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Title: Scope for Activity in the Crayfish Pacifastacus leniusculus: Role of Circulatory and Respiratory Parameters in Limiting It.

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The scope for activity of the crayfish Pacifastacus leniusculus was determined between 5° - 30°C (incipient lethal of 33°C). Standard oxygen consumption rate ($\dot{V}_{O_2}$) was low (eg. 17 ml/kg·hr at 20°C), and it increased with temperature over the entire range (Q_{10}= 2.09 for 5° - 25°C). Active $\dot{V}_{O_2}$ increased with temperature (Q_{10}= 1.76) to a maximum at 20°C ($\dot{V}_{O_2}$ = 176 ml/kg·hr), and then decreased. Scope for activity likewise showed a maximum at 20°C ($\dot{V}_{O_2}$ = 158 ml/kg·hr) and decreased at higher temperatures. Comparison with fish studies revealed that active $\dot{V}_{O_2}$ for crayfish is about two-thirds of that for moderately active fish species such as bass, bullhead, and goldfish between 5° - 20°C; and about twice that for the sluggish gobiid fish, indicating a well developed oxygen uptake ability in this decapod crustacean. And because of the low standard $\dot{V}_{O_2}$ for crayfish, scope for activity of crayfish was almost identical to that of bass and bullhead below 20°C.

To investigate intrinsic factors limiting the scope, crayfish

were acclimated to 10°, 20°, and 25°C. Hemocyanin from animals at these three acclimation temperatures showed distinctly different oxygen binding patterns. At any particular set of test temperature and pH, 10°C acclimated hemocyanin had the lowest oxygen affinity and the greatest cooperativity, while 25°C acclimated hemocyanin had the highest oxygen affinity and the lowest cooperativity. When tested at their own acclimation temperature and at normal pH, however, all three hemocyanins showed P_{50} of 6 - 8 torr. Thus acclimation keeps oxygen affinity centered around a narrow range of values. It was concluded that the acclimation response probably eliminates hemocyanin oxygen affinity as a major factor in the decline of oxygen uptake ability in the crayfish above 20°C.

Heart rate, ventilation rate, $\dot{V}_{O_2}$, hemocyanin concentration, and arterial and venous P_{O_2} and pH were measured in unrestrained crayfish during low-level, routine activity; and during forced (maximal) activity. Hemocyanin percent saturation, oxygen content of arterial and venous blood, cardiac output and stroke volume were calculated from the measured parameters. A procedure for using N-ethylmaleimide as an anticoagulant during blood sampling is described. Experiments were again performed at 10°, 20°, and 25°C. Results showed low arterial P_{O_2} (9 - 12 torr) during routine activity; and this dropped some during maximum activity. Thus hemocyanin is not normally saturated in these animals. Cardiac output is high (eg. at 20°C, 236 ml/kg·min during routine activity and 969 ml/kg·min during forced activity). The implications are discussed, including general circulation strategy in crustaceans. Evidence is presented that

ventilation is the major limiting factor of the scope for activity, both below and above the maximum point at 20°C.

Scope for Activity in the Crayfish *Pacifastacus leniusculus*: Role of Circulatory and Respiratory Parameters in Limiting It

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SCOPE FOR ACTIVITY

IN THE CRAYFISH PACIFASTACUS LENIUSCULUS

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ABSTRACT

The scope for activity of the crayfish Pacifastacus leniusculus was determined between 5° - 30°C (incipient lethal of 33°C). Standard oxygen consumption rate ($\dot{V}O_2$) was low (eg. 17 ml/kg·hr at 20°C), and it increased with temperature over the entire range ($Q_{10} = 2.09$ for 5° - 25°C). Active $\dot{V}O_2$ increased with temperature ($Q_{10} = 1.76$) to a maximum at 20°C ($\dot{V}O_2 = 176$ ml/kg·hr), and then decreased. Scope for activity likewise showed a maximum at 20°C ($\dot{V}O_2 = 158$ ml/kg·hr) and decreased at higher temperatures. Comparison with fish studies revealed that active $\dot{V}O_2$ for crayfish is about two-thirds that for moderately active fish species such as bass, bullhead, and goldfish between 5° - 20°C; and about twice that for the sluggish gobiid fish, indicating a well developed oxygen uptake ability in this decapod crustacean. And because of the low standard $\dot{V}O_2$ for crayfish, scope for activity of crayfish was almost identical to that of bass and bullhead below 20°C.

Index Terms: Standard oxygen consumption, active oxygen consumption, routine activity, Q_{10}, temperature acclimation, oxygen debt.

INTRODUCTION

One of the most promising methods for assessing the influence of environmental factors on the survival of a species is the "scope for activity" method first developed by Fry (10). The scope for activity is the difference in the oxygen uptake rate ($\dot{V}_{O_2}$) between the standard level and the maximum active level. Thus it represents the capacity of the animal to aerobically carry on activities such as locomotion, digestion and growth, which are above the normal short-term maintenance requirements. The concept is generally applied to poikilotherms, being analogous to that of "aerobic capacity" for homeotherms (5). Because of the importance of temperature to a poikilotherm, the scope for activity should be measured for a series of temperatures within the range of temperature tolerance for the species.

To date most studies on scope for activity of aquatic animals have been done with fish (3 - 12). Within this group virtually all studies have been performed with fish which are normally moderately to highly active; that is, they spend most of their time swimming. Brett (5) has pointed out that many species of fish do not normally exhibit this high degree of activity. Also, many other aquatic animals, including most decapod crustaceans, are at best only intermittently active.

Among aquatic invertebrates, only two groups have been studied, the crustaceans and the mollusks. Crustaceans are represented by a single study on the shrimp Palaemonetes vulgaris (15). The mollusks studied include the bivalve Mytilus edulis (21) and the gastropod

Littorina littorina (16). Among invertebrates, criteria for standard and active metabolism have been defined in various ways, sometimes in seemingly arbitrary fashion. For example, in some studies routine activity associated with feeding has been taken as the maximum possible for relatively mobile animals like decapod crustaceans (1, 20).

The objective of this study was to determine the scope for activity over the temperature range compatible with life for the crayfish Pacifastacus leniusculus. This animal was chosen because it is an intermittently active poikilotherm, and a representative member of a large, important subfamily of crustaceans, the decapods. Thus it should provide an instructive comparison with the more continuously active vertebrates (fish), with their closed circulatory system, more efficient gill arrangment, and usually greater blood oxygen carrying capacity.

Animals. Crayfish, Pacifastacus leniusculus Dana, were collected by trapping from Cronemiller Pond, Peavy Arboretum, Benton County, Oregon. Only adult, intermolt (stage C) crayfish weighing between 16 and 33 grams were used. Crayfish were acclimated under controlled conditions of constant temperature and photoperiod for a minimum of 30 days prior to being used in an experiment. Photoperiod was set at 14 hours light and 10 hours dark. Water temperature in the acclimation rooms fluctuated within $\pm$ 0.2°C of the temperature set. Crayfish were kept in divided plastic trays with one animal per section, and all trays were supplied with well aerated running dechlorinated tap water. The acclimating crayfish were fed frogs and beef heart every 5 days, and maple and alder leaves ad lib. All food in an individual's section was removed 2 days prior to testing.

Measurement of standard oxygen consumption. Oxygen consumption ($\dot{V}_{O_2}$) of individual crayfish was measured hourly over a 24 hour period at each acclimation temperature. This series of experiments was performed primarily to obtain standard $\dot{V}_{O_2}$ values, the lowest $\dot{V}_{O_2}$ of the 24 recorded for each crayfish being taken as its standard rate. In practice, there were often 2 - 4 virtually identical low rates of this sort for each crayfish during the 24 hour period.

Measurement of oxygen consumption was done using a flow-through system. Crayfish were contained individually in cylindrical plastic tubes of 250 ml volume each, with inflow tubing at one end and outflow at the other. Usually four crayfish and a blank were run simultaneously. To prevent visual disturbance of the animals by the

experimenter, tubes were covered except for small flaps on the side away from the experimenter, which allowed entrance of some light in conjunction with normal photoperiod. At the start of an experiment, animals were placed in the tubes 3 hours before the first measurements were made to allow the animals to become accustomed to the test situation. Experiments were begun in the afternoon, although not always at the same hour. A water sample of 300 ml was taken every hour from each tube and oxygen content determined with a Yellow Springs Instrument (YSI) Model 54 oxygen meter and self-stirring probe. $\dot{V}_{O_2}$ was calculated from the difference in oxygen content of the water before and after it had passed by the crayfish at a controlled flow rate of 1 liter/hour. Units for $\dot{V}_{O_2}$ are ml O_2/kg wet weight • hour. After 24 hourly measurements, crayfish were removed from their tubes, blotted with paper towels for one minute to remove excess water, and weighed to the nearest 0.01 gm. The tubes were then reassembled for an additional blank run of one hour.

Measurement of active oxygen consumption. In this series of experiments crayfish underwent a period of forced activity. Combined behavioral observations and $\dot{V}_{O_2}$ values taken before, during, and following the forced activity period indicated that $\dot{V}_{O_2}$ values during at least some parts of the forced activity were maximal or very nearly maximal. For each crayfish the highest $\dot{V}_{O_2}$ of those observed over any of the three 5 minute intervals of forced activity was used as the maximum or "active" $\dot{V}_{O_2}$.

Oxygen uptake was measured using a "closed jar" system. An

animal was placed in the circular chamber, which was then sealed. The YSI oxygen probe was inserted and the self-stirrer turned on. A magnetic stirring bar in the bottom of the chamber was also used to ensure good mixing of the water in the chamber (volume approximately 1 liter). Oxygenated water was allowed to flow through the chamber at a rapid rate (about 20 l/hr) for 15 minutes to allow some time for the crayfish to recover from excitement induced by handling. However, in most cases this time period was insufficient for the animal to approach the standard rate. After water flow was shut off, an initial oxygen content reading was taken. Readings were repeated every 5 minutes for 15 minutes total. Then a motorized paddle was turned on to induce activity in the animal. Rotational velocity of the paddle was adjusted so as to keep the crayfish "engaged" continually, but not so fast as to simply sweep the animal around the chamber. Since the velocity adjustments were not continuously variable but rather a series of gear reductions, and since the animals "fought" with the paddle more than they ran or swam, rotational velocity could not be used to quantify activity. Care was taken that the crayfish did not become caught by the paddle for any significant length of time. Oxygen content readings were again made every 5 minutes for a 15 minute total. The paddle motor was then turned off and the oxygen content readings continued for an additional 15 minute recovery period. Throughout the total 45 minute test period the crayfish was observed continuously, and behavioral notes made. At the end of a run the crayfish was removed from the chamber, blotted with a paper towel for one minute, and

weighed to the nearest 0.01 gm.

A blank run of 45 minutes duration was performed at the end of each day of experiments. Changes in oxygen content during this time period were negligible. Response time of the YSI oxygen electrode showed insignificant lag throughout the temperature range under study.

Statistical comparisons were made using Student's *t*-test. Differences were taken to be not significant when $P > 0.05$. Regression lines were fitted using the method of least squares.

24 hour experiments. Mean $\dot{V}_{O_2}$ values at intervals over a 24 hour period are shown for six different temperatures in Figure 1. In general the $\dot{V}_{O_2}$ values are routine rates in the terminology of Fry (10). The rates during the light hours are lower than those observed during the dark ($P < 0.001$); thus the light hours rates are closer to being standard rates. It should be noted that in no case did the rate for an individual crayfish ever approach the mean active rate for that temperature (Table 1). As shown in Figure 1, there is a general increase in level of oxygen consumption as temperature is increased from 5° - 20°C. The overall level of oxygen consumption at 25°C is similar to that at 20°C. At 30°C the $\dot{V}_{O_2}$ values are always higher than those for any other temperature, even 20°C at night.

Active oxygen consumption. $\dot{V}_{O_2}$ values over the three part, 45 minute testing period used in determining active $\dot{V}_{O_2}$ are shown at six temperatures in Figure 2. Values are plotted at 5 minute intervals starting at 2.5 minutes, since the 5 minute readings are an average for the preceding interval.

During the initial 15 minute period most crayfish showed at least a small amount of activity, except at 5°C where most animals were quiescent. There is an increase in $\dot{V}_{O_2}$ between 5° - 20°C followed by a decrease at 25° and 30°C. The $\dot{V}_{O_2}$ values obtained in this initial period are essentially routine rates associated with spontaneous activity.

During the first 5 - 10 minutes of forced activity there is a marked rise in $\dot{V}_{O_2}$, followed by a leveling off. With the onset of

forced activity there is an increase in $\dot{V}_{O_2}$ which is significant ($P < 0.01$) for all temperatures tested. Between the first 5 minute reading and the second 5 minute reading there is another increase, which is significant ($P < 0.05$) at the lower temperatures, but not at 25° and 30°C. There is no significant difference between the second and third 5 minute forced activity $\dot{V}_{O_2}$ values at any temperature.

During the recovery period $\dot{V}_{O_2}$ declined rapidly from the high values observed during the forced activity period (Figure 2). This rapid decrease was no doubt primarily the result of cessation of activity (Table 2). Yet during the first 10 minutes after forced activity the $\dot{V}_{O_2}$ values are still significantly higher ($P < 0.02$) at each temperature than the routine $\dot{V}_{O_2}$ values obtained in the initial measurement period. The $\dot{V}_{O_2}$ differences cannot be due to an increase in activity during the recovery period since there is in fact an overall marked decrease in that period as shown in Table 2. Even after 15 minutes of recovery, the data in Table 2 indicate activity is still below that in the initial 15 minute measurement period. Thus it seems likely that the increased $\dot{V}_{O_2}$ during the recovery period, which occurs despite the decrease in visible activity, is due to the repayment of an oxygen debt incurred during the forced activity period.

Scope for activity. Active and standard $\dot{V}_{O_2}$ values at each temperature tested are given in Table 3 and Figure 3. The scope for activity, which is the difference between the active and standard $\dot{V}_{O_2}$, is indicated by a dashed line in Figure 3. The mean standard rate

increases logarithmically with temperature as expected. After a log transformation of the $\dot{V}_{O_2}$ values, excluding those at 30°C, a regression line was obtained ($R^2 = 0.769$). Its slope, which represents the Q_{10}, is 2.09. At 30°C the standard $\dot{V}_{O_2}$ differs significantly ($P < 0.05$) from that predicted by the regression line obtained for the other five temperatures. This gives rise to a large Q_{10}, 3.40, for the interval from 25° - 30°C.

Mean active $\dot{V}_{O_2}$ increases with temperature from 5° - 20°C. Regression of log $\dot{V}_{O_2}$ on temperature between 5° and 20°C gave Q_{10} = 1.76, with $R^2 = 0.797$. Between 20° and 25°C there is a significant decrease ($P < 0.05$) in $\dot{V}_{O_2}$; and a further decrease from 25° - 30°C which is not statistically significant. The Q_{10} for the active $\dot{V}_{O_2}$ is significantly lower ($P < 0.05$) than the Q_{10} for the standard $\dot{V}_{O_2}$, suggesting that over the temperature range of 5° - 20°C the factor limiting the active $\dot{V}_{O_2}$ is not the same one which sets the standard rate.

The scope for activity (dashed line in Figure 3) increases from 5° - 20°C, but then decreases between 20° - 30°C. The decrease in scope above 20°C is even more dramatic than the one exhibited by the active $\dot{V}_{O_2}$ because the standard $\dot{V}_{O_2}$ continues to increase between 20° - 25°C, and even faster between 25° - 30°C.

DISCUSSION

The scope for activity represents the amount of energy available to an organism through aerobic metabolism beyond that needed for maintenance; for most metazoans this is a good indication of their capacity for sustained work (10). Only brief, occasional anaerobic bursts can exceed this significantly (4, 5). Because of the paramount importance of temperature to poikilothermic animals, the scope for activity is most meaningfully expressed when determined over the full range of temperatures compatable with survival for the particular species. Also, the animals should be acclimated to each temperature used; acutely determined scopes will result in different patterns dependent on the thermal history of the animals prior to testing (15, 21). The active oxygen uptake should be maintained over a significant period, generally one hour (5, 10). In the present study the scope for activity has been determined over the range of 5° - 30°C (incipient upper lethal is about 33°C), with all animals being acclimated to the temperature of experimentation. However, the crayfish were forced to be active for only 15 minutes instead of 60 minutes, mainly because of their inability to swim readily. The values obtained may be reasonably compared to those for fish because of two offsetting factors. The shorter forced activity time might result in slightly high active $\dot{V}_{O_2}$ values (4). On the other hand, as a result of the methodology employed, the water in the respirometer was not fully saturated with oxygen, being usually around 85% saturated during the forced activity period. This would tend to lower maximum $\dot{V}_{O_2}$, as shown for lobsters by Spoek (19).

