, 1979). In tropical regions the differences between the day and night habitats can be extreme, with temperature differences of more than 20°C and O_2 ranging from less than 0.67 kPa at depth during the day to more than 17.50 kPa near the surface at night. The adaptations required for this environment are unique because these animals not only go back and forth between these environmental extremes on a daily basis, but at the same time they must remain continuously active to maintain their position in the water column. In addition to the vertical migrators there are also species which remain continuously at depth, sharing their habitat with the migrators during the day, and they too must remain active to maintain their position in the water column.

Although this environment makes unusual physiological demands on its inhabitants, few data are available on the physiology of these animals. In general, the vertically migrating crustaceans are more robust and more active than the non-migrators and have higher metabolic rates than the migrators, even when measured at the same temperatures (Teal and Carey, 1967; Childress and Nygaard, 1974; Childress, 1975; Cowles, 1987). The midwater crustaceans also generally have little anaerobic capacity, but are able to regulate their oxygen consumption down to the lowest P_{O_2} to which they are exposed, which can be less than 0.67 kPa in some cases (Childress, 1968, 1971, 1975; Cowles, 1987). Although these crustaceans have great abilities to remove O_2 from water and transport it to their tissues, there is virtually no information on the properties of that most critical molecule in this process, haemocyanin.

One would expect that such active, aerobic crustaceans living at a range of temperatures and often at low P_{O_2} would require very specific adaptations of the O_2-binding properties of haemocyanin. The effect of temperature on the oxygen affinity of crustacean haemocyanin is well known (for reviews see Mangum, 1980, 1983; Table 3 of Bridges, 1986), and differences in the oxygen-binding characteristics of haemocyanins have been found between closely related species which inhabit different thermal environments (Burnett *et al.* 1988; Sanders *et al.* 1988; Morris and Bridges, 1989). In crustaceans living in shallow waters, oxygen affinity of haemocyanin is generally lower in species from colder latitudes (Mangum, 1983). However, the few reports of oxygen affinity for haemocyanins from deep-

sea crustaceans which occupy cool (<6°C), thermally stable (<0.2°C range of interannual variation), low-oxygen (continuously <3.32 kPa) environments (Freel, 1978; Arp and Childress, 1985; Sanders and Childress, 1988*a*; Sanders, 1989) have shown that these species have haemocyanins with relatively high O_2 affinities.

The study of the properties of the haemocyanins of vertically migrating and non-migrating crustaceans provides an opportunity to elucidate some aspects of the temperature adaptations of crustacean haemocyanins by allowing one to compare the properties of haemocyanins from animals which share the same environment during the day but have very different environments at night. Such studies can also give insight into haemocyanin adaptations to regularly fluctuating temperatures. The potential effects of temperature, pH and L-lactate on oxygen binding by haemocyanin are of particular interest in the case of vertical migrators because they encounter a wide temperature range, a wide O_2 range and are almost certainly more active when near the surface. Because of the large temperature and O_2 concentration differences between the day and night depths of vertically migratory shrimps off the Hawaiian Islands, we chose to study this problem off Oahu.

Several species of the exclusively pelagic caridean family Oplophoridae, including genera having both migrating and non-migrating species, live at 500–1000 m depth off Oahu during the day. The non-migrators include *Acanthephyra curtirostris* Wood-Mason and *A. acutifrons* Bate, which live continuously below 500 m. The vertical migrators include *A. smithi* Kemp, *Systellaspis debilis* A. Milne-Edwards and *Oplophorus gracilirostris* A. Milne-Edwards, which live below 500 m by day, but at night swim up to depths of 50–300 m (Ziemann, 1975). During daytime, these five species share depths where oxygen partial pressures are between 2.66 and 4.00 kPa, and temperatures range from 3 to 6°C. At night, the three vertical migrators move to waters with oxygen partial pressures above 13.33 kPa and temperatures above 20°C. These vertical migrators have higher metabolic rates at the higher temperatures near the surface and, at the same temperatures, have higher metabolic rates than the non-migrating pelagic crustaceans (Cowles, 1987). All five of these species are active animals which are denser than sea water and must therefore swim continuously to maintain their position in the water column. They are also able to regulate their oxygen consumption down to the lowest environmental P_{O_2} which they encounter (2.66 kPa O_2 at 700 m depth, Cowles, 1987), but have very limited anaerobic abilities. The functional properties of haemocyanins which might support these respiratory characteristics have not been previously studied.

The effects of temperature and pH on haemocyanin oxygen-binding in five species of vertically migrating and non-migrating oplophorid shrimp were examined, as were the effects of L-lactate in three of these species, to determine the role of the respiratory proteins in enabling these species to support their metabolic oxygen demands in the deep-sea and epipelagic environments they encounter.

Materials and methods

Collection and maintenance of animals

Oplophorid shrimps were collected in the summer months of 1986 and 1987 off the leeward side of the Hawaiian island of Oahu (21°15–37′N, 158°15–38′W) from depths of 200–1200 m. Animals were captured in a modified opening–closing Tucker trawl (3.1 m square mouth) and were brought to the surface in a thermally insulated cod end (Childress *et al.* 1978) which kept the samples near the depth temperature. Shrimps were kept alive on board ship in individual 1 l containers (5.5 °C) until used in experiments.

Initial sampling and ion measurement

For pH measurements, haemolymph samples were removed *via* hypodermic syringe from the ventral sinus of individual shrimps. Without air exposure, the sample was immediately injected into a Radiometer glass capillary electrode (Radiometer America G298A) in a water-jacketed chamber held at the experimental temperature, in conjunction with a reference electrode (Radiometer K171). Precision buffers were used to calibrate the electrode (Radiometer S1500 and S1510). Samples of haemolymph for oxygen equilibrium curves were either dialyzed for immediate use or kept frozen at −80 °C for 12–15 months for later use in experiments.

For each species, the absorbance maximum near 340 nm was measured in a 1:100 dilution of haemolymph in 50 mmol l^{-1} Tris buffer (pH 8.9) with EDTA (50 mmol l^{-1}). Haemocyanin concentration was calculated using an extinction coefficient of $2.69\,^{1\%}_{1\,cm}$ (Nickerson and Van Holde, 1971). The oxygen-carrying capacity was estimated from the haemocyanin concentration, assuming a subunit mass of 75 000 Da.

The concentrations of the major inorganic solutes of the haemolymph (Na^+, K^+, Ca^{2+}, Mg^{2+}, SO_4^{2-}, Cl^- and NH_4^+) as well as trimethylamine were measured with single-column ion chromatography (Sanders and Childress, 1988*b*), using Wescan cation and anion columns with a conductivity detector. L-Lactate concentrations in fresh, native haemolymph were measured with a Sigma L-lactate test kit (kit no. 826-UV, Sigma Chemical Co.), using the modifications suggested by Graham *et al.* (1983).

Temperature/pH experiments

To examine the short-term effects of temperature changes during vertical migration on haemolymph pH *in vivo*, specimens of *Acanthephyra smithi* (4–6 g animals) and *Oplophorus gracilirostris* (3–5 g animals) were placed, five per container, in 4 l jars of aerated sea water at 5, 10, 15, 20 or 25 °C. After 4 h, the haemolymph pH of each animal was measured. When a large enough haemolymph sample could be obtained, the concentration of L-lactate in the haemolymph of these individuals was also measured. These animals had been maintained for 24–48 h at 5.5 °C prior to their use, to allow them to recover from the stress of

capture in a trawl. Because these animals encounter all the experimental temperatures during the course of their migrations each day and we wished to simulate the effect of vertical migration, we did not consider acclimation at each temperature necessary or desirable for these studies.