The pattern of scope for activity observed for Pacifastacus leniusculus is one of increasing scope with increasing temperature up to a maximum at 20°C, followed by a decrease until the upper lethal is reached. This is the most common pattern observed to date, at least for fish (5). The scope pattern is intrinsically dependent mainly on the active $\dot{V}_{O_2}$ profile. In animals that show a maximum scope below the lethal temperature, the influence of the standard $\dot{V}_{O_2}$ is most obvious above the maximum scope temperature (see Figure 3), contributing to a rapidly decreasing scope since the active $\dot{V}_{O_2}$ is decreasing while the standard $\dot{V}_{O_2}$ is still increasing.

The active $\dot{V}_{O_2}$ values for Pacifastacus leniusculus are lower than those found for most of the well studied species of fish (salmonids, bullhead, bass, and goldfish); but they are still about two-thirds the active rates of goldfish, bullhead, and bass up to 20°C. Since these fish are moderately to highly active fish, they probably represent the upper range for fish in general (5). Thus these crayfish appear remarkably competitive with even fairly active fish in oxygen uptake ability, contrary to some previously held views concerning crustaceans (1). Because standard $\dot{V}_{O_2}$ for crayfish is quite low, the scope for activity itself is even closer to the scopes for fish than when comparing active $\dot{V}_{O_2}$ values. In fact, between 5° - 20°C, the scopes are virtually identical in bass, bullhead, and crayfish. Furthermore, when compared with the rather sluggish gobiid fish Pomatoschistus studied by Fonds and Veldhuis (9), the crayfish shows active $\dot{V}_{O_2}$ values approximately twice those of the most active

of the four gobiid species tested.

The standard $\dot{V}_{O_2}$ is dependent on the activities of certain key allosteric enzymes in metabolic pathways within the cells of the animals, hormone levels, and ultimately upon the general energy demand by the tissues for maintenance (17). Factors which influence the energy demand or the operation of the metabolic pathways will change the standard rate. The active rate is probably under somewhat different control. This can be inferred from the Q_{10} differences between active and standard $\dot{V}_{O_2}$ for the crayfish. Fish also usually show different Q_{10} values for standard and active rates; although in a few cases, as for bass (3), the active rate has the higher Q_{10}. What limits the active $\dot{V}_{O_2}$? There are two general areas to consider. One is oxygen demand by the tissues; the other is the oxygen delivery system. Limitations within the somatic muscle fibers as to how much work can be performed could set the active $\dot{V}_{O_2}$ by limiting demand. However, many animals are capable of short-term (less than one minute), largely anaerobic bursts of work performance significantly higher than sustained aerobic maximum levels (4). Artificially increasing blood flow or arterial oxygen tension can also increase active $\dot{V}_{O_2}$ (13). It is more likely, therefore, that the active $\dot{V}_{O_2}$ is limited by one or more factors in the oxygen delivery system. This topic is dealt with in more detail in a subsequent paper (18).

In discussions of scope for activity it is generally assumed that the absolute magnitude of the scope is the critical characteristic. Whether one is assessing the influence of environmental factors on the

long-term survival of an animal, or determining an optimum temperature in a set of conditions for culturing of a species, it is important to know if this assumption is valid. In the present study there is some conflicting evidence. At 30°C, despite good care, there was about 40% mortality during the acclimation period of one month. This contrasts to less than 5% mortality from 5° - 20°C, and about 10% at 25°C. This would indicate that at temperatures higher than the maximum scope, crayfish are not in good condition to resist stress. Yet the actual scope at 30°C is greater than that at either 5° or 10°C. Thus, although the absolute magnitude of the scope is similar at high and lower temperatures in the physiological range, the "value" of this scope to the animal may be different. Pathogens will alter the ability of the crayfish to resist them through changes in their own growth rates, which are greater at higher temperatures. This, coupled with reductions in efficiency of circulatory and respiratory pumps at higher temperatures (see 18), means that in this region above the maximum scope, relatively minor decreases in the active $\dot{V}_{O_2}$ could be critical to the animal.

Brett (5) has pointed out that salmon do not spontaneously show $\dot{V}_{O_2}$ values greater than one-half the forced active rate, even when feeding. He maintains that animals in general do not need such a large "buffer zone"; and using data from Idler and Clemens (14), he calculates that in the case of salmon this capacity has evolved to meet needs during upstream migration, where about 70 - 75% of the active rate is sustained for long periods of time. The crayfish in the

present study likewise show routine $\dot{V}_{O_2}$ values considerably below the forced activity rates. Only about one-third of the active rates is shown, except at 30°C where the value goes up to about one-half. This would again raise the possibility that these animals do need a large buffer zone between a "normal" maximum and their true active rate. This could be due to the energetics of pumping water or blood. Since energy cost of pumping increases logarithmically with volume pumped (2, 21), it may be advantageous for an animal to be required to approach its true maximum only very occasionally, especially for any length of time. In crustaceans, the periods when their respiratory pigment concentration is very reduced, as in starvation or after the molt, may require well developed oxygen uptake ability. Finally there is the matter of short-term bursts of activity. With large active $\dot{V}_{O_2}$ capability, an animal can minimize the accumulation of oxygen debts; and speed repayment of debts when they do occur.

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LITERATURE CITED

1. Ansell, A.D. Changes in oxygen consumption, heart rate and ventilation accompanying starvation in the decapod crustacean Cancer pagurus. Neth. J. Sea Res. 7: 455-475, 1973.
2. Ballintijn, C.M. Efficiency, mechanics and motor control of fish respiration. Resp. Physiol. 14: 125-141, 1972.
3. Beamish, F.W.H. Oxygen consumption of largemouth bass, Micropterus salmoides, in relation to swimming speed and temperature. Can. J. Zool. 48: 1221-1228, 1970.
4. Brett, J.R. The respiratory metabolism and swimming performance of young sockeye salmon. J. Fish. Res. Bd. Can. 21: 1183-1226, 1964.
5. Brett, J.R. The metabolic demand for oxygen in fish, particularly salmonids, and a comparison with other vertebrates. Resp. Physiol. 14: 151-170, 1972.
6. Brett, J.R., M. Hollands and D.F. Alderdice. The effect of temperature on the cruising speed of young sockeye and coho salmon. J. Fish. Res. Bd. Can. 15: 587-605, 1958.
7. Dickson, I.W. and R.H. Kramer. Factors influencing scope for activity and standard metabolism of rainbow trout (Salmo gairdneri). J. Fish. Res. Bd. Can. 28: 587- 596, 1971.
8. Dwyer, W.P. and R.H. Kramer. The influence of temperature on scope for activity in cutthroat trout, Salmo clarki. Trans. Am. Fish. Soc. 104: 552-554, 1975.

9. Fonds, M. and C. Veldhuis. The oxygen consumption of four pomatoschistus species (Pisces, Gobiidae) in relation to water temperature. Neth. J. Sea Res. 7: 376-386, 1973.

10. Fry, F.E.J. Effects of the environment on animal activity. Univ. Toronto Stud. Biol. Ser. 55, Pub. Ont. Fish. Res. Lab. 68: 1-62, 1947.

11. Fry, F.E.J. and J.S. Hart. Cruising speed of goldfish in relation to water temperature. J. Fish. Res. Bd. Can. 7: 169-175, 1948.

12. Gibson, E.S. and F.E.J. Fry. The performance of the lake trout, Salvelinus namaycush, at various levels of temperature and oxygen pressure. Can. J. Zool. 32: 252-260, 1954.

13. Horstman, D.H., M. Gleser, and J. Delehunt. Effects of altering O_2 delivery on $\dot{V}O_2$ of isolated, working muscle. Am. J. Physiol. 230: 327-334, 1976.

14. Idler, D.R. and W.A. Clemens. The energy expenditures of Frazer River sockeye salmon during the spawning migration to Chilko and Stuart Lakes. Int. Pacific Salmon Fish. Com., Prog. Rept. 80 pp. in Brett, J.R. Resp. Physiol. 14: 151-170, 1972.

15. McFarland, W.N. and P.E. Pickens. The effect of season, temperature, and salinity on standard and active oxygen consumption of the grass shrimp, Palaemonetes vulgaris (Say). Can. J. Zool. 43: 571-585, 1965.

16. Newell, R.C. and V.I. Pye. Quantitative aspects of the relationship between metabolism and temperature in the winkle Littorina littorina (L). Comp. Biochem. Physiol. 38B: 635-650, 1971.

17. Prosser, C.L. Comparative Animal Physiology. Philadelphia: W.B. Saunders, 1973. 966 pp.

18. Rutledge, P.S. Circulation and oxygen transport during activity in the crayfish. In review to Am. J. Physiol.: Regulatory Integrative Comp. Physiol., 1978.

19. Spoek, G.L. The relationship between blood haemocyanin level, oxygen uptake, and the heart-beat and scaphognathite-beat frequencies in the lobster Homarus gammarus. Neth. J. Sea Res. 8: 1-26, 1974.

20. Wallace, J.C. Activity and metabolic rate in the shore crab, Carcinus maenas (L). Comp. Biochem. Physiol. 41A: 523-533, 1972.

21. Widdows, J. The effects of temperature on the metabolism and activity of Mytilus edulis. Neth. J. Sea Res. 7: 387-398, 1973.

Table 1. Maximum spontaneous $\dot{V}_{O_2}$ shown over a 24 hour period, and a comparison with active $\dot{V}_{O_2}$.

Temperature (°C)	$\dot{V}_{O_2}$ (ml/kg·hr)	Range (ml/kg·hr)	% of Active $\dot{V}_{O_2}$
5°	28.7 ± 5.4 (8)	12.5 - 56.5	38.1%
10°	32.9 ± 7.2 (11)	13.6 - 69.0	33.7%
15°	40.2 ± 5.0 (12)	26.0 - 76.9	33.0%
20°	63.1 ± 5.5 (11)	37.6 - 91.3	36.0%
25°	57.6 ± 5.2 (12)	37.2 - 99.0	36.6%
30°	77.2 ± 6.2 (8)	57.3 - 109.6	52.5%

$\dot{V}_{O_2}$ values in second column are means of individual spontaneous maximums observed for each crayfish ± SE, with number of individuals in parentheses. Range of third column shows high and low individual maximum $\dot{V}_{O_2}$ values at each temperature. "Active" of fourth column refers to maximum forced active $\dot{V}_{O_2}$ (see Table 3).

Table 2. Comparison of spontaneous activity before and after 15 minutes forced maximal activity at different temperatures.

Condition	Elapsed Time (min)	Temperature (°C) 5°	10°	15°	20°	25°	30°
Before	5	53%	100%	100%	94%	92%	100%
Forced	10	60%	92%	100%	100%	100%	100%
Activity	15	67%	92%	100%	100%	100%	78%
	5	7%	31%	21%	25%	0%	0%
Recovery	10	27%	31%	57%	69%	31%	11%
	15	40%	62%	71%	81%	38%	0%

Values shown represent the number of crayfish showing some movement of entire body expressed as percent of the total number tested at that temperature. Number at each temperature can be found in Table 3 under "Active".

Table 3. Standard and active $\dot{V}_{O_2}$ as a function of temperature.

Condition	Temperature (°C)	$\dot{V}_{O_2}$ (ml/kg·hr)
Standard	5°	5.6 ± 0.6 (8)
	10°	8.4 ± 0.8 (11)
	15°	10.0 ± 0.9 (12)
	20°	17.4 ± 0.9 (11)
	25°	23.4 ± 1.4 (12)
	30°	43.2 ± 2.1 (8)
Active	5°	75.3 ± 3.6 (15)
	10°	97.7 ± 4.5 (13)
	15°	124.9 ± 5.9 (14)
	20°	175.5 ± 5.5 (16)
	25°	157.5 ± 7.1 (13)
	30°	147.1 ± 7.4 (9)

$\dot{V}_{O_2}$ values are means ± SE, with number of individuals in parentheses.

Figure 1. $\dot{V}_{O_2}$ of crayfish at different temperatures over a 24 hour period. Values shown are means, and represent routine rates. Animals were acclimated to their test temperature for one month prior to measurement. The temperatures are 5° (□), 10° (○), 15° (▲), 20° (●), 25° (△), and 30°C (■).

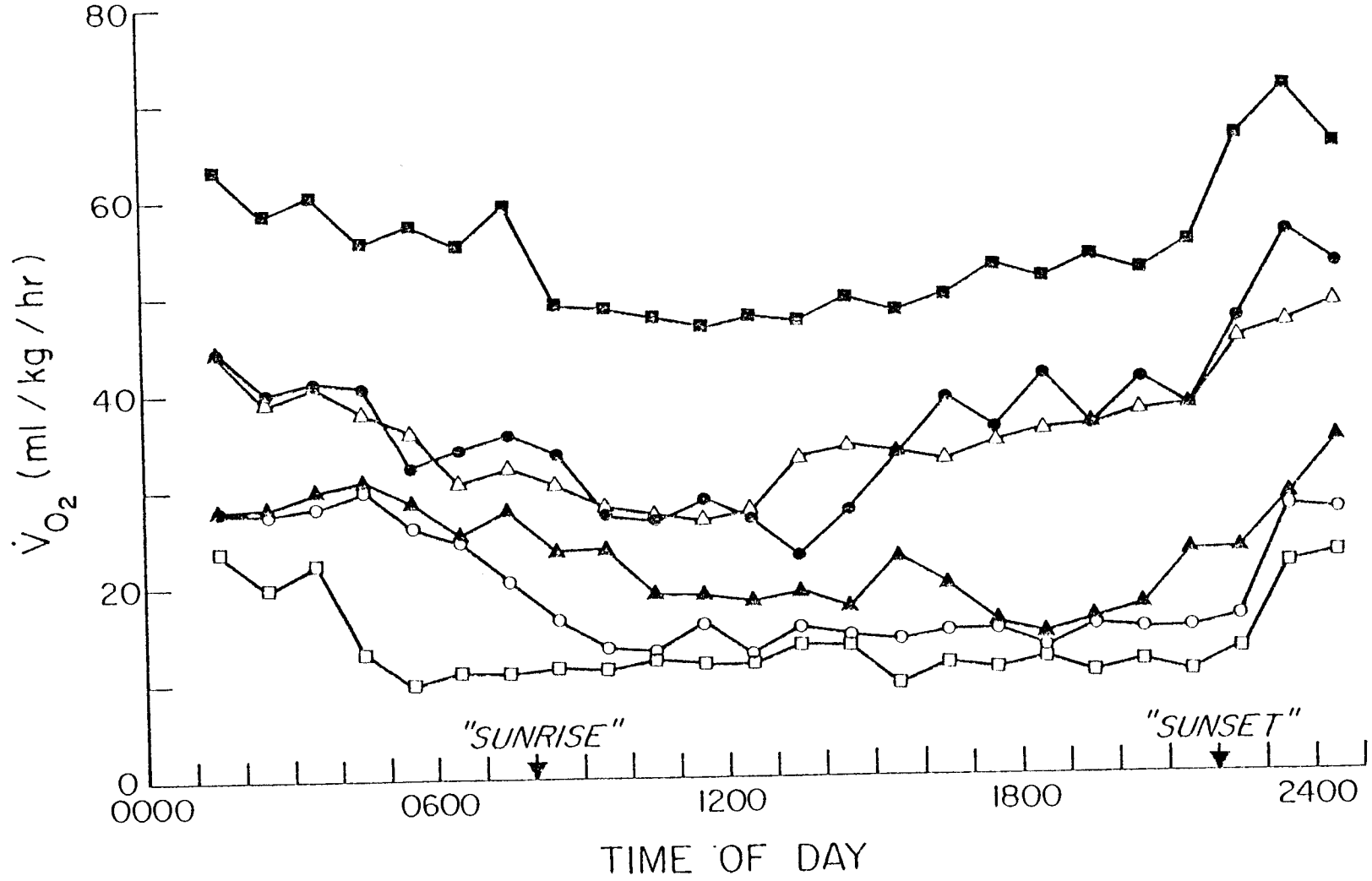

80
60
40
20
0
$\dot{V}_{O_2}$ (ml / kg / hr)
"SUNRISE"
"SUNSET"
0000
0600
1200
1800
2400
TIME OF DAY

Figure 2. $\dot{V}_{O_2}$ measurements over the 45 minute activity procedure period. After an initial 15 minute period in the test chamber, crayfish were forced to be as active as possible through use of a motorized paddle for 15 minutes; and their recovery was followed for an additional 15 minutes after the forced activity. Animals were acclimated to their test temperature as in Figure 1. The symbols used are the same in both figures: 5° (□), 10° (○), 15° (▲), 20° (●), 25° (△), and 30°C (■).