Oxygen equilibrium curves

To determine the effects of pH and temperature on oxygen binding by haemocyanin, haemolymph samples were used in a thin-layer spectrophotometric system (Childress *et al.* 1984; Sanders *et al.* 1988). A small sample of haemolymph (5–20 μl) was sandwiched between two layers of 0.006 mm Teflon membrane, and the absorbance at 347 nm of gas-equilibrated samples was recorded with a Tracor Northern diode array spectrophotometer at successively higher oxygen partial pressures. Gas partial pressures (O_2 and N_2) were controlled with a Union Carbide mass flow controller. Oxygen concentrations within the gas-tight sample chamber were monitored with a Systech Instruments zirconium cell oxygen analyzer (Systech model ZR891). The calibration of the oxygen analyzer was verified with 99.99 % oxygen gas, air and 99.998 % nitrogen gas. A second sample of haemolymph from the same sample was maintained in a gas-tight, water-jacketed tonometer at the same temperature and gas concentrations as the oxygen equilibrium curve sample; the pH of this haemolymph sample was measured near the 50 % saturation point.

Samples used to determine the effects of temperature and pH on oxygen binding by haemocyanin were dialyzed in a buffered, physiological saline prepared from the inorganic ion concentrations measured in the haemolymph of each species. The use of buffered, dialyzed samples removed the possibility of variation between haemolymph samples caused by varying levels of modulators of haemocyanin function and provided control of pH during the determination of O_2 equilibria. A separate dialysis was carried out for each O_2 equilibrium determination. Possible effects of Tris buffer on the haemocyanin properties were evaluated by comparing the $\log P_{50}$–pH relationship of frozen, dialyzed, Tris-buffered *A. smithi* haemolymph at 5.0 °C with the same relationship determined on frozen, dialyzed *A. smithi* haemolymph whose pH was controlled by varying the P_{CO_2} in the equilibration gases (method of Sick and Gersonde, 1969, carried out with help of S. Morris).

The salines were prepared from the formulae given (see Table 3) and then titrated with NaOH to the desired pH. Although ammonia and/or trimethylamine were present at low concentrations in the haemolymphs of some oplophorid species, these ions were not used in dialysis media. Since these ions are volatile, their concentrations cannot be maintained in a haemolymph sample used in oxygen-binding experiments, unless the gas mixtures contain ammonia and trimethylamine gases. Although there are few data that show an effect of ammonia or trimethylamine on oxygen binding by haemocyanin (Sanders, 1989), the absence of these ions in dialyzed samples may induce functional differences in

haemocyanins. Because of the low concentrations found in these species, these effects are likely to be small in the species studied here.

Samples were dialyzed for 15–18 h in three changes of saline buffered with 0.05 mol l^{-1} Tris at a ratio of 1000 parts saline to one part sample. Each pH required a separate dialysis with the saline adjusted to the desired pH with NaOH. Since the salines were acidified with a fixed amount of HCl (see Table 3) and then titrated to the desired pH with NaOH, [Cl^-] was the same at all pH values. The effects of L-lactate on haemocyanin oxygen-binding were determined by adding 10 μl of 15 or 150 mmol l^{-1} L-lactate in saline to dialyzed haemolymph samples and completing spectral analyses, as described above to generate oxygen equilibrium curves. The lactate concentrations in these dialyzed samples were measured after each experiment using a Boehringer L-lactate test kit. Haemolymph samples used in L-lactate experiments had been stored frozen at −80 °C for 12–15 months prior to use.

Statistics

Results are reported as mean and standard deviation unless otherwise noted. Linear regression analysis was used to fit the data in the figures. Values of n_{50} were taken from the equations describing the Hill plots between 25 % and 75 % saturation. Analysis of covariance (ANCOVA) was used to test the significance of differences in elevation of regression lines having slopes which were not significantly different. Oxygen equilibrium curves at particular environmental temperatures were constructed using interpolated (for appropriate values of pH) values of P_{50} and n_{50}. Some custom software provided by S. Morris was used for these calculations.

Results

Initial haemolymph parameters

The concentration of haemocyanin, estimated oxygen-carrying capacity of the haemocyanin, mean haemolymph pH and L-lactate concentrations in samples of haemolymph drawn from animals maintained alive on board ship at 5.5 °C for between 24 and 48 h prior to sampling are shown in Table 1. In all cases the animals were swimming in an apparently unexcited manner prior to sampling and the sampling was executed quickly. Higher haemocyanin concentrations were found in the haemolymphs of vertical migrators than in non-migrators (P=0.083, Mann–Whitney U-test). Lactate concentrations varied considerably among the species, but for all except *A. smithi* showed little variation within species.

The concentrations of the major inorganic ions in the haemolymph of each of the five species are reported in Table 2. In addition to the usually detected ions, NH_4^+ and trimethylamine were detected in all species. Trimethylamine did not exceed the trace level (about 0.1 mmol l^{-1}) in any species. NH_4^+ was present at trace levels in two species of vertical migrators (*A. smithi* and *O. gracilirostris*) and at higher concentrations in the other three species. The measurements in Table 2

Table 1. Haemocyanin concentration ([Hc]), estimated haemocyanin oxygen-carrying capacity ($HcCCO_2$) of pooled samples (N=5), mean haemolymph pH and L-lactate concentrations in haemolymph samples from five animals of each species maintained for 24–48 h at 5.5°C prior to sampling

Species	Habit	[Hc] ($mg\,ml^{-1}$)	$HcCCO_2$ ($mmol\,l^{-1}$)	pH	[L-lactate] ($mmol\,l^{-1}$)
Acanthephyra curtirostris	Non-migrator	26.0	0.36	7.71±0.14	1.80±0.08
Acanthephyra acutifrons	Non-migrator	36.4	0.50	7.74±0.10	0.05±0.02
Acanthephyra smithi	Migrator	46.8	0.65	7.78±0.06	6.42±0.64
Systellaspis debilis	Migrator	39.4	0.55	7.82±0.09	2.75±0.06
Oplophorus gracilirostris	Migrator	57.6	0.80	7.85±0.02	1.16±0.14

Values for pH and L-lactate are reported as means±one standard deviation.

were used to prepare dialysis media having the ion concentrations reported in Table 3 but without NH_4^+ or trimethylamine.

In vivo *pH*

Results from experiments to determine *in vivo* haemolymph pH in *Acanthephyra smithi* and *Oplophorus gracilirostris* at different experimental temperatures are shown in Fig. 1. The coefficients of the regressions of haemolymph pH as a function of temperature were similar to the slope of the neutral pH of water (Reeves, 1977) and within the range reported by other authors for crustaceans (McMahon and Burggren, 1981; Morris *et al.* 1985, 1988; Morris and Bridges, 1989).