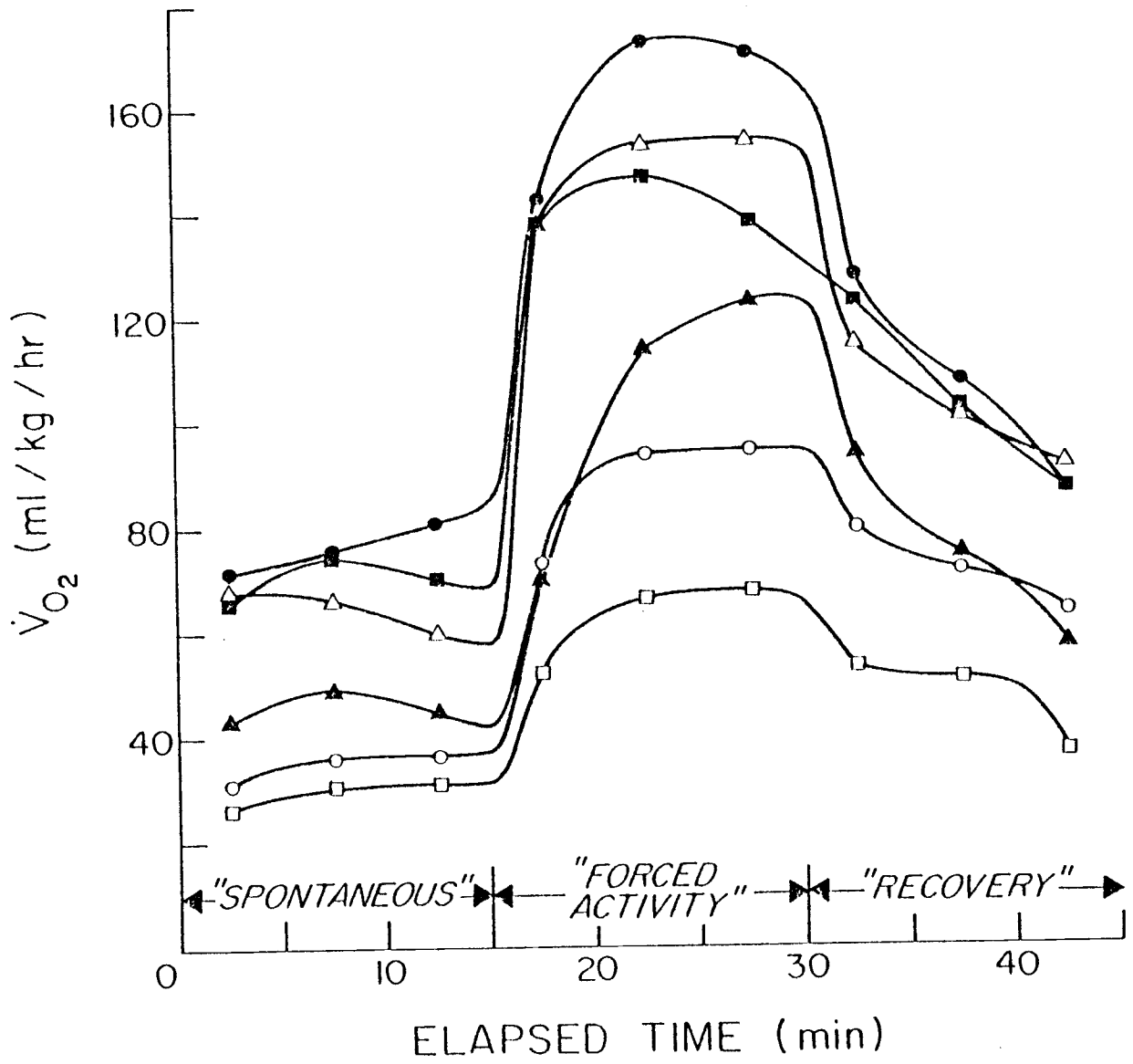
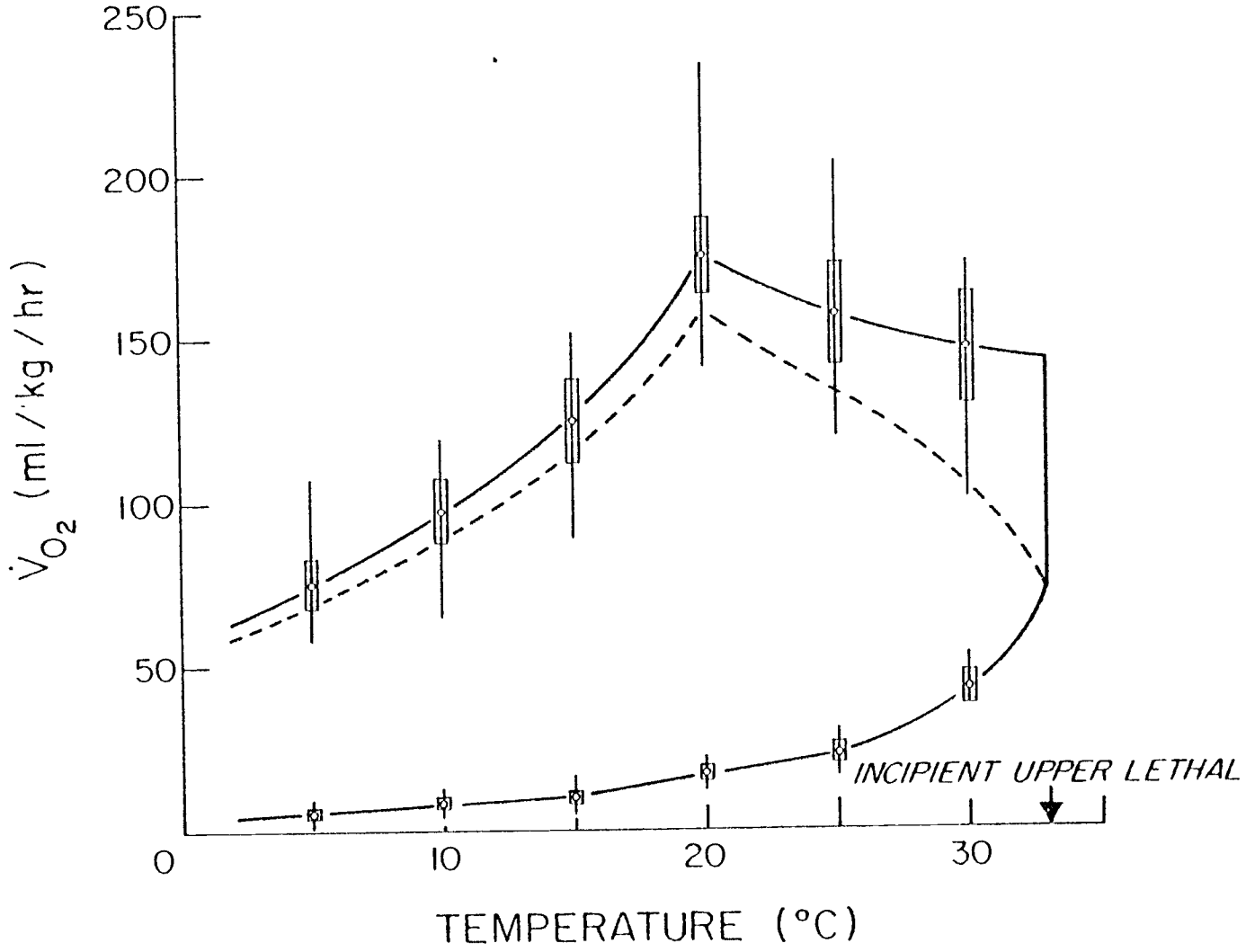

160
120
80
40
0
10
20
30
40
$\dot{V}_{O_2}$ (ml / kg / hr)
"SPONTANEOUS"
"FORCED ACTIVITY"
"RECOVERY"
ELAPSED TIME (min)

Figure 3. Active $\dot{V}_{O_2}$, standard $\dot{V}_{O_2}$ and scope for activity of the crayfish Pacifastacus leniusculus over most of its viable temperature range. Upper solid curve is active rate, lower solid curve is standard rate, and dashed line is scope for activity. Vertical bars represent 95% confidence limits; vertical lines are the ranges. All animals were acclimated to test temperatures for one month.


250
200
150
100
50
0
10
20
30
$\dot{V}_{O_2}$ (ml / kg / hr)
INCIPIENT UPPER LETHAL
TEMPERATURE (°C)

TEMPERATURE ACCLIMATION RESPONSE OF CRAYFISH HEMOCYANIN

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Running head: Temperature Acclimation of Hemocyanin

Crayfish, Pacifastacus leniusculus, were acclimated to 10°, 20°, and 25°C for one month. Hemocyanin from animals at these three acclimation temperatures showed distinctly different oxygen binding patterns. At any particular set of test temperature and pH, 10°C acclimated hemocyanin had the lowest oxygen affinity and the greatest cooperativity, while 25°C acclimated hemocyanin had the highest oxygen affinity and the lowest cooperativity. When tested at their own acclimation temperature, and at normal pH for that temperature, all three hemocyanins showed P_{50} of 6 - 8 torr. Thus acclimation keeps oxygen affinity centered around a narrow range of values. It was concluded that the acclimation response probably eliminates hemocyanin oxygen affinity as a major factor in the decline of oxygen uptake ability in the crayfish above 20°C. The structural basis for the observed functional changes in the hemocyanin is not clear. Crayfish hemocyanin exists in two aggregation states (17 S and 25 S), and has three subunit types; but neither of these structural levels appeared to change composition or proportion with acclimation. Column chromatography purification also produced no change in oxygen binding.

Index Terms: Oxygen affinity, pH, Bohr effect, relative alkalinity theory, cooperativity, R and T states, heat of reaction, subunit heterogeneity.

In the crayfish Pacifastacus leniusculus scope for activity (the difference between active and standard oxygen consumption) increases with temperature to a maximum at 20°C, then decreases gradually until the upper lethal temperature is reached (39). This seems to be the general pattern for those few groups of aquatic poikilotherms studied (4, 9, 26). Jones (17) has hypothesized that in fish the cardiac pump is limiting the maximum uptake at the lower temperatures (below maximum scope), while the respiratory pump is limiting at the higher temperatures. These pumps correspond to the heart and scaphognathites, respectively, in the crayfish.

The presence of a respiratory pigment, such as the hemocyanin found in crayfish, can greatly alter the rate of gas exchange at both the respiratory surface and the tissues, as well as increase the amount of oxygen carried in the blood (34). Thus the properties of the pigment can modify the effectiveness of the respiratory and circulatory pumps. Although hemocyanin may not be necessary for short-term survival in crustaceans (40, 50), it does influence the amount of aerobic activity by the animal (40). A similar situation is true for some fish (1, 12).

Crayfish experience large fluctuations (0° - 25°C or more) in temperature during the course of a year, although the temperature changes for Pacifastacus leniusculus are generally not rapidly fluctuating as they are for marine intertidal crustaceans. Hemocyanins, like hemoglobins, show in vitro decreases in oxygen affinity as temperature is increased (16, 19, 33, 34, 49). Considering

this affinity change together with the problems of aquatic respiration as temperature is increased, including lower oxygen solubility in water (31), one might expect some compensatory change in properties of the respiratory pigment to take place with acclimation. Among crustaceans only Truchot (43, 44), working with the intertidal crab Carcinus maenas, has attempted to demonstrate temperature acclimation differences and their basis. Functional differences in hemocyanins from this crab were observed only at higher temperatures of acclimation ($\geq 25^{\circ}C$); they were apparently caused by small modulators because the differences could be abolished by dialysis.

The present study was undertaken to determine the influence of temperature acclimation on hemocyanin oxygen binding properties in the crayfish Pacifastacus leniusculus; and to attempt to determine the mechanism of the change. The temperatures of 10°, 20°, and $25^{\circ}C$ were chosen since $20^{\circ}C$ is the approximate temperature of maximum scope for activity in the crayfish, while 10° and $25^{\circ}C$ represent points below and above the maximum, respectively (39).

Preparation of hemocyanin. The northwest crayfish, Pacifastacus leniusculus Dana, was trapped in Cronemiller Pond, Benton County, Oregon, returned to the lab and acclimated to a particular temperature (10°, 20°, or $25^{\circ}C$) for one month as described previously (39). Hemolymph sampling was done via syringe using a 20 gauge needle inserted into the pericardial sinus. The hemolymph (about 1 - 2 ml) was withdrawn rapidly and expelled into a beaker where it was allowed to clot. For most experiments, hemolymph from animals acclimated to a common temperature was pooled, although in some cases individual samples were kept separate as noted later. After clotting was complete, the clot was mechanically broken up, forced through nylon gauze, and the sample centrifuged at 10,000 rpm (12,100 X G) for 15 minutes at $2^{\circ}C$ in a Sorvall RC2-B Superspeed centrifuge. The supernatant was then filtered using mild suction through a 0.45 u pore size membrane filter. This solution was designated "filtered hemolymph". It was stored at $3^{\circ}C$ under toluene.

Solutions. Because divalent cations, especially in concentrations below the levels normally found in crayfish, have been shown to alter hemocyanin oxygen binding properties (19), all solutions used in this study contained 15 mM $CaCl_2$ and 4 mM $MgCl_2$. These values are approximately those normally found in crayfish hemolymph (18).

TRIS·HCl buffer (0.1 ionic strength), prepared according to Long (20), was used throughout the experiments. The dissociation of TRIS is very temperature sensitive, so different buffer series were prepared for each experimental temperture, and used only at their

particular temperature. The pH of each was verified at operational temperature using a Radiometer Blood Gas Analyzer PHM 71 Mk 2 with thermostatted pH microelectrode (E5021).

Absorption properties of hemocyanin. For initial determination of some of the physical properties of the crayfish hemocyanin, it was pelleted from samples of filtered hemolymph by centrifugation at 64,000 rpm for 3 hours (2°C) in a Beckman L2-65 ultracentrifuge using a no. 65 rotor. The supernatant contained only very small amounts of protein, including carotenoid pigments. The pellets were resuspended in TRIS buffer and shown by subsequent sucrose density gradient centrifugation (7) to be essentially pure hemocyanin. From these and other measurements involving absorption spectra and analytical ultracentrifugation it appears that for fed, intermolt (stage C) crayfish the hemocyanin accounts for at least 95% of the total protein dissolved in the hemolymph. This predominance of hemocyanin agrees with other studies on crustacean hemocyanins (37).

Spectrophotometric measurements of hemocyanin were made using matched quartz cuvets in either a Beckman DU-2 or Gilford - modified DU spectrophotometer. The absorption spectrum was measured between 240 and 620 nm for both filtered hemolymph and the resuspended hemocyanin pellet. The resulting spectra were similar to those for other crustacean hemocyanins (24), with a protein peak at 280 nm and oxy-hemocyanin peaks at 337 and 565 nm. The only notable difference between the filtered hemolymph and the resuspended pellet was the presence of some additional absorption in the region of approximately

440 to 560 nm, probably by carotenoid pigments. This might interfere with oxy-hemocyanin measurements using the 565 nm peak, but not the 337 nm peak. Largely for this reason the 337 nm wavelength was used for oxy-hemocyanin measurements in this study.

Extinction coefficients were determined by a dry weight method as described by Ellerton et al. (7). For well oxygenated solutions at pH 7.7 the extinction coefficients are $E^{1\%}_{1cm} = 13.7$ at 280 nm, $E^{1\%}_{1cm} = 3.00$ at 337 nm, and $E^{1\%}_{1cm} = 0.14$ at 565 nm.

Analytical ultracentrifugation. Sedimentation velocities were measured in a Spinco Model E analytical ultracentrifuge equipped with an ultraviolet optical scanner. Scans were made at 337 nm, so hemocyanin concentrations were approximately the same as those used in the oxygen binding experiments (3 - 4 mg/ml). Only two components were observed, and they were well resolved. Sedimentation coefficients were calculated from the point of half maximum absorbance at a series of time intervals with the aid of a computer program from the Department of Biochemistry and Biophysics, Oregon State University. Coefficients were corrected to 20°C; but often only partial corrections were made for buffer viscosity, and no corrections were made for radial dilution. The proportion of the two components was calculated from the absorbance values recorded by the scanner.