Concentrations of lactate were low and varied little in *O. gracilirostris* haemolymph (range 1.01–1.70 $mmol\,l^{-1}$, mean 1.29±0.26 $mmol\,l^{-1}$, N=6, at least one data point from each temperature), showing no significant variation with

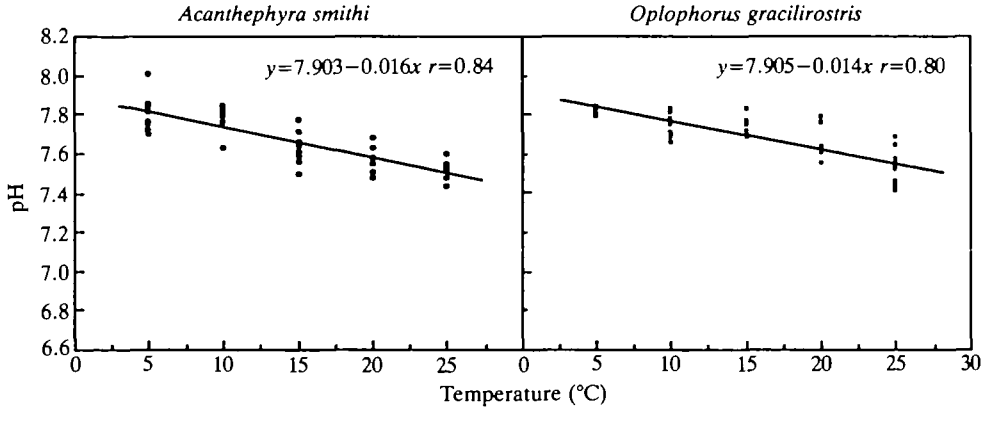

Fig. 1. The change with temperature of haemolymph pH *in vivo* of *Acanthephyra smithi* and *Oplophorus gracilirostris*.

Table 2. *Concentrations of the major inorganic solutes measured by single-column ion chromatography in the haemolymph of five species of oplophorid shrimps*

Species	Solute concentration ($mmol\,l^{-1}$)							
	Na^+	K^+	NH_4^+	Me_3NH^+	Ca^{2+}	Mg^{2+}	SO_4^{2-}	Cl^-
Acanthephyra curtirostris	451 (11)	15.4 (0.7)	12.1 (1.1)	Trace	8.9 (0.3)	11.5 (1.4)	9.4 (0.6)	467 (14)
Acanthephyra acutifrons	456 (21)	19.7 (0.8)	12.3 (0.3)	Trace	5.2 (0.4)	26.3 (2.4)	17.7 (2.6)	545 (13)
Acanthephyra smithi	446 (9)	17.8 (0.7)	Trace	Trace	6.5 (0.3)	16.7 (1.8)	17.6 (2.5)	485 (17)
Systellaspis debilis	497 (12)	15.6 (0.3)	23.2 (0.9)	Trace	6.8 (0.6)	25.0 (2.4)	17.8 (2.1)	531 (18)
Oplophorus gracilirostris	480 (12)	14.0 (1.6)	Trace	Trace	3.9 (0.3)	8.1 (0.7)	15.5 (1.8)	541 (19)

Concentrations are the mean of three samples from individual animals for each species (±1 standard deviation).
Trace refers to a detectable, but non-integratable, peak.

Table 3. *Molecular concentrations ($mmol\,l^{-1}$) used to prepare dialysis media*

Species	NaCl	KCl	$CaCl_2$	$MgCl_2$	Na_2SO_4	HCl	Tris
Acanthephyra curtirostris	360	15.4	8.9	11.5	9.4	50	50
Acanthephyra acutifrons	416	19.7	5.2	26.3	17.7	50	50
Acanthephyra smithi	369	17.8	6.5	16.7	17.6	50	50
Systellaspis debilis	409	15.6	6.8	25.0	17.8	50	50
Oplophorus gracilirostris	392	14.0	3.9	8.1	15.5	50	50

pH was adjusted to the desired value by adding NaOH so the actual Na^+ values were somewhat higher.

temperature in these experiments. This pattern is in agreement with the data from animals kept a longer time at 5.5°C (Table 1). In contrast, the haemolymph L-lactate concentrations in *A. smithi* were relatively high and variable (range 1.39–9.34 mmol l^{-1}, mean 4.25±2.56 mmol l^{-1}, N=17, at least one data point from each temperature). These data are comparable to those presented for individuals of this species kept at 5.5°C for a longer period (Table 1) and showed no significant relationship between temperature and L-lactate. Although the shrimps were swimming in an unexcited fashion before sampling and haemolymph samples were taken quickly from each animal (within seconds of removal from the experimental chamber), handling stress might have played some role in producing the high levels of L-lactate in *A. smithi*. However, given the similar manner in which the other four species behaved and were treated and the lower, less-variable L-lactate concentrations found in these species (Table 1), this seems unlikely to be the explanation. These data, along with those in Table 1, demonstrate that these species produce different amounts of L-lactate under similar conditions.

Oxygen-binding properties of the haemocyanins

The effects of pH and temperature on oxygen binding by haemocyanin in dialyzed haemolymph samples (never frozen) are shown in Fig. 2 for vertical migrators and non-migrators. Moderate Bohr effects ($\Delta \log P_{50}/\Delta pH$) were found for vertical migrators at all experimental temperatures (Fig. 2). Analysis of covariance confirmed the significance of the large decreases in oxygen affinities of the haemocyanins of vertical migrators at higher temperatures (Fig. 2, $P \leq 0.005$ for all three species, comparing P_{50} values from 5 to 15°C, 15 to 25°C, 5 to 25°C). Oxygen affinities of haemocyanins in dialyzed haemolymph samples from non-migrators were also moderately affected by changes in pH, but the decreases in oxygen affinities of haemocyanins at higher temperatures were smaller and not highly significant (Fig. 2; *A. acutifrons*, P_{50} values from 5 to 25°C and *A. curtirostris*, P_{50} values from 5 to 15°C, $P \leq 0.05$, ANCOVA; *Acanthephyra acutifrons*, comparing P_{50} values from 5 to 15°C, 15 to 25°C and *A. curtirostris* from 5 to 25°C, $P \leq 0.10$, ANCOVA). Cooperativity of oxygen binding by haemocyanin in dialyzed haemolymph was not significantly affected by temperature ($P \geq 0.25$, ANCOVA) for vertical migrators or non-migrators (Fig. 2), except for a slight increase in cooperativity with temperature found for *Oplophorus gracilirostris* at 5°C ($P<0.01$, ANCOVA).

The effects of temperature on oxygen affinity of haemocyanins from vertical migrators and non-migrators, at three constant pH values, were analyzed by van't Hoff plots (Fig. 3). Relative to other crustacean species (see Mangum, 1983; Table 3 of Bridges, 1986), the ΔH values (kJ mol^{-1}) at a constant pH (Fig. 3) are moderate for vertical migrators and low for non-migrators.

Effects of freezing, Tris buffer and CO_2

Comparisons of the oxygen-binding properties of haemocyanin in 'fresh' (never frozen) dialyzed haemolymph, and haemolymph which had been dialyzed after