Oxygen binding curves. Oxygen binding curves were determined using standard spectrophotometric tonometry procedures (36). The main chamber of the tonometer with the attached 1 cm path length quartz cuvet had a volume of 103.9 ml, while the sidearm had a volume of

0.564 ml. Hemocyanin solutions in TRIS buffer were used, the concentration of pigment being 3 - 4 mg/ml. The volume of this solution was 3.00 ml in all cases. Absorbance measurements were made using the 337 nm oxy-hemocyanin peak. Three evacuations followed by nitrogen addition (after the first two only) and equilibration sufficed to fully deoxygenate the pigment. Additions of air were then made, using equilibration times of 12 minutes in a thermostatted water bath between additions. Fully oxygenated readings were made before and after each run; if these two differed by more than 3%, the run was discarded. Oxygen partial pressures were calculated from an equation similar to that given by Spoek et al. (41). Data obtained by the spectrophotometric method, when compared with data obtained using a mixing method (6) where the hemocyanin concentration was approimately that normally found in vivo, revealed no significant differences.

Chromatography. Chromatographic purification of the hemocyanin and separation of the monomer and dimer components were accomplished using a Bio-Gel A5M column as described by Roxby et al. (37). To improve resolution the filtered hemolymph was concentrated to approximately 120 mg/ml in each case before loading, using an Amicon Model 12 filtration system. 5 ml fractions were collected and checked spectrophotometrically at both 280 and 337 nm.

Separation of subunits. Initially basic polyacrylamide gel electrophoresis was done as described by Miller et al. (22), except higher pH (10.2) was used. This caused dissociation of perhaps 10 - 15% of the molecules, resulting in a reaction mixture of interacting

subunits with unidentifiable pattern.

Subsequently, urea - acrylamide gel electrophoresis was used in a procedure modified from Panyim and Chalkley (25). 7% acrylamide and 6 M urea at pH 4 were used in the present case. Gels were electrophoresed at 130 volts and 11 mAmps; electrophoresis lasted for about 26 hours due to unfolding of the polypeptide chains. Staining and destaining were done as for the regular polyacrylamide gels.

Figure 1 shows the oxygen binding curves over physiologically significant ranges of pH values for hemocyanin from crayfish acclimated to 10°, 20°, and 25°C; each set of curves was determined at its temperature of acclimation. The important oxygen binding parameters for the curves in Figure 1, as well as for curves determined at temperatures other than that of acclimation, are given in Table 1.

The most striking difference between the three sets in Figure 1 is that of binding cooperativity[1]. Hemocyanin from animals acclimated to 10°C has the highest cooperativity, $n = 3.68$ at pH 7.8; 20°C hemocyanin is intermediate ($n = 2.33$ at pH 7.8); and 25°C hemocyanin has the lowest cooperativity, $n = 1.63$ at pH 7.8. This difference is due to properties of the hemocyanin from the different acclimation temperatures, rather than being a function of experimental temperature; the cooperativity differences remain when the hemocyanin preps are tested at the other (non-acclimation) temperatures (Table 1). In fact, there seems to be a trend in the opposite direction when the influence of experimental temperature is considered; cooperativity increases slightly at higher temperatures for all three acclimation preps.

Cooperativity varies with pH and P_{50}[2] for each acclimation temperature (Figures 2 and 3, respectively). Hemocyanin from 10°C acclimated animals shows by far the most sensitivity of the three.

The Bohr effect of crayfish hemocyanin is normal, at least over the range of pH tested (Figure 4). ϕ values, calculated from the slopes in Figure 4, for the three acclimation preps were quite similar, being between - 0.50 and - 0.54. When the preps were tested at non-

acclimation temperatures, the Bohr effect was still normal, although the ϕ values were somewhat different in magnitude (Table 1). These values are all within the range previously reported for crustaceans, as well as the majority of vertebrates (29).

The response of hemocyanin to <u>in vitro</u> change in temperature is typical of respiratory pigments; the oxygen affinity decreases with increasing temperature (29) (Table 1). Heat of reaction (ΔH) values (47), which provide a measure of the sensitivity of oxygen affinity to temperature change, are given in Table 2. In the present case, there is a noticable influence of acclimation temperature on the ΔH values; temperature sensitivity of oxygen affinity increases with decreasing acclimation temperature.

The data presented thus far indicate a difference in the oxygen binding properties of hemocyanin dependent on the temperature of acclimation. A good example of this is shown in Figure 5. Similar relationships between the three acclimation preps hold at any specific temperature and pH; the 25°C hemocyanin exhibits the highest oxygen affinity and the lowest cooperativity while the 10°C hemocyanin exhibits the lowest oxygen affinity and the highest cooperativity. The 20°C hemocyanin occupies an intermediate position.

Figure 6 shows the probable significance of these changes in hemocyanin oxygen binding behavior with acclimation temperature. In this example, if the water temperature of a crayfish acclimated to 10°C (hemolymph pH 7.84) is raised to 25°C, the P_{50} would increase from 6.2 torr to 10.0 torr. The pH also tends to drop at the higher

temperature (15, 38), down to 7.6 or less, leading to a P_{50} of at least 11.9 torr. This is a rather large change in oxygen affinity (see discussion), especially considering the decrease in oxygen solubility of water at the warmer temperature. The dashed line in Figure 6 shows that acclimation does indeed result in lowering of the hemocyanin P_{50}, approaching the value found at 10°C and pH 7.84.

One question which can be raised with data of this sort is the reliability of P_{50} and n values. Since the hemolymph of about 15 crayfish were pooled for each acclimation temperature, the resulting prep should be fairly representative of the hemocyanin at that temperature. This could still depend on the amount of individual variation present. Oxygen binding curves for six indiviual crayfish, all from the same acclimation temperature (10°C) and tested at 25°C, pH 7.8, showed mean values of $P_{50} = 9.8$ torr $\pm$ 0.08 ($\pm$ SEM) with a range of 0.5 torr and $n = 3.54 \pm 0.049$. These samples were taken at a different time of year than those in Table 1; yet their mean P_{50} is within 0.2 torr, and cooperativity within 0.15, of the pooled sample at the same conditions. Moreover, when two consecutive curves were determined on the same sample, the P_{50} agreement was generally within 0.1 torr. This sort of repeatability would indicate that the above differences between hemocyanin oxygen binding from different acclimation temperatures are real.

Hemolymph hemocyanin concentration varies greatly regardless of acclimation temperature (Table 3). Mean values for crayfish at the three acclimation temperature do seem to show a downward trend as

temperature is increased; but there is no significant difference between 20° and 25°C.

Table 4 shows the results of analytical ultracentrifugation to determine the aggregation states of hemocyanin present and their proportions. There were two aggregation sizes present at all temperatures, 25 S and 17 S.[4] These values are only approximate since no concentration series was run; and only partial corrections for buffer viscosity were made. However, the values obtained are ones corresponding to the usual crustacean hemocyanin monomers and dimers (24). The proportion of these two states was approximately 85% 25 S and 15% 17 S, which agrees well with data for the European crayfish at corresponding temperature and pH (8). In the present case the proportion did not change significantly with either acclimation temperature or with acute temperature change. Thus the two aggregation states do not appear to be in equilibrium with each other; and do not play a role in observed changes in oxygen binding.

Separation of monomer and dimer was not entirely complete after column chromatography, but was good enough to yield pure dimer at the top of the major peak, as confirmed by analytical ultracentrifugation. After column purification, the three acclimation preps showed oxygen binding properties identical to those shown before purification; that is, the affinity and cooperativity differences did not exceed 0.1 torr and 0.05, respectively.

Hemocyanin subunit separation by urea gel electrophoresis showed three subunits present in all preps, with no significant

differences in the proportions between different acclimation temperatures (Table 5).

Changes in oxygen affinity. The data from this study show that the crayfish Pacifastacus leniusculus is able to respond to different temperature regimes by altering the oxygen binding pattern of its hemocyanin. There is no concomitant adaptive change in the hemolymph concentration of hemocyanin. Over the temperature range of 10° - 25°C the oxygen affinity is maintained in the range of P_{50} = 6 - 8 torr at normal physiological pH, in contrast to a range of P_{50} = 3 - 12 torr which would occur without an acclimation response (Table 1). As seen in Figure 6, 10°C hemocyanin goes from a P_{50} of 6 torr at 10°C to a P_{50} of 12 torr at 25°C. Thus it easy to see why it would not be advantageous for an aquatic animal to keep this same hemocyanin over a wide range of temperatures, especially considering the lower solubility of oxygen in water at the higher temperatures. The 25°C hemocyanin would also not be particularly suitable over a wide range of temperatures. Its cooperativity is low, and it appears to have a very high affinity at lower temperatures; this combination would hinder efficient unloading at the tissues. Thus the acclimation changes observed keep the oxygen affinity of the hemocyanin centered around a narrow range of values, which are probably dictated by the need for a balance between loading oxygen at the gills and unloading it at the tissues. This acclimation response probably prevents hemocyanin oxygen affinity from being a major contributing factor to the decline of active oxygen consumption above 20°C (39).

There is very little data from crustaceans to compare with this study. Truchot (43, 44) has studied the effects of temperature

acclimation on oxygen affinity of Carcinus maenas hemocyanin. His results show patterns somewhat different from those for crayfish. While he observed an acclimation response qualitatively similar to that for the crayfish at higher temperatures ($\geq 25^{\circ}C$), below $21^{\circ}C$ acclimation temperature he found no change in oxygen affinity. He did not determine hemocyanin cooperativity. This different pattern may be related to the intertidal habitat of Carcinus maenas where it would experience rapid fluctuations in temperature. Thus, acclimation would not be relevant over much of the range encountered. Only at prolonged high temperature would there be an advantage because of the much lower inherent affinity.

Changes in hemoglobin oxygen affinity with temperature acclimation qualitatively similar to those in the crayfish have been reported for the bullhead (10). Qvist et al. (30) have also found a similar acclimation response in the Antarctic cod, although over a very restricted temperature range. The changes associated with the acclimation response in all cases return the pigment affinity towards a narrow range of oxygen affinities. Other studies on fish, however, do not provide evidence for an acclimation response over the range of temperatures tested (2, 46).

Changes in cooperativity. Hemocyanin cooperativity increases with increasing experimental temperature in all temperature acclimation groups. Recently Hlastala et al. (11) have drawn attention to a similar relationship with experimental temperature for human hemoglobin. How might this change in cooperativity with temperature be brought

about? Possibly it involves the charges of the subunits, particularly the imidazole groups so important in changes in pH with temperature (15, 32, 35). Figure 2 shows that cooperativity varies with pH for the different acclimation hemocyanins in crayfish. Although the less cooperative 20° and 25°C hemocyanins show broad, relatively pH independent curves, the 10°C hemocyanin (open circles and squares) shows a greater dependence of cooperativity on pH, so that when tested at different experimental temperatures, fairly well defined optima for both 10° and 25°C can be discerned. It is interesting that these optima occur at the pH values normally found in the animals at those two temperatures (pH = 7.9 at 10°C and pH = 7.65 at 25°C) (38). More important is the fact that the change in pH optimum is approximately that predicted according to the relative alkalinity theory of Rahn and his coworkers (15, 32). Thus the same degree of ionization would be present for the hemocyanin molecules at these two temperatures when at the respective pH values. This would argue that degree of ionization of the molecules plays a major role in the overall cooperativity shown.

The cooperativity optima themselves seem to be shifted upwards with higher experimental temperatures (see Table 1 for increases in "n" with increasing test temperature). The binding kinetics of the pigment-oxygen reaction, once oxygen molecules start to be bound, may not be as temperature sensitive as the initial binding phase. This would probably involve mainly changes in the dissociation reaction (3).

The allosteric transition theory of Monod et al. (23) suggests

that cooperativity as a function of P_{50} should pass through a maximum where the presence of transition states between R and T is maximal. As the curve approaches n = 1 (no cooperativity) at high and low P_{50} values, the hemocyanin is becoming pure R or T state. Miller and Van Holde (21) have shown that this model satisfactorily describes the oxygen binding behavior of Callianassa californiensis hemocyanin. In the present case it appears that hemocyanin is becoming stabilized in hybrid transition states (5) as the acclimation temperature is increased, as indicated by the lower, broader curves (Figure 3). This could be caused by an effector binding to the hemocyanin in increasing amounts. In any case the stabilization is associated with lower but more pH independent cooperativity, greater temperature independence, and generally higher oxygen affinity. The cooperativity changes are probably consequences dictated by the changes occurring to increase the affinity of the pigment.

Possible nature of the acclimation response. The possibility of hemocyanin subunit heterogeneity playing a role in temperature acclimation response has been discussed by several workers (3, 22, 42). In one case (42) different subunits have been isolated and shown to have different oxygen binding behaviors. Similar structural and functional heterogeneity has been shown for fish hemoglobins (27, 28, 45, 48). Relative amounts of hemoglobin subunits have also been shown to change with temperature acclimation in some fish (13, 14). However, parallel changes in both subunit composition and changes in oxygen binding behavior have not yet been demonstrated in either

hemocyanins or hemoglobins with acclimation. In the present situation, crayfish do show subunit heterogeneity, and there is a definitive temperature acclimation effect. But since there is no consistent change observed in the relative amounts of the three subunit species with acclimation, it appears unlikely that subunit composition is involved in the acclimation response of Pacifastacus leniusculus.

There is a possibility that the distribution and arrangment of the subunits is not random, and that the arrangment is being changed during acclimation. If the different subunits do have distinct association and dissociation kinetics, as has been demonstrated for Limulus polyphemus (42), then the arrangment of subunits could determine the overall binding behavior through changes in cooperativity of each hemocyanin molecule. This possibility was not investigated in the present study.

Miller and Van Holde (21) have found some difference in oxygen binding behavior between the two hemocyanin aggregation states in hemolymph of Callianassa californiensis. It is possible that the observed functional changes in crayfish hemocyanin could be caused by changes in the proportion of the two states. This does not seem to be the case, since the proportion of monomer and dimer remained constant for the three acclimation temperatures tested. The data might be misleading, however, since aggregation states were determined only for oxy-hemocyanin. In any case, there may not be much difference in the binding behavior of the two states in crayfish. Although oxygen binding curves were not determined for purified

monomer, largely because of the small amounts obtained, the fact that the oxygen binding curves for hemolymph and column purified dimer were virtually identical would imply only slight differences at most.

The most common method of altering oxygen binding behavior actually demonstrated to date is that of changing amounts of an allosteric effector, which then modifies the pigment behavior without any change in primary structure. Possibly this is the method employed by the crayfish, even though column purification produced no change in oxygen binding. The effector may simply be rather tightly bound, perhaps covalently. This would seem to be a more efficient method than having to resynthesize the respiratory pigment during acclimation, especially if the turnover time for hemocyanin (which is unknown) is long. Truchot (44) has found that the acclimation response observed in Carcinus maenas was caused by an allosteric effector (not Ca^{++}). His data show a change in ϕ value with an acute change in temperature, as well as a change in P_{50}, which agrees with the findings of the present study. However, he gets only "translation" of the ϕ plots with acclimation, while in Pacifastacus leniusculus there is an additional rotation of the ϕ plots so that ϕ values of acclimated animals are virtually the same. It is possible there are both short-term and long-term acclimation changes, since his acclimation period was only 3 - 5 days. These might involve different effectors, or an effector together with some other structural change. Only isolation and identification of the effectors, followed by controlled addition experiments, can resolve this problem.

Concluding remarks. The regulation of hemocyanin oxygen affinity within the very narrow range observed is noteworthy. It might appear that a change in P_{50} of only 6 torr when the temperature of 10°C acclimated hemocyanin is raised from 10° to 25°C is a rather minor one. However, the P_{50}, which represents overall oxygen affinity, is a function of the ratio of dissociation and association constants between the pigment and oxygen (3). A doubling of the P_{50} will then represent an approximate halving of the overall reaction rate for binding oxygen, probably through an increase in the dissocation reaction. Thus for a relatively high affinity pigment such as we have here, small changes in P_{50} will have more significance than in the case of lower affinity pigments. These changes may be crucial for a water breather, where it is often difficult to saturate the pigment as it passes through the gills, let alone reach an equilibrium as happens in the lungs of mammals.

The contention by some workers (34) that low P_{50} values are useful because they confer a sort of temperature independence is incorrect. As pointed out above, small changes in P_{50} for high affinity pigments are significant since they represent relatively large changes in the overall reaction rate. The degree of temperature dependence is given by the heat of reaction (ΔH), which is a function of the change in P_{50} with temperature, not of P_{50} itself. For example, crayfish hemocyanin in the present study has notably lower ΔH values when compared with other crustacean hemocyanins (16, 19, 34, 49), yet it has affinities well within the range of these other crustaceans.

This means adaptations in the direction of temperature independence will be indicated by low ΔH values, not low P_{50}. Thus the relatively high affinity pigments in water breathers must be mostly a consequence of the viscosity of the respired medium and diffusion problems at the respiratory surface. And the present study shows that temperature acclimation causes an adaptive response of crayfish hemocyanin, keeping the high oxygen affinity regulated within a very narrow range.

FOOTNOTES

1. Oxygen binding cooperativity of respiratory pigments is generally taken as an indication of the extent of interaction between subunits of the protein. The Hill coefficient (n) is calculated from the slope in the region of $\bar{Y} = 0.5$, when $\log \bar{Y}/1 - \bar{Y}$ is plotted versus $\log P_{O_2}$.

2. P_{50} is the partial pressure of oxygen required to half-saturate the hemocyanin; it is inversely related to oxygen affinity.

3. Heat of reaction is an approximation of the term from the Van't Hoff equation, substituting P_{50} for k.

4. S = Svedberg units, 1×10^{-13} sec.

5. R and T states from the Monod model refer to two states of the same protein with different binding affinities for a particular ligand, such as oxygen. The two states are in equilibrium.

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LITERATURE CITED

1. Anthony, E.H. Survival of goldfish in the presence of carbon monoxide. J. Exp. Biol. 38: 109-129, 1961.
2. Black, E.C., D. Kirkpatrick and H.H. Tucker. Oxygen dissociation curves of the blood of brook trout (Salvelinus fontinalis) acclimated to summer and winter temperatures. J. Fish. Res. Bd. Can. 23: 1-13, 1966.
3. Bonaventura, J., C. Bonaventura and B. Sullivan. Hemoglobins and hemocyanins: Comparative aspects of structure and function. J. Exp. Zool. 194: 155-174, 1975.
4. Brett, J.R. The metabolic demand for oxygen in fish, particularly salmonids, and a comparison with other vertebrates. Resp. Physiol. 14: 151-170, 1972.
5. Buc, H., K.-J. Johannes and B. Hess. Appendix to Johannes, K.-J. and B. Hess. Allosteric kinetics of pyruvate kinase of Saccharomyces carlsbergensis. J. Mol. Biol. 76: 181-205, 1973.
6. Edwards, M.J. and R.J. Martin. Mixing technique for the oxygen-hemoglobin equilibrium and Bohr effect. J. Appl. Physiol. 21: 1898-1902, 1966.
7. Ellerton, H.D., D.E. Carpenter and K.E. Van Holde. Physical studies of hemocyanins. V. Characterization and subunit structure of the hemocyanin of Cancer magister. Biochemistry. 9: 2225-2232, 1970.
8. Eriksson-Quensel, I.-B. and T. Svedberg. The molecular weights and pH - stability regions of the hemocyanins. Biol. Bull. 71: 498-547, 1936.

9. Fry, F.E.J. Effects of the environment on animal activity. Univ. Toronto Stud. Biol. Ser. 55, Pub. Ont. Fish. Res. Lab. 68: 1-62, 1947.

10. Grigg, G.C. Temperature induced changes in the oxygen equilibration curve of the blood of the brown bullhead, Ictalurus nebulosus. Comp. Biochem. Physiol. 28: 1203-1223, 1969.

11. Hlastala, M.P., R.D. Woodson and B. Wranne. Influence of temperature on hemoglobin-ligand interaction in whole blood. J. Appl. Physiol: Respirat. Environ. Exercise Physiol. 43: 545-550, 1977.

12. Holeton, G.F. Gas exchange in fish with and without hemoglobin. Resp. Physiol. 14: 142-150, 1972.

13. Houston, A.H. and D. Cyr. Thermoacclimatory variation in the hemoglobin systems of goldfish (Carassius auratus) and rainbow trout (Salmo gairdneri). J. Exp. Biol. 61: 455-461, 1974.

14. Houston, A.H., K.M. Mearrow and J.S. Smeda. Further observations upon the hemoglobin systems of thermally-acclimated freshwater teleosts: pumpkinseed (Lepomis gibbosus), white sucker (Catostomus commersoni), carp (Cyprinus carpio), goldfish (Carassius auratus) and carp-goldfish hybrids. Comp. Biochem. Physiol. 54A: 267-273, 1976.

15. Howell, B.J., H. Rahn, D. Goodfellow and C. Herreid. Acid-base regulation and temperature in selected invertebrates as a function of temperature. Amer. Zool. 13: 557-563, 1973.

16. Johansen, K., C. Lenfant and T.A. Mecklenburg. Respiration in the crab, Cancer magister. Z. Vergl. Physiol. 70: 1-19, 1970.

17. Jones, D.R. Theoretical analysis of factors which may limit the maximum oxygen uptake of fish: The oxygen cost of the cardiac and branchial pumps. J. Theor. Biol. 32: 341-349, 1971.

18. Kerley, D.E. and A.W. Pritchard. Osmotic regulation in the crayfish, Pacifastacus leniusculus, stepwise acclimated to dilutions of sea water. Comp. Biochem. Physiol. 20: 101-113, 1967.

19. Larimer, J.L. and A.F. Riggs. Properties of hemocyanins. I. The effects of calcium ions on the oxygen equilibrium of crayfish hemocyanin. Comp. Biochem. Physiol. 13: 35-46, 1964.

20. Long, C. Biochemists Handbook. Princeton: Van Norstrand, 1961. p. 28.

21. Miller, K.I. and K.E. Van Holde. Oxygen binding by Callianassa californiensis hemocyanin. Biochemistry. 13: 1668-1674, 1974.

22. Miller, K.I., N.W. Eldred, F. Arisaka and K.E. Van Holde. Structure and function of hemocyanin from thalassinid shrimp. J. Comp. Physiol. B. 115: 171-184, 1977.

23. Monod, J., J. Wyman and J.-P. Changeux. On the nature of allosteric transitions: A plausible model. J. Mol. Biol. 12: 88-118, 1965.

24. Nickerson, K.W. and K.E. Van Holde. A comparison of molluscan and arthropod hemocyanin. I. Circular dichroism and absorption spectra. Comp. Biochem. Physiol. 39B: 855-872, 1971.

25. Panyim, S. and R. Chalkley. High resolution acrylamide gel electrophoresis of histones. Arch. Biochem. Biophys. 130: 337-346, 1969.

26. Newell, R.C. and V.I. Pye. Quantitative aspects of the relationship between metabolism and temperature in the winkle Littorina littorina (L). Comp. Biochem. Physiol. 38B: 635-650, 1971.

27. Perez, J.E. and N. Maclean. The haemoglobins of the fish Sarotherodon mossambicus (Peters): Functional significance and ontogenetic changes. J. Fish. Biol. 9: 447-455, 1976.

28. Powers, D.A. Hemoglobin adaptation for fast and slow water habitats in sympatric catostomid fishes. Science. 177: 360-362, 1972.

29. Prosser, C.L. Comparative Animal Physiology. Philadelphia: Saunders, 1973. 966 pp.

30. Qvist, J., R.E. Weber, A.L. DeVries and W.M. Zapol. pH and haemoglobin oxygen affinity in blood from the Antarctic cod Dissostichus mawsoni. J. Exp. Biol. 67: 77-88, 1977.

31. Rahn, H. Aquatic gas exchange: Theory. Resp. Physiol. 1: 1-12, 1966.

32. Rahn, H., R.B. Reeves and B.J. Howell. Hydrogen ion regulation, temperature and evolution. Am. Rev. Resp. Dis. 112: 165-172, 1975.

33. Redmond, J.R. The respiratory function of hemocyanin in Crustacea. J. Cell. Comp. Physiol. 46: 209-248, 1955.

34. Redmond, J.R. Oxygen-hemocyanin relationships in the land crab, Cardisoma guanhumi. Biol. Bull. 122: 252-262, 1962.

35. Reeves, R.B. An imidazole alphastat hypothesis for vertebrate acid-base regulation: Tissue carbon dioxide content and body temperature in bullfrogs. Resp. Physiol. 14: 219-236, 1972.

36. Rossi-Fanelli, A. and E. Antonini. Studies on the oxygen and carbon monoxide equilibria of human myoglobin. Arch. Biochem. Biophys. 77: 478-492, 1958.

37. Roxby, R., K. Miller, D.P. Blair and K.E. Van Holde. Subunits and association equilibria of Callianassa californiensis hemocyanin. Biochemistry. 13: 1662-1668, 1974.

38. Rutledge, P.S. Circulation and oxygen transport during activity in the crayfish. In review to Am. J. Physiol.: Regulatory Integrative Comp. Physiol., 1978.

39. Rutledge, P.S. and A.W. Pritchard. Scope for activity in the crayfish Pacifastacus leniusculus. In review to Am. J. Physiol.: Regulatory Integrative Comp. Physiol., 1978.

40. Spoek, G.L. The relationship between blood haemocyanin level, oxygen uptake, and the heart-beat and scaphognathite-beat frequencies in the lobster Homarus gammarus. Neth. J. Sea Res. 8: 1-26, 1974.

41. Spoek, G.L., H. Bakker and H.P. Wolvekamp. Experiments on the haemocyanin-oxygen equilibrium of the blood of the edible snail (Helix pomatia L). Comp. Biochem. Physiol. 12: 209-221, 1964.

42. Sullivan, B., J. Bonaventura and C. Bonaventura. Functional differences in the multiple hemocyanins of the horseshoe crab, Limulus polyphemus L. Proc. Natl. Acad. Sci. 71: 2558-2562, 1974.

43. Truchot, J.-P. Fixation et transport de l'oxygène par le sang de Carcinus maenas: Variations en rapport avec diverses conditions de température et de salinité. Neth. J. Sea Res. 7: 482-495, 1973.

44. Truchot, J.-P. Factors controlling the in vitro and in vivo oxygen affinity of the hemocyanin in the crab Carcinus maenas (L). Resp. Physiol. 24: 173-189, 1975.

45. Weber, R.E., G. Lykkeboe and K. Johansen. Physiological properties of eel haemoglobin: Hypoxic acclimation, phosphate effects and mutiplicity. J. Exp. Biol. 65: 75-88, 1976.

46. Weber, R.E., S.C. Wood and J.P. Lomholt. Temperature acclimation and oxygen-binding properties of blood and multiple haemoglobins of rainbow trout. J. Exp. Biol. 65: 333-345, 1976.

47. Wyman, J. Linked functions and reciprocal effects in haemoglobin: A second look. In Adv. Protein Chem., Editors: C.B.Anfinsen, M.L. Anson, J.T. Edsall and F.M. Richards. Vol. XIV: 223-286. New York: Academic Press, 1964.

48. Yamaguchi, K., Y. Kochiyama, K. Hashimoto and F. Matsuura. Studies on multiple hemoglobins of eel. II. Oxygen dissociation curve and relative amount of components F and S. Bull. Jap. Soc. Sci. Fish. 28: 192-200, 1962.

49. Young, R.E. The physiological ecology of haemocyanin in some selected crabs. II. The characteristics of haemocyanin in relation to terrestrialness. J. Exp. Mar. Biol. Ecol. 10: 193-206, 1972.

50. Zuckerkandl, E. Hémocyanin et cuivre chez un crustacé décapode, dans leurs rapports avec le cycle d'intermue. Ann. Inst. Ocean. 38: 1-122, 1960.

Table 1. Summarized oxygen binding data.

Acclimation T (°C)	Experimental T (°C)	pH	P_{50}	n	ϕ
10°	10°	7.42	10.2	2.56	
10°	10°	7.62	8.2	3.07	
10°	10°	7.84	6.2	3.68	- 0.53
10°	10°	8.04	4.9	3.50	
10°	10°	8.20	4.0	3.26	
10°	20°	7.84	8.9	3.68	
10°	25°	7.44	14.9	3.71	
10°	25°	7.64	11.9	3.88	- 0.47
10°	25°	7.82	10.0	3.69	
20°	20°	7.30	13.4	2.04	
20°	20°	7.48	11.0	2.22	
20°	20°	7.65	8.9	2.24	- 0.50
20°	20°	7.84	7.2	2.33	
20°	20°	8.06	5.6	2.34	
20°	10°	7.65	6.8	1.89	
20°	10°	7.84	5.6	1.97	- 0.44
20°	10°	8.04	4.6	2.14	
20°	25°	7.63	9.8	2.65	- 0.32
20°	25°	7.84	8.3	2.55	
25°	25°	7.26	11.0	1.66	
25°	25°	7.44	8.7	1.74	
25°	25°	7.63	7.0	1.67	- 0.54
25°	25°	7.82	5.6	1.63	
25°	25°	8.02	4.2	1.55	
25°	10°	7.64	5.6	1.42	
25°	10°	7.84	4.3	1.46	- 0.63
25°	10°	8.04	3.1	1.47	
25°	20°	7.84	5.1	1.53	

"n" refers to the Hill coefficient, measured at half saturation. "ϕ" is a measure of the Bohr effect. Values correspond to a pH series done at a particular experimental temperature.

Table 2. Heat of reaction values as a function of acclimation temperature.

Acclimation T (°C)	ΔH (kcal/mole)
10°	- 5.95
20°	- 4.86
25°	- 3.12

ΔH values were determined from slopes of Van't Hoff plots (log P_{50} vs $1/T^{\circ}K$).

Table 3. Hemolymph hemocyanin concentration of crayfish acclimated to temperature.

Acclimation T (°C)	Hcy (mg/ml)
10°	62.6 ± 5.0 (17)
20°	50.9 ± 5.0 (18)
25°	48.8 ± 4.0 (17)

Hemocyanin (Hcy) concentration given as mean ± SE, with number of crayfish sampled in parentheses. There is a significant difference only between 10°and 25°C acclimated animals ($P < 0.05$).

Table 4. Presence of hemocyanin 25 S aggregation state as a function of temperature.

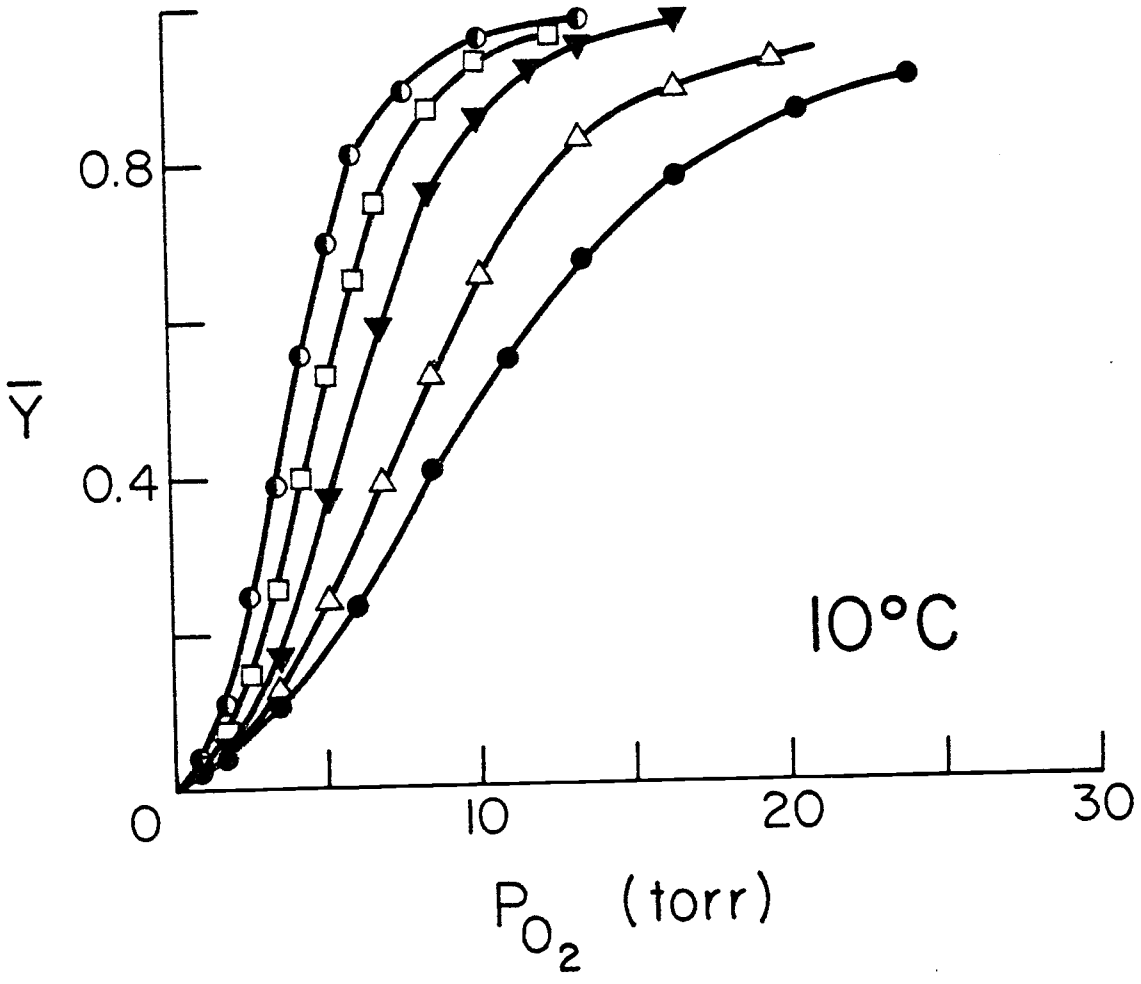
Acclimation T (°C)	Experimental T (°C) 5°	10°	20°	25°	30°
10°	79.1% (1)	82.6% (3)			82.1% (1)
20°	87.9% (1)		88.1% (3)		88.2% (1)
25°	92.9% (1)			84.9% (3)	92.3% (1)