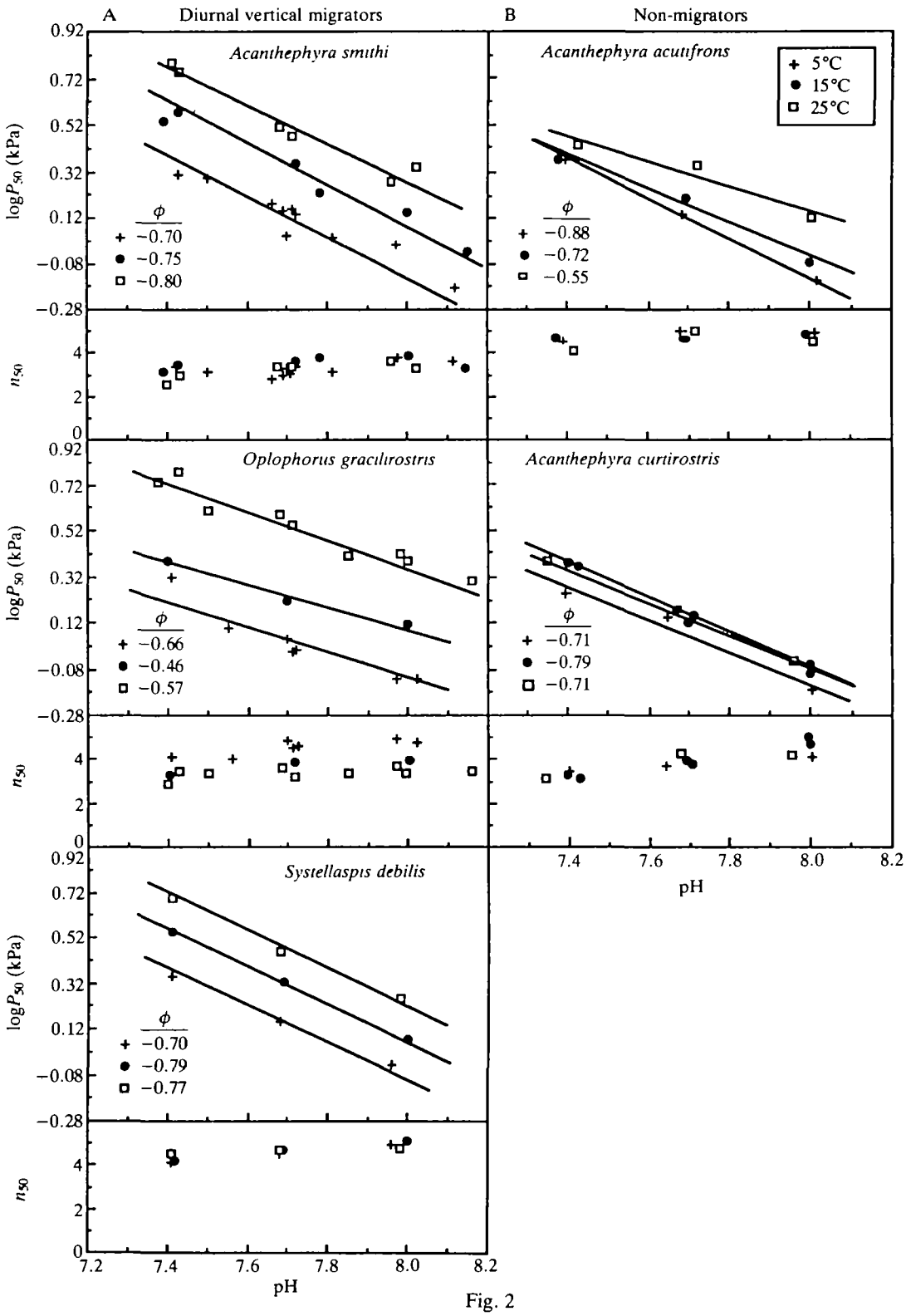
A
Diurnal vertical migrators
B
Non-migrators
Acanthephyra smithi
Acanthephyra acutifrons
Oplophorus gracilirostris
Acanthephyra curtirostris
Systellaspis debilis
+ 5°C
● 15°C
□ 25°C
φ
+ −0.70
● −0.75
□ −0.80
+ −0.88
● −0.72
□ −0.55
+ −0.66
● −0.46
□ −0.57
+ −0.71
● −0.79
□ −0.71
+ −0.70
● −0.79
□ −0.77
log P50 (kPa)
n50
pH

Fig. 2

Fig. 2. Effects of pH and temperature on affinity (P_{50}) and cooperativity (n_{50}) of oxygen-binding by haemocyanin from five species of oplophorid shrimps. The Bohr effect (ϕ) is shown for each temperature over the experimental pH range shown. (A) vertical migrators; (B) non-migrators. Equations for lines: *A. smithi* 5°C, $\log P_{50}$ (kPa)=5.524−0.699 pH, r^2=0.92; 15°C, $\log P_{50}$ (kPa)=6.175−0.752 pH, r^2=0.98; 25°C, $\log P_{50}$ (kPa)=6.660−0.796 pH, r^2=0.95; *O. gracilirostris* 5°C, $\log P_{50}$ (kPa)= 5.172−0.664 pH, r^2=0.91; 15°C, $\log P_{50}$ (kPa)=3.811−0.463 pH, r^2=0.98; 25°C, $\log P_{50}$ (kPa)=4.966−0.573 pH, r^2=0.94; *S. debilis* 5°C, $\log P_{50}$ (kPa)=5.508−0.696 pH, r^2=0.99; 15°C, $\log P_{50}$ (kPa)=6.416−0.791 pH, r^2=1.00; 25°C, $\log P_{50}$ (kPa)= 6.384−0.768 pH, r^2=0.99; *A. acutifrons* 5°C, $\log P_{50}$ (kPa)=6.919−0.883 pH, r^2=0.99; 15°C, $\log P_{50}$ (kPa)=5.743−0.724 pH, r^2=0.96; 25°C, $\log P_{50}$ (kPa)=4.518−0.546 pH, r^2=0.90; *A. curtirostris* 5°C, $\log P_{50}$ (kPa)=5.574−0.714 pH, r^2=0.96; 15°C, $\log P_{50}$ (kPa)=6.266−0.792 pH, r^2=0.98; 25°C, $\log P_{50}$ (kPa)=5.644−0.713 pH, r^2=1.00.

being frozen and thawed once (frozen at −80°C for 12–14 months) are shown in Fig. 4 for *Acanthephyra smithi*, *A. acutifrons* and *Oplophorus gracilirostris*. For *A. smithi*, ANCOVA showed no significant differences in cooperativity (n_{50} values) and affinity (P_{50} values) of oxygen binding (P>0.25, 5 and 25°C). Significant decreases in haemocyanin cooperativity (P<0.005, ANCOVA) and increases in haemocyanin oxygen-affinity (P<0.005, ANCOVA) were found for frozen haemolymph from *A. acutifrons* and *O. gracilirostris* (5 and 25°C) (Fig. 4).

The effects of Tris buffer and CO_2 on the O_2 affinity of the haemocyanin of *A. smithi* were evaluated by comparing the $\log P_{50}$–pH relationship generated at 5°C using Tris–buffered, previously frozen samples with no CO_2 present (Fig. 4) with one generated using previously frozen samples buffered with 0.002 mol l^{-1} bicarbonate whose pH was controlled by varying P_{CO_2} (Fig. 4). Both data sets had six points at pH values ranging from 7.1 to 8.1. ANCOVA showed that they did not differ significantly in slope (P>0.25) or elevation (P=0.73). Thus, neither Tris nor CO_2 have apparent specific effects (independent of pH) on the affinity of this haemocyanin for oxygen.

Effects of L-*lactate*

The effects of L-lactate on haemocyanin oxygen-affinity were measured in frozen, dialyzed haemolymph of *Acanthephyra smithi*, *Oplophorus gracilirostris* and *A. acutifrons* (Fig. 5). The oxygen affinity of the haemocyanin was increased slightly in *A. acutifrons* ($\Delta\log P_{50}/\Delta\log$[lactate]=−0.08 at pH 7.4 and −0.05 at pH 7.9) and *O. gracilirostris* haemocyanin ($\Delta\log P_{50}/\Delta\log$[lactate]=−0.03 at pH 7.4 and 7.9, 5°C). These increases in affinity were, however, significant only at high concentrations of lactate for both species (P<0.005, ANCOVA). Lactate had a moderate, but significant (P<0.005, ANCOVA), effect on the oxygen affinity of the haemocyanin of *A. smithi* at 5°C, and a smaller, but significant (P<0.01, ANCOVA), effect at 25°C ($\Delta\log P_{50}/\Delta\log$[lactate]=−0.11 at pH 7.4 and −0.17 at pH 7.9, 5°C; −0.04 at pH 7.4 and 7.9, 25°C). The cooperativity of oxygen binding by haemocyanin was not significantly altered by the presence of L-lactate (P>0.25, ANCOVA) in any of the three oplophorid species examined (Fig. 5).