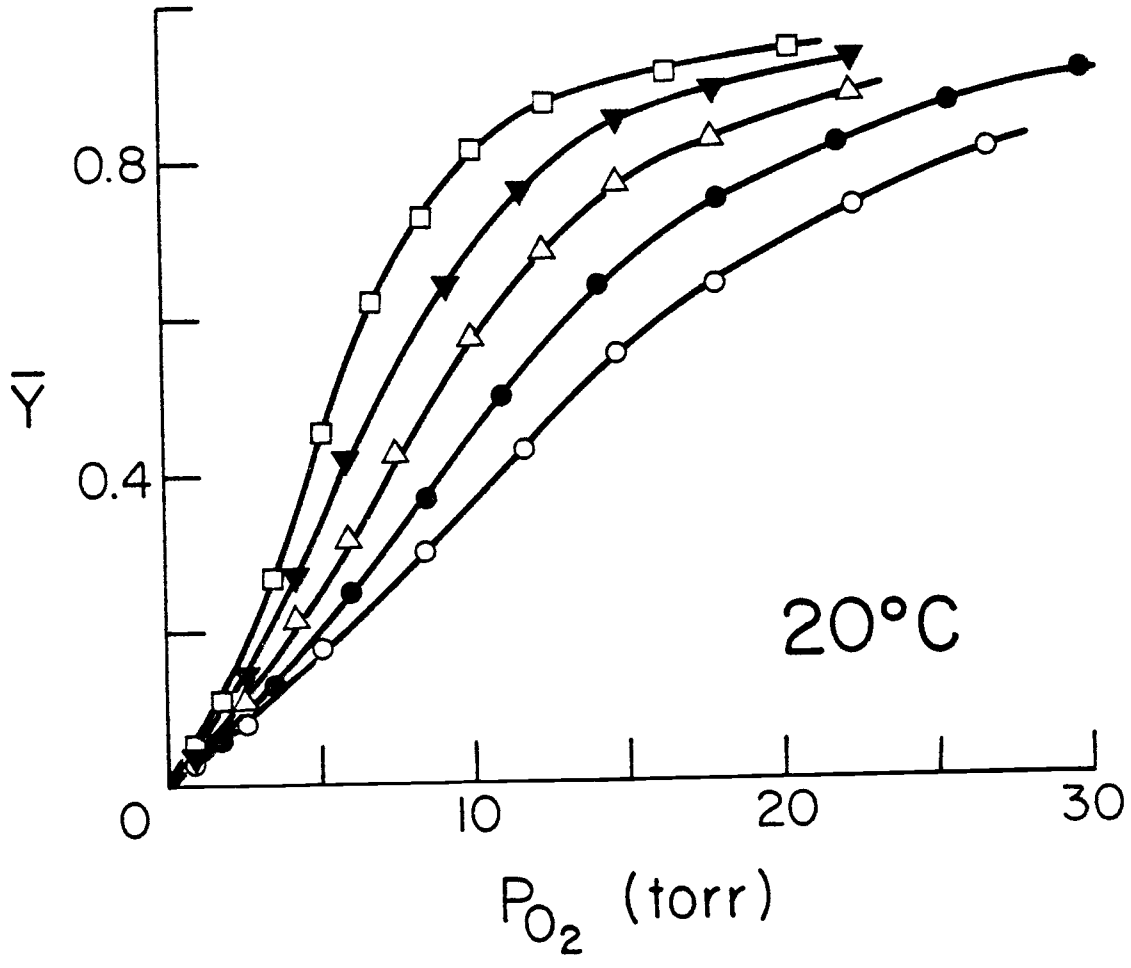
Values are expressed in terms of percent of the total hemocyanin being 25 S; the remainder was all 17 S in each case. The number of individuals tested is in parentheses. There was no significant difference between acclimation temperatures.

Table 5. Hemocyanin subunit proportions as percent of total hemocyanin present.

Acclimation T (°C)	Peak I	Peak II	Peak III
10°	21%	18%	61%
20°	21%	18%	61%
25°	21%	17%	62%

Figure 1. Hemocyanin oxygen binding curves over the physiological pH range at three temperatures as indicated (10°, 20°, and 25°C). $\bar{Y}$ refers to the fraction of hemocyanin oxygen binding sites in the oxy - state. Crayfish were acclimated to the respective temperatures for one month prior to sampling; hemocyanin samples were tested only at their temperature of acclimation. Symbols used correspond to the following approximate pH values: pH 8.2 (◐), pH 8.0 (□), pH 7.8 (▲), pH 7.6 (△), pH 7.4 (●), and pH 7.3 (○). See Table 1 for exact pH values, as well as oxygen affinity (P_{50}) and cooperativity (n) values.


0.8
0.4
0
$\bar{Y}$
10°C
10
20
30
P_{O_2} (torr)


0.8
0.4
0
$\bar{Y}$
10
20
30
P_{O_2} (torr)
20°C

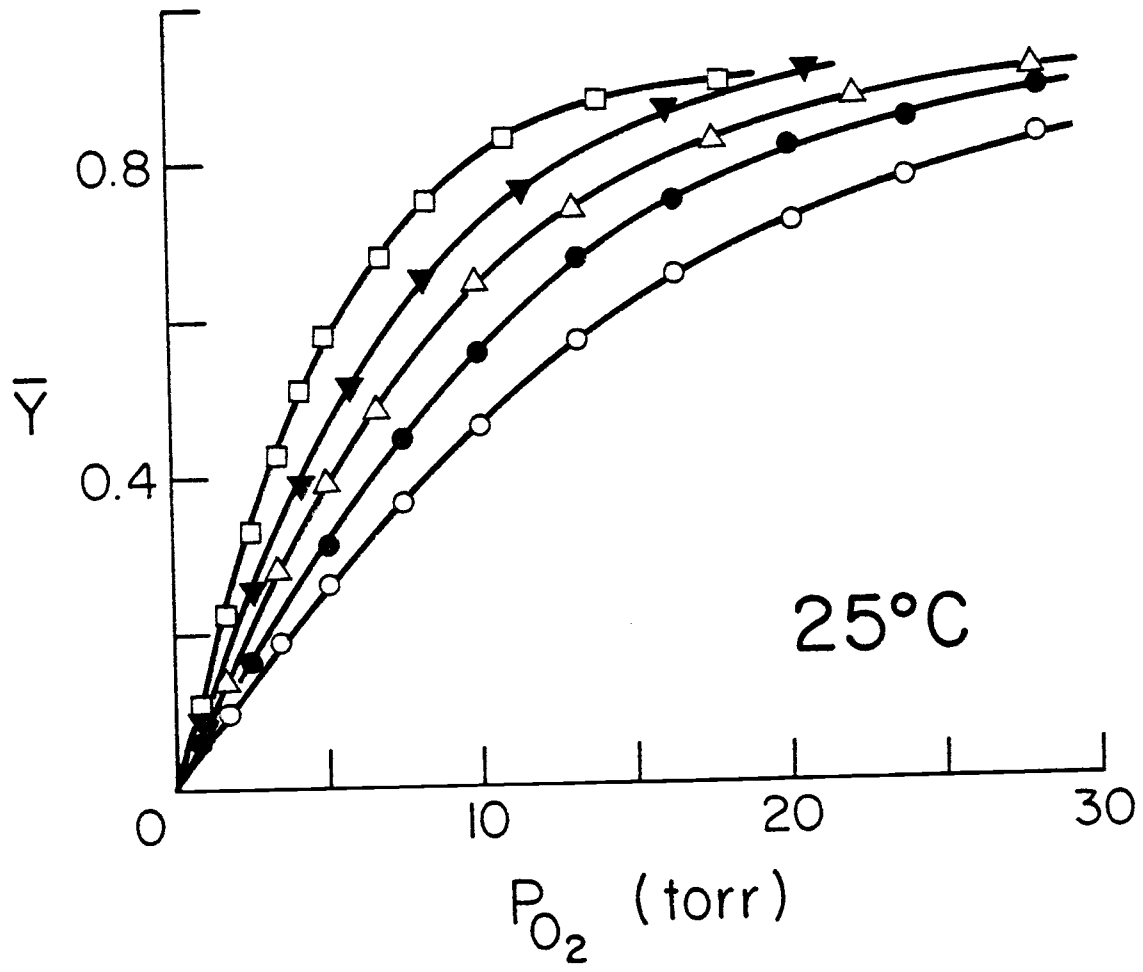

0.8
0.4
$\bar{Y}$
25°C
0
10
20
30
P_{O_2} (torr)

Figure 2. Cooperativity (n is Hill coefficient) as a function of pH. The symbols used represent the following: 10°C acclimated hemocyanin tested at 10°C (○) and at 25°C (□); 20°C acclimated hemocyanin tested at 20°C (●); and 25°C acclimated hemocyanin tested at 25°C (△).

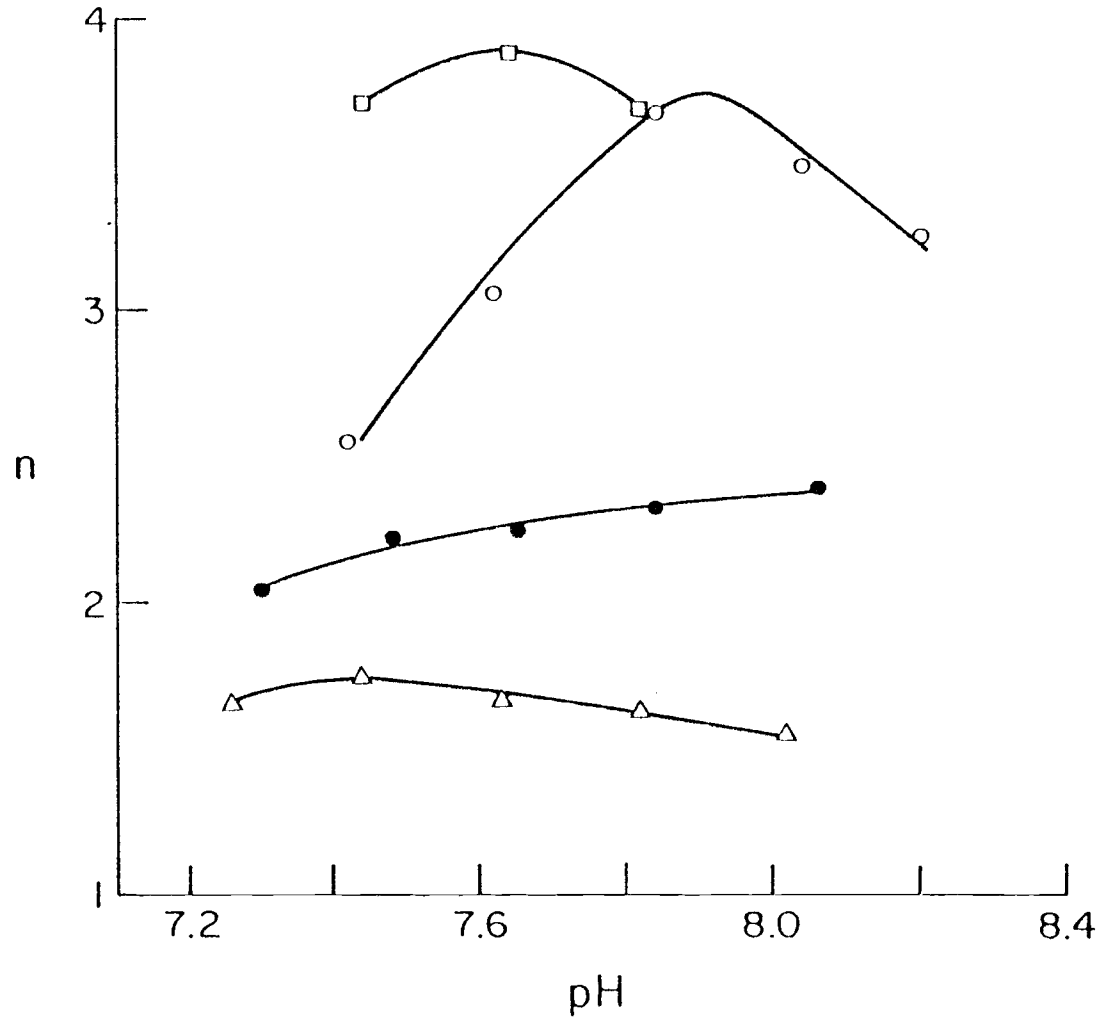

4
3
2
1
n
7.2
7.6
8.0
8.4
pH

Figure 3. Cooperativity (n) as a function of hemocyanin oxygen affinity (P_{50}). Symbols are the same as used in Figure 2.

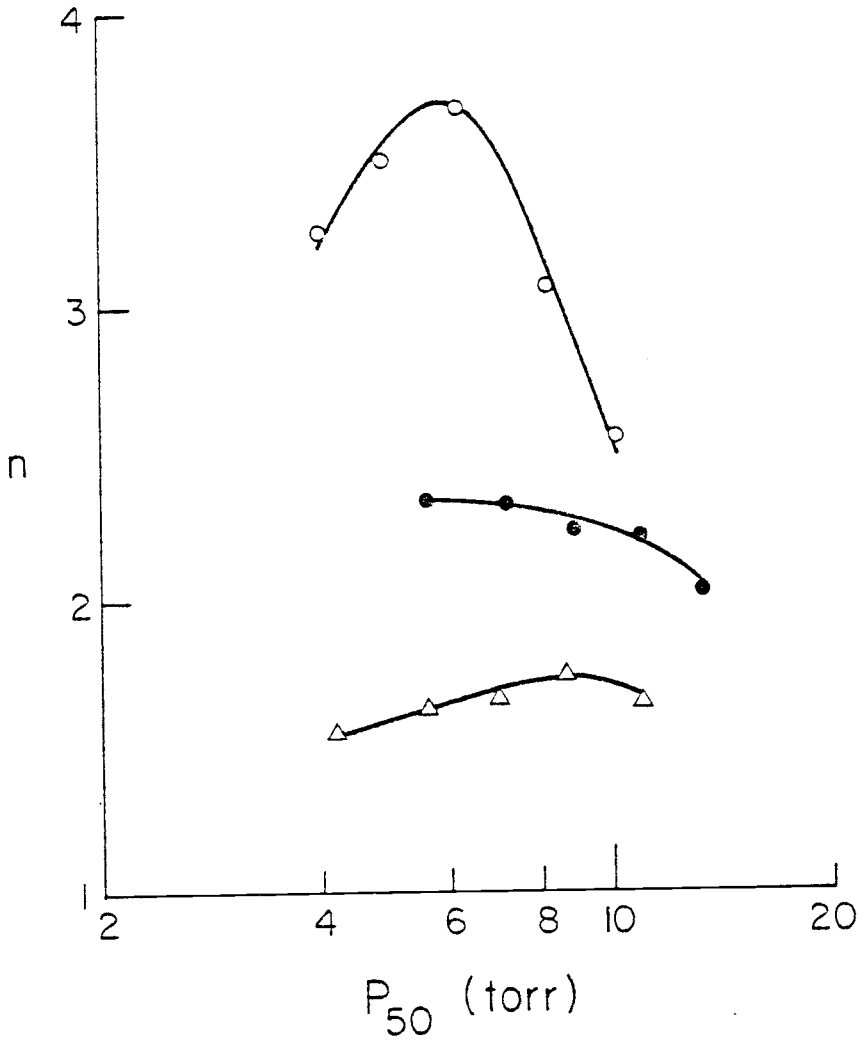

4
3
2
1
n
2
4
6
8
10
20
P50 (torr)

Figure 4. The change in oxygen affinity (P_{50}) with pH (Bohr effect) for crayfish hemocyanin. The slope of the lines is the value ϕ. The hemocyanin was tested only at the temperature corresponding to its acclimation temperature in this case. 10°C hemocyanin (○), 20°C hemocyanin (●), and 25°C hemocyanin (△). ϕ values are - 0.53 at 10°C, - 0.50 at 20°C, and - 0.54 at 25°C.