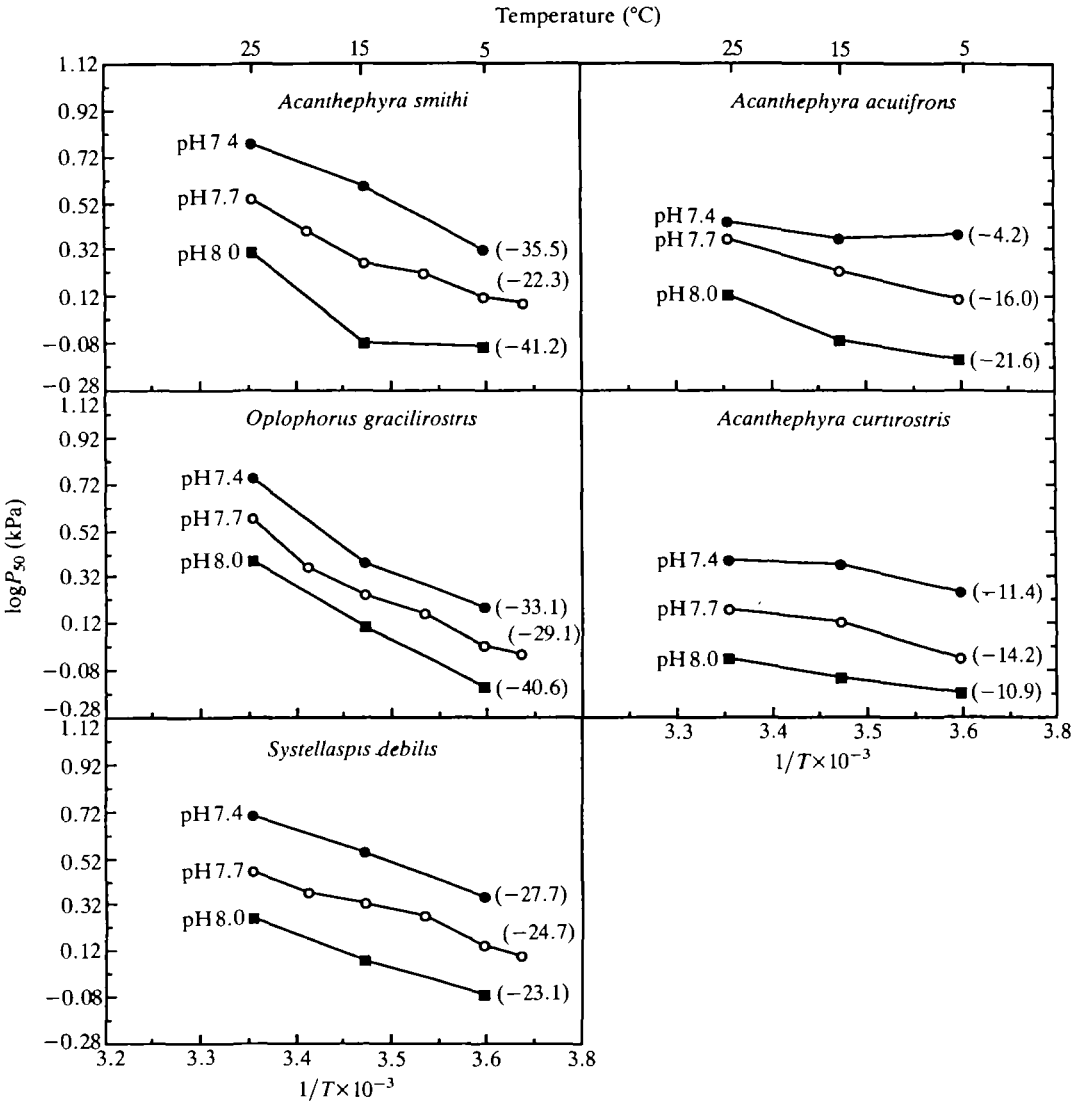

Fig. 3. van't Hoff plots showing the effects of temperature on haemocyanin oxygen-affinity at three fixed pH values. Numbers in brackets are values for ΔH (kJ mol^{-1}) at each pH. T is temperature (K).

Discussion

Freezing effects

It is now well established that freezing haemolymph samples prior to their use in oxygen-binding experiments can result in changes in haemocyanin cooperativity (Bridges *et al.* 1984; Morris *et al.* 1985; Morris, 1988), but freezing often does not significantly alter affinity. In the present study, haemocyanin oxygen-affinity, as well as cooperativity, was significantly altered by freezing for two of the three

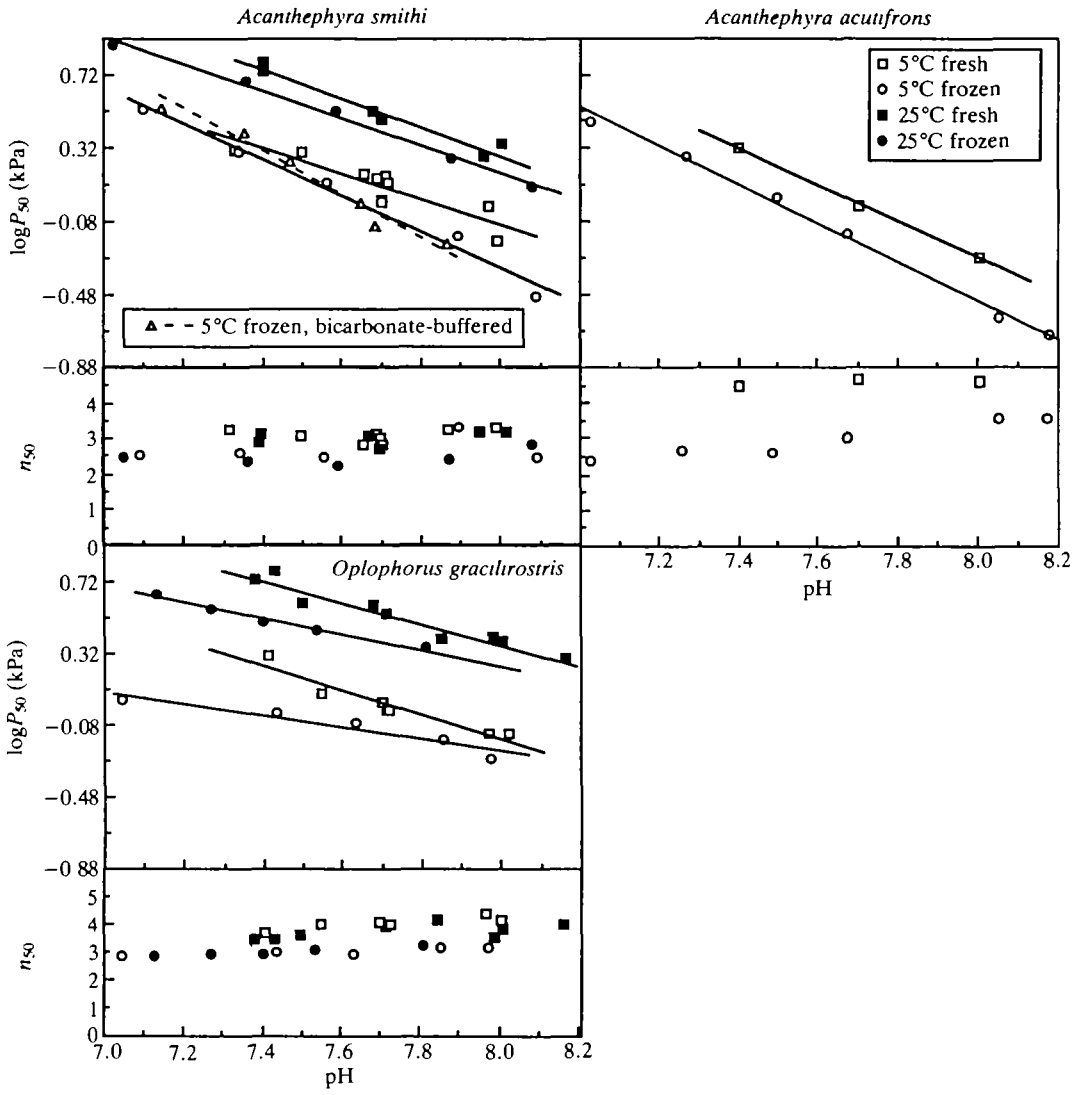

Fig. 4. Effects of freezing on affinity and cooperativity of haemocyanin in Tris-buffered, dialyzed haemolymph samples and the effect of CO_2 on the affinity of one haemocyanin at 5°C. These haemolymph samples from oplophorid shrimps had been stored for 12–15 months at −80°C. Equations: *A. smithi* 5°C, fresh, logP_{50} (kPa)= 5.361−0.681 pH, r^2=0.80; 5°C, frozen, logP_{50} (kPa)=7.519−0.981 pH, r^2=0.98; 5°C, frozen, bicarbonate-buffered, logP_{50} (kPa)=9.148−1.192 pH, r^2=0.98 (dashed line); *A. smithi* 25°C, fresh, logP_{50} (kPa)=6.582−0.787 pH, r^2=0.96; 25°C, frozen, logP_{50} (kPa)=6.162−0.747 pH, r^2=0.99. *O. gracilirostris* 5°C, fresh, logP_{50} (kPa)= 5.172−0.664 pH, r^2=0.91; 5°C, frozen, logP_{50} (kPa)=2.268−0.346 pH, r^2=0.92; *O. gracilirostris* 25°C, fresh, logP_{50} (kPa)=4.966−0.573 pH, r^2=0.94; 25°C, frozen, logP_{50} (kPa)=3.753−0.437 pH, r^2=0.98; *A. acutifrons* 5°C, fresh, logP_{50} (kPa)= 6.919−0.883 pH, r^2=0.99; 5°C, frozen, logP_{50} (kPa)=7.152−0.938 pH, r^2=0.99.

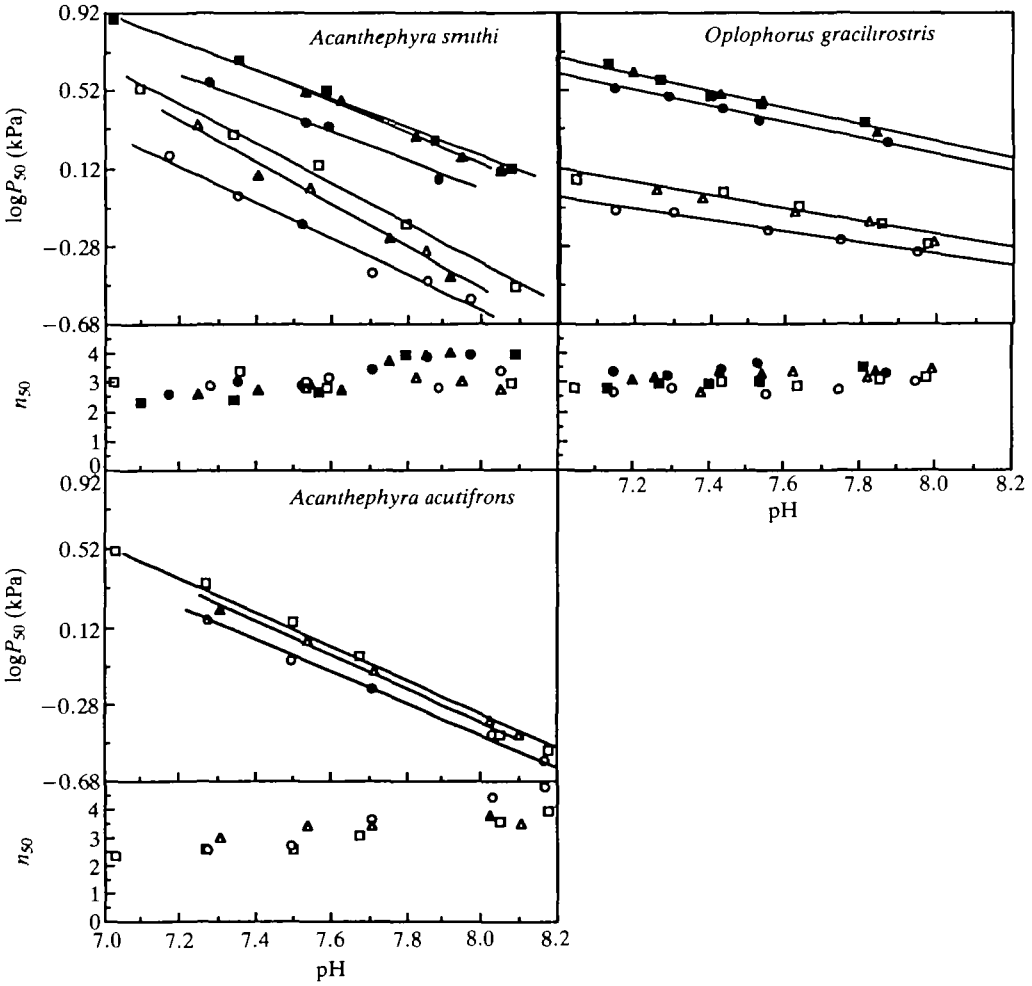

Fig. 5. The effects of L-lactate on oxygen binding by haemocyanin from two species of vertically migrating oplophorid shrimps. Haemolymph had been stored frozen (−80°C) for 12–15 months prior to use, and was thawed once only. Oxygen equilibrium curves were generated at 5 (open symbols) and 25°C (closed symbols). Regression equations for *Acanthephyra smithi*: 0.14 mmol l^{-1} lactate (squares), 5°C, logP_{50} (kPa)=7.852−1.027 pH, r^2=0.99; 25°C, logP_{50} (kPa)=6.162−0.747 pH, r^2=0.99; 1.23 mmol l^{-1} lactate (triangles), 5°C, logP_{50} (kPa)=8.156−1.084 pH, r^2=0.99; 25°C, logP_{50} (kPa)=6.675−0.816 pH, r^2=0.99; 12.80 mmol l^{-1} lactate (circles), 5°C, logP_{50} (kPa)=6.816−0.929 pH, r^2=0.98; 25°C, logP_{50} (kPa)=5.284−0.651 pH, r^2=0.93. Regression equations for *Oplophorus gracilirostris*: 0.02 mmol l^{-1} lactate (squares), 5°C, logP_{50} (kPa)=2.536−0.345 pH, r^2=0.92; 25°C, logP_{50} (kPa)=3.753−0.437 pH, r^2=0.98; 1.78 mmol l^{-1} lactate (triangles), 5°C, logP_{50} (kPa)=2.602−0.355 pH, r^2=0.98; 25°C, logP_{50} (kPa)=4.026−0.472 pH, r^2=0.99; 16.19 mmol l^{-1} lactate (circles), 5°C, logP_{50} (kPa)=2.055−0.297 pH, r^2=0.99; 25°C, logP_{50} (kPa)=3.522−0.417 pH, r^2=0.99. Regression equations for the non-migrating *A. acutifrons* at 5°C: 0.17 mmol l^{-1} lactate, logP_{50} (kPa)=7.152−0.938 pH, r^2=0.99; 1.21 mmol l^{-1} lactate, logP_{50} (kPa)=6.326−0.835 pH, r^2=0.99; 15.84 mmol l^{-1} lactate, logP_{50} (kPa)=5.906−0.791 pH, r^2=0.99.