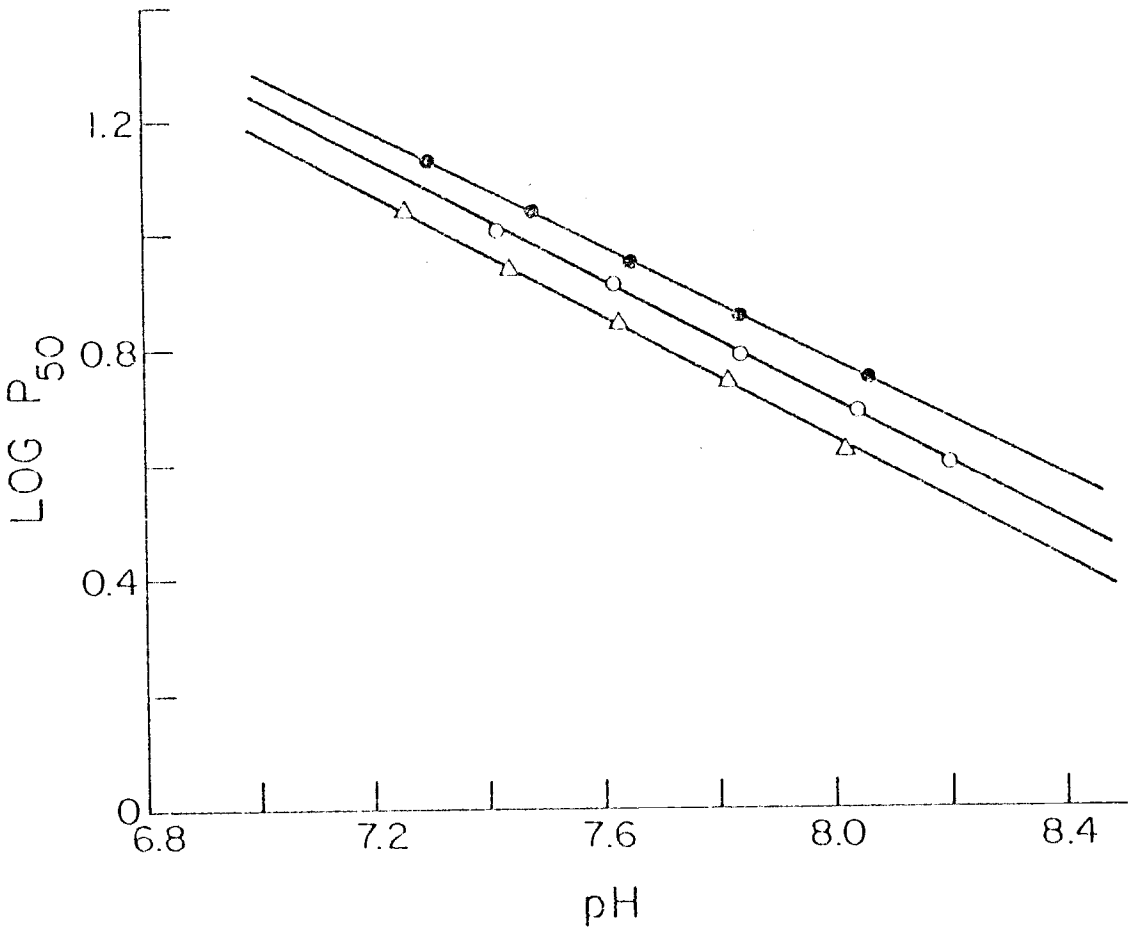

LOG P_{50}
1.2
0.8
0.4
0
6.8
7.2
7.6
8.0
8.4
pH

Figure 5. Effect of acclimation temperature on hemocyanin oxygen binding. In this case, hemocyanin from the three acclimation temperatures, 10°C (○), 20°C (●), and 25°C (△) have all been run at 20°C, pH 7.8.

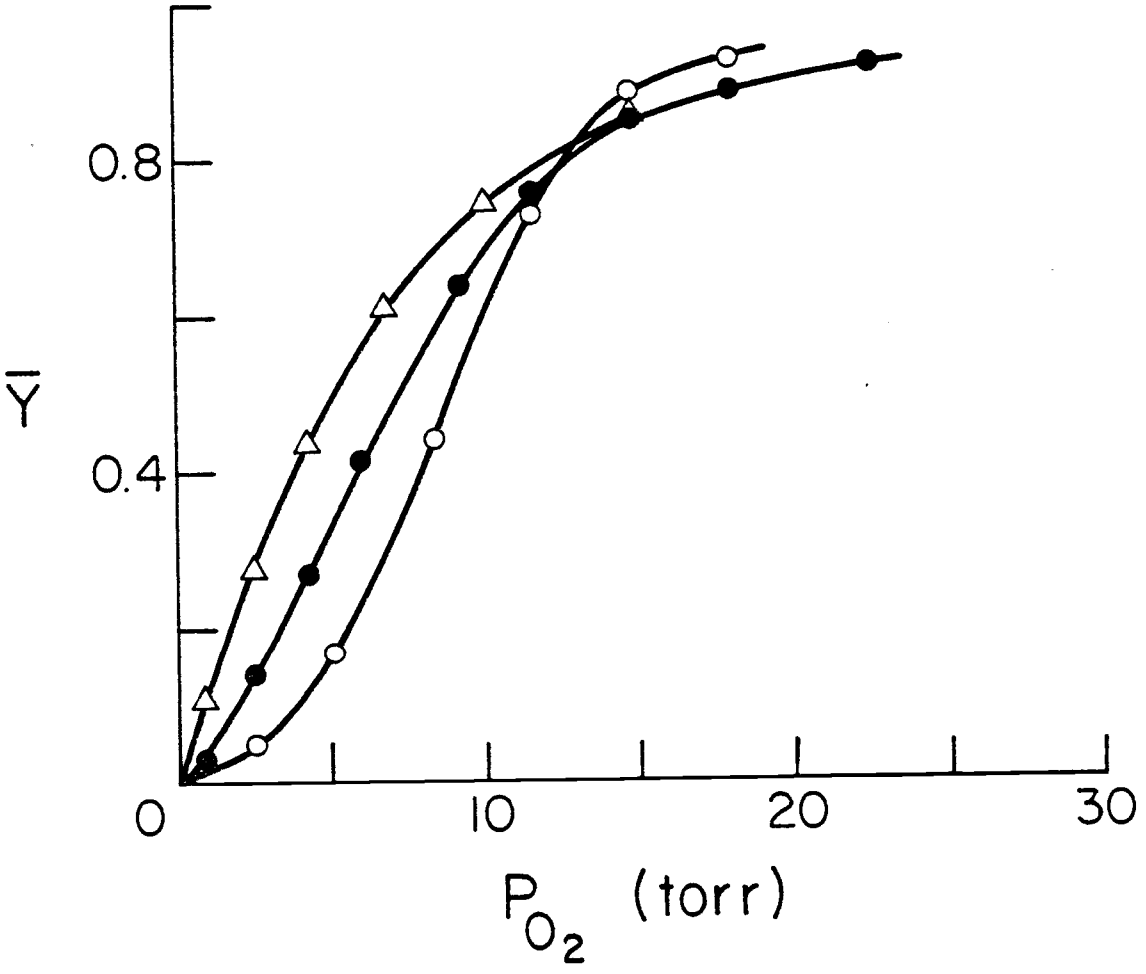
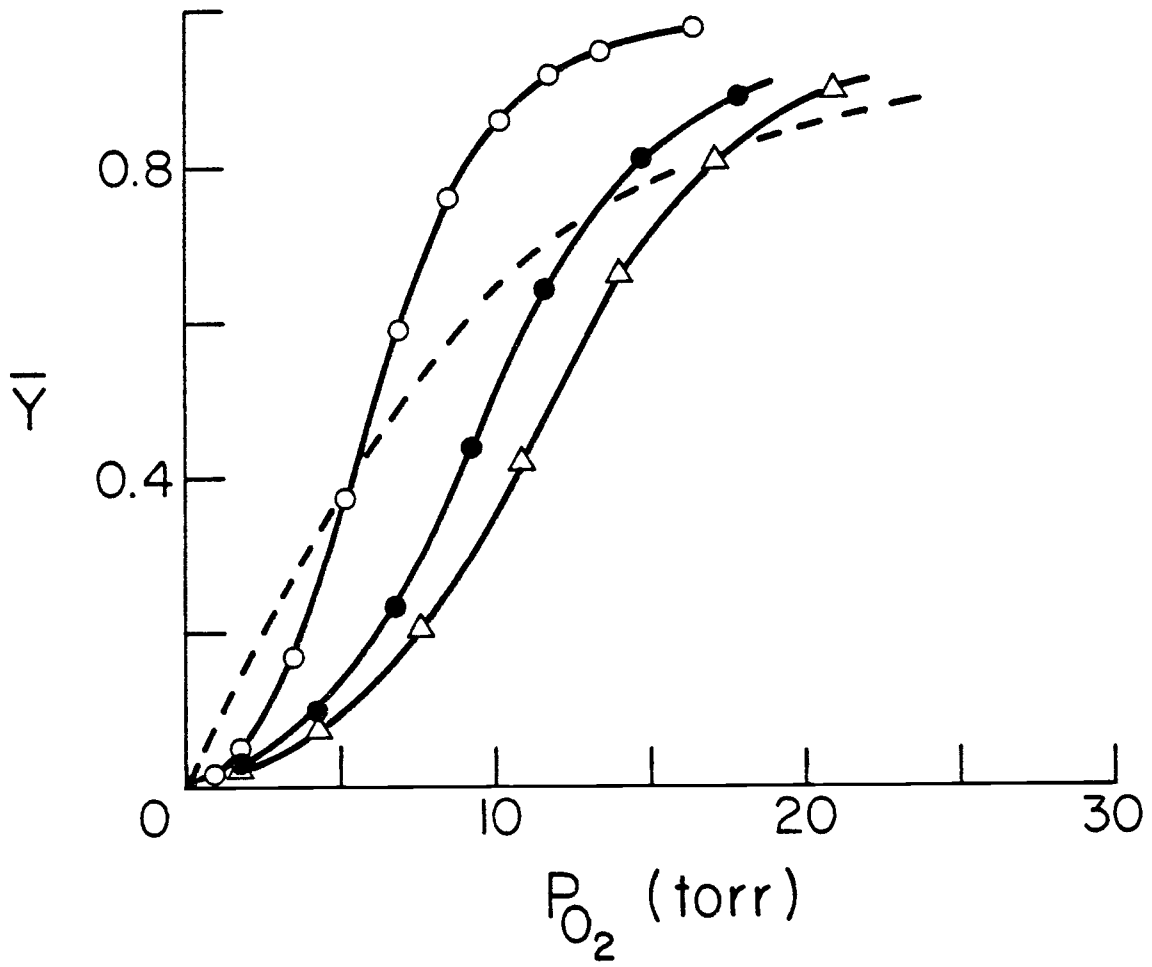

0.8
0.4
$\bar{Y}$
0
10
20
30
P_{O_2} (torr)

Figure 6. Effect of experimental temperature, alone and in combination with the temperature induced change in pH, on the oxygen binding of 10°C acclimated hemocyanin. Curves are 10°C hemocyanin at 10°C, pH 7.84 (O); at 25°C, pH 7.82 (●), and at 25°C, pH 7.63 (Δ). The dashed line shows the binding curve of 25°C acclimated hemocyanin at 25°C, pH 7.63. This indicates that acclimation returns the affinity to a narrow range, so that P_{50} is about 6 - 8 torr at normal pH at the various temperatures.


0.8
0.4
0
10
20
30
$\bar{Y}$
P_{O_2} (torr)

CIRCULATION AND OXYGEN TRANSPORT DURING ACTIVITY IN THE CRAYFISH

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Running head: Oxygen transport in the crayfish.

ABSTRACT

Heart rate, ventilation rate, oxygen consumption, hemocyanin concentration, and arterial and venous P_{O_2} and pH were measured in unrestrained crayfish (Pacifastacus leniusculus) during low-level, routine activity: and during forced (maximal) activity. Hemocyanin percent saturation, oxygen content of arterial and venous blood, cardiac output, and stroke volume were calculated from the measured parameters. A procedure for using N-ethylmaleimide as an anti-coagulant during blood sampling is described. Experiments were performed at 20°C, which is the temperature of maximum scope for activity of this crayfish; and at 10° and 25°C. Results showed low arterial P_{O_2} (9 - 12 torr) during routine activity; and this dropped some during maximum activity. Thus hemocyanin is not normally saturated in these animals. Cardiac output is high (eg. at 20°C, 236 ml/kg.min during routine activity and 969 ml/kg·min during forced activity). The implications are discussed, including general circulation strategy in crustaceans. Evidence is presented that ventilation is the major limiting factor of the scope for activity, both below and above the maximum point.

Index Terms: Oxygen consumption, oxygen tensions in blood, oxygen delivery, pH, temperature acclimation, hemocyanin, oxygen capacity, cardiac output, heart rate, ventilation rate, crustaceans, water breathers, blood sampling, anticoagulant, N-ethylmaleimide.

The scope for activity of the crayfish Pacifastacus leniusculus has been shown (31) to follow the pattern most commonly observed to date for aquatic poikilotherms, namely an increase in scope up to a maximum level as temperature is increased, followed by a decrease in scope until eventually the lethal high temperature is reached (5). Little experimental work has been done on the underlying mechanisms. Since the standard rate of oxygen uptake ($\dot{V}_{O_2}$) usually increases with temperature up to the lethal limit, the important and generally unknown feature concerns the limitations on active $\dot{V}_{O_2}$.

It is unlikely that active $\dot{V}_{O_2}$ is determined by limitations of the somatic musculature itself (5, 14). Rather, something within the oxygen delivery system is probably setting the maximum $\dot{V}_{O_2}$. There are three basic parts to this: the respiratory pump, the circulatory pump, and the respiratory pigment. Rahn (27) has theoretically related ventilation volume, solubilities of oxygen in blood and water, cardiac output, and oxygen tensions in blood and water to $\dot{V}_{O_2}$; and discussed some of the implications, including the effect of temperature. Building on his analysis, Jones et al. (18) and Jones (17), working with data from fish, have postulated that the cardiac pump may be limiting the active $\dot{V}_{O_2}$ (and thus the scope for activity) at low temperatures, while the respiratory pump may become limiting at higher temperatures and thus be the cause for the ultimate decline in both active $\dot{V}_{O_2}$ and scope for activity. Characteristics of the pigment can influence oxygen uptake at the respiratory surface and oxygen delivery at the tissues, as well as directly determine the

amount of oxygen carried by the blood. In the crayfish, hemocyanin may play only a minor role in the limitations of the oxygen exchange system as temperature is increased since its affinity shows a temperature acclimation response (30).

The most important deficiency in previous studies on oxygen exchange and transport in aquatic poikilotherms is that only a single temperature has been used in each study. The effects of exercise on various blood and water parameters have been examined at 5°C in rainbow trout (28, 35). Piiper et al. (25) have measured a wide range of parameters in an elasmobranch, Scyliorhinus stellaris. However, the fish in this study were not induced to be maximally active as necessary for scope determinations, but only to an intermediate routine level of activity. Recently there have been a few studies on the effect of exercise on oxygen exchange and transport in crustaceans as well (16, 21, 33). But the extent of activity has usually not been well defined; and the data are again insufficient to determine limiting factors over an entire scope.

This paper presents data on circulatory and respiratory parameters in the crayfish Pacifastacus leniusculus during low level (routine) activity and forced maximum activity (see 31 for further explanation of these levels). Experiments were performed at the temperature of maximum scope (20°C), as well as at one temperature on either side (10° and 25°C). Results are discussed in terms of limitations to active oxygen uptake by the crayfish; and in terms of oxygen exchange and transport in crustaceans in general.

MATERIALS AND METHODS

Animals. Crayfish (Pacifastacus leniusculus) were captured and acclimated for one month as described previously (31). In the present study groups of crayfish were acclimated to 10°, 20°, or 25°C. Each animal was tested only at its temperature of acclimation.