species examined, emphasizing the importance of using, whenever possible, fresh haemolymph samples in descriptions of oxygen-binding properties of haemocyanins from species whose haemocyanins have not been previously studied. A comparison of the oxygen affinity of the haemocyanin of fresh *Oplophorus gracilirostris* haemolymph at 5°C and once-frozen haemolymph at 25°C, for example, would obscure the actual effects of temperature on affinity shown by direct comparison in fresh haemolymph samples for this species (Figs 2 and 4). As shown by Morris (1988), the effects of freezing vary among species, making examination of the oxygen-binding properties of haemocyanin in fresh haemolymph samples necessary prior to evaluation of the oxygen-binding properties of haemocyanin in frozen haemolymph samples.

Effects of temperature

It is evident from Figs 2 and 3 that oxygen affinities of the haemocyanins from the vertical migrators *Acanthephyra smithi*, *Systellaspis debilis* and *Oplophorus gracilirostris* are temperature dependent. Values for ΔH at constant values of pH are comparable to those of other crustaceans from environments with variable temperature (Mangum, 1983; Morris *et al.* 1985; Bridges, 1986; Burnett *et al.* 1988). When the changes in haemolymph pH with temperature are taken into account, the predicted changes with increasing temperature in oxygen affinities of the haemocyanins of vertical migrators are even greater (Figs 6 and 7).

These temperature-induced changes in the oxygen affinities of the haemo-

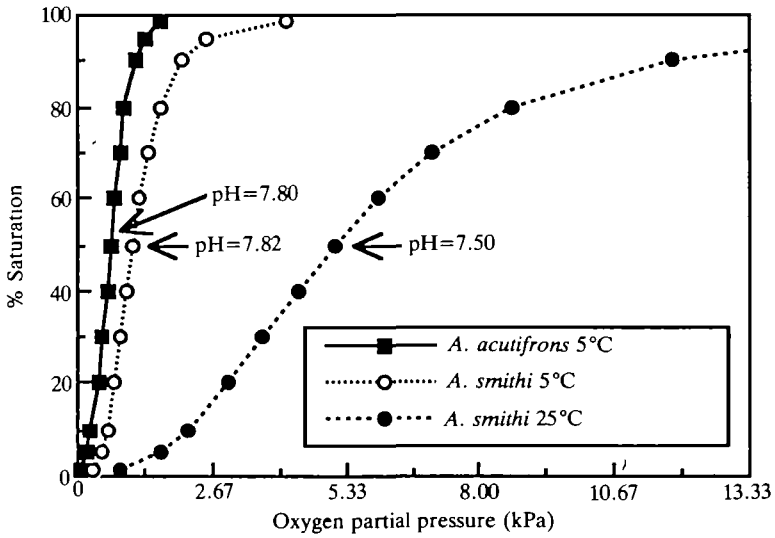

Fig. 6. Oxygen equilibrium curves interpolated from measured data to show curves applicable at environmental conditions for *Acanthephyra smithi* (5 and 25°C) and *A. acutifrons* (5°C). Curves were generated from measured values of P_{50} and n_{50} (Fig. 3), at a predicted *in vivo* pH (Fig. 1).

cyanins are adaptive for the vertical migrators in the context of diurnal changes in their oxygen consumption rates and habitat (Cowles, 1987). At daytime depths off Hawaii, environmental P_{O_2} values are in the range 2.66–4.00 kPa, and temperatures are 3–6 °C. The haemocyanin of *Acanthephyra smithi* can, however, be 95 % saturated at an internal P_{O_2} of 2.66 kPa at 5 °C, pH 7.82 (Fig. 6), allowing it to be effective, we suggest, in taking up oxygen from the lower-P_{O_2} water. Conversely, at night-time depths, the vertical migrators are more active in warmer, high-P_{O_2} surface waters (P_{O_2}>13.33 kPa above 400 m), and the greater demand for oxygen requires a haemocyanin with a lower oxygen affinity. This facilitates off-loading at the tissues while not jeopardizing maximal saturation at the gills due to the increased environmental P_{O_2}. At 25 °C the haemocyanin of *A. smithi* will be highly saturated with oxygen at intermediate O_2 levels (Fig. 6). If a temperature effect resulting in increased oxygen affinity at reduced temperatures were not present, the haemocyanin of *A. smithi* could be only poorly saturated at the low temperatures found at this species' daytime depths (Fig. 6). Therefore, the substantial temperature effects on the haemocyanins of the vertical migrators result in the same haemocyanins exhibiting very different, yet adaptive, properties under the diverse environmental conditions they encounter at day and night depths.

The situation for non-migrators is somewhat different. Oxygen consumption rates and *in vivo* pH measurements could not be made at higher temperatures because these animals do not survive in temperatures above 10–15 °C (Cowles, 1987). At 5 °C, *Acanthephyra acutifrons* and *A. curtirostris* are less active and have lower oxygen consumption rates compared to vertical migrators at the same temperature. This is reflected, for non-migrators, in the presence of haemocyanins with higher oxygen affinities (Figs 2 and 7), which are adaptive for the uptake of oxygen from the low-temperature, low-P_{O_2} waters in their environment. At the predicted *in vivo* pH of 7.8 at 5 °C (Fig. 1), the haemocyanin of *A. acutifrons* can be fully saturated at 2.66 kPa O_2 (Fig. 6). Because they are relatively temperature insensitive, however, these haemocyanins do not have the greatly reduced affinities at higher temperatures (Fig. 7) which would be necessary to support the oxygen demand present in vertical migrators when they move to near-surface waters. Low temperature sensitivity may be a general property of deeper-living crustaceans which do not migrate into the surface waters. Similar low-temperature effects have been found in a physiological pH range for the deep-sea benthic glyphocrangonid shrimp *Glyphocrangon vicaria* Faxon (Arp and Childress, 1985) and the pelagic lophogastrid mysid *Gnathophausia ingens* Dohrn (Sanders, 1989), which also live in thermally stable environments.

Effects of L-*lactate*

Low levels of L-lactate were found in haemolymph of four of these oplophorids, while high concentrations were measured in *Acanthephyra smithi*. The absence of higher L-lactate concentrations at higher temperatures in *A. smithi* and *O.*

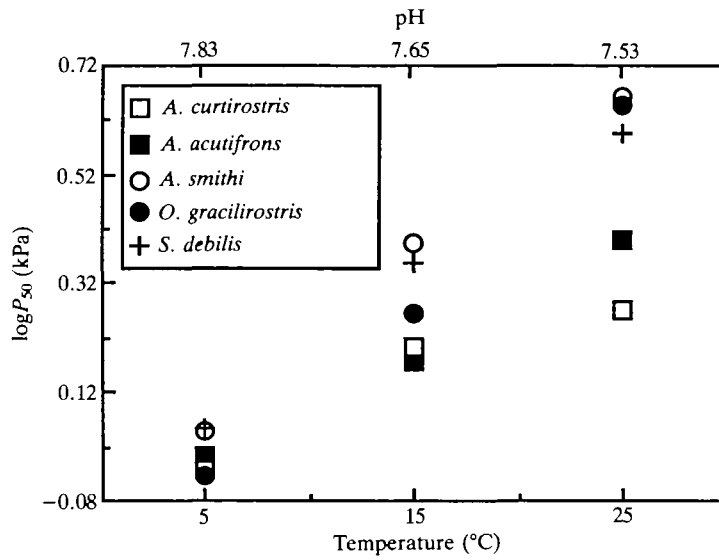

Fig. 7. The relationship between temperature, pH and oxygen affinity for the haemocyanins of five species of oplophorid shrimps. The values for predicted *in vivo* pH at the given temperatures were calculated from data for *Acanthephyra smithi* given in Fig. 1. logP_{50} data were calculated from regressions of $\Delta\log P_{50}/\Delta$pH given in Fig. 2.

gracilirostris suggests that L-lactate is not produced in response to the higher metabolic demands at higher temperatures.

Among the three species for which the specific effects of L-lactate on haemocyanin oxygen-binding were investigated using frozen material, only in *A. smithi* was a moderate effect of lactate ($\Delta\log P_{50}/\Delta\log[\text{lactate}]=-0.17$ at 5°C, pH 7.9) present. Although the effect of freezing on lactate sensitivity is unknown, this value is at the lower end of the range of lactate coefficients measured under physiological conditions of temperature and pH for other active crustacean species (−0.63 for *Palaemon elegans*, Bridges *et al.* 1984; −0.50 for *Hyas araneus*, Morris and Bridges, 1989; −0.25 for *Cancer magister*, Graham *et al.* 1983; −0.096 for *Carcinus maenas*, Truchot, 1980; see Bridges and Morris, 1986, for a review). Oxygen affinity of the haemocyanin of *A. smithi* was significantly increased by L-lactate, particularly at 5°C, and this may be important in allowing sufficient oxygen uptake when the animal descends to its daytime depths. In contrast, a significant effect of L-lactate on the oxygen affinity of the haemocyanin was present only at very high concentrations in *O. gracilirostris* and *A. acutifrons* (Fig. 5), suggesting that L-lactate is not an important modulator of haemocyanin function in these species which apparently produce little L-lactate. These data also suggest that there is considerable variation in the importance of lactate as a metabolite and as a modulator of haemocyanin O_2-affinity in midwater crustaceans.

Adaptive basis of haemocyanin properties

Data are available on the oxygen-binding properties of the haemocyanins of crustaceans from a wide variety of habitats characterized by different physical and chemical conditions (Jokumsen *et al.* 1981; Jokumsen and Weber, 1982; Mangum, 1982, 1983; Arp and Childress, 1985; Morris *et al.* 1985; Bridges, 1986; Burnett *et al.* 1988; Sanders *et al.* 1988; Morris and Bridges, 1989; Sanders, 1989). In those cases where the biology of the subject species has been sufficiently studied, the authors have generally been able to put forward quite plausible explanations of the adaptive value of the haemocyanin properties found. More general trends in haemocyanin properties have also been enunciated, based upon data from shallow-living, non-pelagic species. In particular, it has been suggested that there is an inverse relationship between affinity and environmental temperature, so that higher O_2 affinities are found in species from warmer habitats and lower affinities in species from colder ones (Redmond, 1968; Mangum, 1982; Mauro and Mangum, 1982). It has also been suggested that there is an inverse relationship between the temperature sensitivity and pH sensitivity of haemocyanin O_2-binding, so that animals with large Bohr coefficients have small ΔH values and *vice versa*, minimizing the indirect effect of temperature on O_2 affinity *via* haemolymph pH changes (Burnett *et al.* 1988). The species upon which these generalizations are based are benthic animals which live at shallow depths. Although they are often exposed to low O_2 concentrations or variable temperatures, they are able to survive under unfavourable conditions by becoming quiescent and/or relying on anaerobic metabolism for a short time, options that are usually not available to midwater crustaceans.

The data presented here, while they appear to indicate highly adaptive properties for the haemocyanins studied, do not readily fit into the generalizations derived from studies on shallow-living benthic species. These pelagic crustaceans experience very different conditions from those experienced by shallow-living benthic species, and this has apparently resulted in selection for different combinations of adaptive properties. The oplophorids considered here are active animals which must swim continuously to maintain their position in the water column and live continuously at low values of P_{O_2} when at depth. As a result they must regulate their O_2 consumption down to environmental P_{O_2} values since they are dependent on aerobiosis, having, like most other midwater crustaceans (Childress, 1971, 1975), little anaerobic capacity (Cowles, 1987). Thus, the adaptive value of the high O_2 affinities of the haemocyanins of these species is clear. Belman and Childress (1976) have, in fact, demonstrated that another active, pelagic deep-sea crustacean, the mysid *Gnathophausia ingens*, requires a high-affinity oxygen-binding protein to survive at the low P_{O_2} values in its environment. The adaptive value of the haemocyanin properties described here is underscored by the finding of similar properties of high affinity and low temperature sensitivity (under depth conditions) in this distantly related crustacean from the same midwater habitat (Freel, 1978; Sanders, 1989). In this context, and given the aerobic nature of these midwater crustaceans, the higher

oxygen affinities of the haemocyanins of these species under the conditions prevailing at depth appear to be specifically adapted to the low P_{O_2} values found in the deep-sea oxygen minimum layer environment. The higher affinities found for the non-migrators indicate that adaptation to an aerobic lifestyle at low O_2 has taken precedence over the general trend of lower affinity at lower temperature found in studies of shallower species.

The trend of an inverse relationship between the pH sensitivity and the temperature sensitivity of haemocyanin O_2-affinity is also not found in these midwater shrimps, all of which have moderate Bohr coefficients but vary considerably in temperature sensitivity. This may be the result of the Bohr shift having an important functional role in these continuously active animals so that it cannot readily be reduced in the migrators. The moderate size of the Bohr effect in all these species may represent an adaptation to limit indirect temperature effects which would be more important if the animals had larger Bohr effects.

This comparison of the data presented in the literature leads one to the conclusion that generalizations about the adaptive value of haemocyanin properties in particular environments are now beginning to become apparent. Generalizations which span the full range of environments, however, require considerably more knowledge about the habitats, habits, metabolic poise and respiratory adaptations of crustaceans from as wide a range of habitats as possible. We would suggest that in addition to temperature range and magnitude it is essential to consider the activity level, the reliance on anaerobic metabolism, the environmental O_2 partial pressures and the pattern of environmental fluctuation of the crustaceans studied to evaluate the adaptive nature of the properties of their haemocyanins. In this effort the study of closely related species from different habitats or with different habits is likely to be especially rewarding. It is also essential to study species from as wide a range of habits and habitats as can be found.

The marked differences in oxygen-binding properties of the haemocyanins of vertically migrating and non-migrating oplophorids reflect the lifestyles and habitats of each. Both groups have haemocyanins with moderately high oxygen affinity and moderate Bohr shifts at low temperature, enabling both the binding of oxygen from low-P_{O_2} waters, and the off-loading of oxygen to the tissues *via* a reduction in haemolymph pH. The non-migrators, which are limited by other physiological constraints to the low temperatures at depth, appear to maintain stable respiratory function within the narrow limits of their temperature range by having haemocyanins whose oxygen affinity is almost independent of temperature. In contrast, vertical migrators, which encounter very different environments and have much higher metabolic rates at night, achieve suitable, but very different, haemocyanin properties in both of these habitats by means of large temperature effects on the oxygen affinity of their haemocyanins.

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