Blood parameters. Because crayfish hemolymph clots rapidly, it clogs microelectrode and sample chamber assemblies before sufficient time has elapsed for reliable measurements to be made; thus a suitable anticoagulant had to be found. Calcium chelators are effective anticoagulants in crustaceans (37); but they are unsuitable for blood gas measurement studies since altering the calcium concentration of crustacean hemolymph would change the hemocyanin oxygen affinity (20), and thus change the hemolymph sample P_{O_2}. Sulfhydryl blockage by N-ethylmaleimide (NEM) has also been reported to effectively prevent clotting in Limulus polyphemus (6) and a hermit crab (2). NEM was therefore investigated as to its effectiveness as an anticoagulant in crayfish for use in blood sampling and found to be satisfactory. A concentration of about 10mM was found to be necessary to prevent clotting in all individuals. NEM does not alter the oxygen binding curve of hemocyanin. Using a decapod crustacean whose hemolymph does not clot to any significant extent (Callianassa californiensis), samples taken with and without NEM showed no significant difference in either pH or P_{O_2}, providing samples were analyzed soon after obtaining them. pH slowly dropped in all samples, due to spontaneous imide hydrolysis of NEM to N-ethylmaleamic acid (1, 13); but the rate was slow enough, even at 25°C, that no appreciable change occurred

if samples were analyzed within 8 - 10 minutes after withdrawing them. Because of the somewhat nonpolar nature and relatively low water solubility of NEM, the best procedure for capillary sampling with NEM was to rinse the capillary tubes with a 1 M solution (using absolute ethanol as solvent) just before sampling, leaving enough in the tube to give a final concentration of about 10 mM NEM.

In this series of experiments oxygen uptake rate ($\dot{V}_{O_2}$) was measured in a circular chamber equipped with a motorized paddle as described previously (31). Hemolymph was sampled from an individual animal either after the initial 15 minute period in the chamber (routine activity), or after 10 minutes of forced activity. Blood sampling was done in the following way. At the end of the $\dot{V}_{O_2}$ measurement period, the chamber top was flipped up, the crayfish removed, and its cephalothorax covered with a towel. With the abdomen gently held in flexed position, the "arterial" sample of 150 ul was taken into a capillary from the pericardial sinus by puncture of the dorsal membrane between the cephalothorax and abdomen. This was followed by a 150 ul "venous" sample taken from the base of a walking leg. Total time for obtaining the two samples was 10 - 12 seconds after removal of the animal from the water. A third sample was then taken for determination of hemocyanin concentration; the absorbance peak at 337 nm was used for analysis. The arterial and venous samples were analyzed immediately after sampling was completed for pH and P_{O_2} in a Radiometer Blood Gas Analyzer PHM 71 Mk 2. The pH microelectrode (E5021) and oxygen microelectrode (E5046) were

thermostatted to the temperature of the crayfish. Percent saturation of hemocyanin and the oxygen content of hemolymph were calculated using the hemocyanin oxygen binding curves reported in a previous paper (30) and solubilities corresponding to the normal crayfish osmolarity (19). Cardiac output was estimated using the Fick principle.

Heart and ventilation rates. In a separate series of experiments heart and ventilation rates were measured simultaneously on individual, unrestrained crayfish under conditions of routine and forced activity. Although a larger container was used, forced activity produced similar behavioral responses to those observed in the previous section.

Heart rate was calculated from ECG recordings obtained using two electrodes connected via a high gain preamp to a Narco Biosystems Physiograph amplifier and recorder (Model 4). The electrodes were made from very flexible multistranded aluminum wire from which the last 4 mm of insulation had been removed. Two grooves were made transversely half-way into the exoskeleton over the anterior and posterior margins of the heart. The uninsulated leads were then fixed into these grooves with cyanoacrylate glue. A third wire from the water to the preamp acted as ground.

Ventilation rate was recorded from two additional aluminum wire electrodes, using a Narco Biosystems Impedance Pneumograph (Model 4) connected to an amplifier and recorder as above. The wire was stripped of insulation only 1 mm in this case. The end of one wire was hooked around the anterior edge of the right branchiostegite so

that it protruded into the right anterior branchial opening for about 3 mm, with the end facing the scaphognathites. The other wire was inserted into a hole drilled into the mid-lateral area of the right branchiostegite so that it protruded into the mid-branchial chamber. Both leads were again held in place with cyanoacrylate glue. No ground was needed.

The two sets of wires were attached to a cork float to keep the crayfish from becoming entangled in them. After placement of the electrodes, the crayfish were allowed to recover overnight. The following day crayfish were transferred individually to their testing containers. Recordings were made immediately, and again after 20 - 25 minutes. These latter values were considered to be representative of routine activity, as in the experiments where blood parameters were determined. The crayfish was then stimulated to be maximally active for 10 minutes. Recordings were made briefly after 3 and 6 minutes, and again after 10 minutes of activity.

Statistical comparisons were made using Student's *t*-test. Differences were taken to be not significant when $P > 0.05$.

Blood parameters and oxygen consumption ($\dot{V}_{O_2}$) measured during both routine and forced activity, as well as additional parameters calculated from them, are shown in Table 1. A striking feature is the relatively low pericardial P_{O_2} values at all temperatures during both routine and forced activity, accompanied by incomplete saturation of the hemocyanin. In the routine category, at all temperatures, individuals showed $\dot{V}_{O_2}$ values from about one-third maximum to ones approaching standard. Yet only two individuals at 10°C had pericardial hemocyanin saturation values approaching 100%. Since this range of activity is the most prevalent one in crayfish (31), it appears that the crayfish does not normally operate at oxygen tensions high enough to fully saturate its respiratory pigment. In this situation, hemocyanin carries at least 95% of the oxygen delivered to the tissues. As a comparison, several crayfish which were not fed during the one month acclimation period were sampled during routine activity. Hemocyanin concentrations were 3 - 18 mg/ml. Hemocyanin saturation was >96% in all cases, with arterial oxygen tensions approaching 100 torr.

During forced activity the hemocyanin saturation falls even further. The decline is caused in part by a general drop in P_{O_2}; but more important is the drop in pH during activity. A similar decline in saturation and pH has been observed in the blue crab (21). There is a decrease in the amount of oxygen delivered to the tissues (A-V difference) with increasing temperature during forced activity. This decrease is not statistically significant between 10° and 20°C;

but it is highly significant ($P < 0.01$) between 20° and 25°C. The decrease is only partially compensated for by an increase in cardiac output. This appears critical to the decline of oxygen uptake at 25°C.

Another way of looking at the problem encountered at higher temperature is by plotting cardiac output as a function of $\dot{V}_{O_2}$ (Figure 1). This shows how much cardiac output is necessary to maintain a particular oxygen uptake under certain conditions. Contrary to a previous report on Cancer magister (11), for either routine or active animals there is an increase in cardiac output with temperature. There is also an increase from routine to active levels at any particular temperature. The important point is that cardiac output for 25°C active animals is higher but $\dot{V}_{O_2}$ is lower than for 20°C active animals. This means crayfish at 25°C are expending more energy circulating the blood and yet are delivering less oxygen to the tissues.

pH decreases with increasing temperature during routine activity, as has been found for other poikilotherms (15). The pH of 7.63 at 25°C is somewhat lower than expected, though, on the basis of pH values at the other two temperatures.

During forced activity, heart rate increases significantly ($P < 0.05$) between both 10° - 20°C and 20° - 25°C (Table 2). However, with ventilation rate there is a significant increase ($P < 0.05$) only between 10° and 20°C. Between 20° and 25°C there is essentially no change in ventilation.

Although heart rate increases with temperature at routine levels,

the ventilation rate change is rather minor (Table 2). It appears that ventilation is normally minimized. Occasional pauses in ventilation (9, 22) were noted in resting crayfish at all temperatures, the frequency and duration of the pauses decreasing with increasing temperature. Parallel pauses in heart rate were not observed.

Stroke volume of the heart (Table 2) calculated for a hypothetical 25 gm crayfish from the cardiac output and heart rate data showed a noticable increase when going from routine to forced activity. The stroke volume at all three temperatures during forced activity is remarkably similar (about 160 ul), probably representing the maximum possible for a heart from a 25 gm crayfish.

Figure 2 shows a comparison of the effect of temperature on $\dot{V}_{O_2}$, ventilation rate, heart rate, and cardiac output, all during forced activity. The Q_{10} values between 10° and 20°C for $\dot{V}_{O_2}$ and ventilation are very similar (1.76 and 1.75, respectively); and neither show an increase between 20° and 25°C. Q_{10} values for heart rate and cardiac output are somewhat higher, being on the order of 2.0 between 10° and 20°C. Also, while heart rate and cardiac output values still increase between 20° and 25°C, their increase is not as much as one might expect from their performance between 10° and 20°C.

The present study shows that crayfish (Pacifastacus leniusculus) usually do not have arterial P_{O_2} values high enough to fully saturate their respiratory pigment, even under resting or near standard $\dot{V}_{O_2}$ conditions. Since the oxygen affinity of crayfish hemocyanin is high (P_{50}= 6 - 8 torr) (30), this means there is a high oxygen gradient between the hemolymph and external medium. This situation qualitatively agrees with data from Redmond (29) for decapod crustaceans, a study which has been challenged in recent years (16). Studies on several other crustaceans have shown fully or nearly fully saturated respiratory pigments (16, 21, 22, 36, 38). Thus results for Pacifastacus leniusculus suggest that some crustaceans may operate normally at relatively low oxygen tensions, while others operate at higher tensions; and that the discrepancy between Redmond's data (29) and the others may not be due solely to faulty technique. In fact, the situation for crustaceans may be similar to that found in fish. Under resting conditions trout show full saturation of their hemoglobin, with arterial P_{O_2} = 80 torr (35). Tench show slightly less than fully saturated hemoglobin (10); carp are about 75% saturated (12); and eel only 50% saturated (34). This occurs despite the fact that the last three species have relatively high oxygen affinity hemoglobins.

Factors influencing arterial P_{O_2} and hemocyanin % saturation. Data from the present study on Pacifastacus leniusculus emphasize that one must be careful when comparing data from studies on various species of crustaceans, since a number of factors can markedly alter the oxygen

exchange and transport, including the arterial P_{O_2}. Activity by the animal is a key factor. In this study the crayfish were generally not at a true standard condition, but rather in a situation where they exhibited routine activity. It is possible this modest amount of activity contributed to the only partially saturated condition of the hemocyanin. For the blue crab, Mangum and Weiland (21) reported almost fully saturated hemocyanin for resting animals. They noted that these crabs become "unusually quiet" when confined, a response different from that found for crayfish. However, as Mangum and Weiland (21) have suggested, and long-term measurements of $\dot{V}_{O_2}$ in the crayfish have indicated (31), the routine state of activity is the most common state of these decapods. Therefore, the oxygen exchange profile during this routine activity condition may be considered relevant.

Vigorous, maximal activity caused a decrease in both arterial and venous hemocyanin % saturation levels in crayfish, as well as in the blue crab (21). This also indicates that activity state will alter the degree of oxygenation of the hemolymph in crustaceans. Unfortunately, the state of maximal activity has not been adequately defined in most studies.

Data from the crayfish indicate that temperature has a strong influence on oxygenation, at least at routine levels. The hemocyanin is much better saturated at 10°C than at 20° or 25°C, even though the animals were at roughly corresponding levels of activity. Since animals at cooler temperatures become more quiescent than those at

warmer temperatures when left undisturbed for a few hours or more (31), these lower levels of activity might be responsible for the higher saturation of hemocyanin in some cases (16, 22).

Hemolymph hemocyanin concentration appears to alter the oxygen tension and % saturation. Data from a few starved Pacifastacus leniusculus, where hemocyanin levels dropped to 3 - 18 mg/ml, indicate that below about 20 mg/ml the maximum possible amount of oxygen bound to the pigment becomes so low that the animal must have arterial tensions well above the saturation point for the pigment to carry enough oxygen to meet its needs. This might explain the high oxygen tensions found by Zuckerkandl (38) and Taylor and Butler (36), since the crabs in both studies had hemocyanin concentrations of less than about 20 - 25 mg/ml.

The osmotic and ionic problems faced by a freshwater animal may also play a role in how well the hemolymph is oxygenated at the gills (34). To reduce the flux of water and ions, the crayfish gill may have a reduced permeability compared to marine crustaceans; this would cause a reduced permeability to oxygen as well. Furthermore, under well oxygenated conditions ventilation rates in lobsters are greater than heart rates, whereas the reverse is true for the crayfish (23, 33) (Table 2). In aquatic animals such as these, where the hemolymph and respiratory medium are probably not in equilibrium at the respiratory surface, an increase in ventilation accompanied by a relative decrease in blood flow would result in greater oxygenation of the hemolymph. Indeed, McMahon and Wilkens (22) have reported

pericardial oxygen tensions for Homarus americanus of 50 - 60 torr, corresponding to 98 - 100% saturation.

Ventilation during activity. Except at rather low temperatures, accompanied by low $\dot{V}_{O_2}$ values, it seems energetically wasteful for a crustacean to ventilate at rates which would achieve an arterial P_{O_2} beyond the saturation level of its pigment. Energy cost of pumping water over the gills increases exponentially as volume pumped increases arithmetically (17, 27), and ventilatory dead space probably increases as flow rate increases in crustaceans (7, 24). Thus in situations where oxygen demand is higher, such as with increased temperature, it becomes less advantageous for a crustacean to expend the additional energy required to fully saturate the pigment. Above about 90 - 95% saturation the advantages of the pigment in oxygen exchange and transport become minimal, considering the relatively low oxygen capacity blood of crustaceans, so much of the added increment of oxygen will simply be in solution. Data from crayfish support this, where under routine conditions the hemocyanin was noticably better saturated at low temperature (sometimes approaching 97% at 10°C) than it was at 20° or 25°C; and ventilation rates increased less than heart rates or $\dot{V}_{O_2}$.

Characteristics of the ventilatory pump appear crucial in the crayfish during maximum activity. It is clear that the decrease in active $\dot{V}_{O_2}$ from 20° to 25°C results mainly from the inability of the crayfish to increase ventilation (Figure 2). Decrease of oxygen solubility in water between 20° and 25°C undoubtedly amplifies the

problem. Heart rate and cardiac output actually increase between 20° and 25°C, whereas the A-V difference in the blood decreases significantly, with most of the decrease due to lower arterial saturation and oxygen content (Table 1). Since the hemocyanin oxygen affinity remains approximately constant (30), this further suggests that the ventilation is falling behind the circulation.

Between 10° and 20°C ventilation may also be the major limiting factor of the active $\dot{V}_{O_2}$ in Pacifastacus leniusculus since its Q_{10} value is similar to that for active $\dot{V}_{O_2}$, while Q_{10} of heart rate and cardiac output are noticably higher (Figure 2). This would agree with evidence from the lobster Homarus gammarus at 15°C, where active $\dot{V}_{O_2}$ varies directly with P_{O_2} of the inspired water (33). It would, however, contradict the hypothesis that the heart is limiting active $\dot{V}_{O_2}$ below the maximum scope (17); but this hypothesis may still be true for fish since their ventilatory pump gets help from the body musculature via ram ventilation during active swimming.

Circulatory responses during activity. Johansen et al. (16) have reported a cardiac output value of 30 ml/kg·min for the crab Cancer magister at 10°C. Cardiac output values for the crayfish are considerably higher, even at the routine levels of activity (94 ml/kg·min at 10°C, 237 ml/kg·min at 20°C, and 496 ml/kg·min at 25°C). Other recent studies on decapod crustaceans report values closer to those for the crayfish (3, 8, 21, 32). The low value for Cancer magister is probably due to the low test temperature and large body size. A 25 gm crayfish should have a weight specific cardiac output

approximately three times that of a 1 kg crab (32). If this is considered, then the observed values at 10°C of 30 ml/kg·min for Cancer magister and 94 ml/kg·min for Pacifastacus leniusculus are quite comparable.

The cardiac output values found in Pacifastacus leniusculus during forced activity are the highest yet reported for a crustacean (496 ml/kg·min at 10°C, 969 ml/kg·min at 20°C, and 1202 ml/kg·min at 25°C); they are also the only truly active values reported to date for crustaceans. Considering the rather high cardiac output values at routine levels, these high output values are reasonable, though, since cardiac output should increase even more than $\dot{V}_{O_2}$ with activity (17). It would seem, therefore, that this high cardiac output enables the crayfish at attain high active $\dot{V}_{O_2}$ values.

Strategy of circulation in crustaceans. Cardiac output values at warmer temperatures (20°C or higher) for Pacifastacus leniusculus, as well as other decapod crustaceans (8, 21, 32), are on the same order as those for mammals and birds of similar size and at rest; and much greater than for poikilothermic vertebrates (26). This remarkable situation is probably a consequence of the "open" circulatory systems of crustaceans. Recent data on a large number of crustaceans show pulse pressures (which indicate driving force of the heart) of at least 10 mmHg (4); this is about three times less than those found in small mammals, a difference much smaller than previously thought (26). Thus, although the force developed by a mammalian heart is somewhat greater, the crustacean heart still seems capable of providing the

force necessary for a fast circulation. And the discrepancy in force is probably offset by the lower resistance to flow in an open circulatory system.

Development of a fast circulation through relatively large channels, instead of slower flow through smaller bore capillaries as found in fish (whose oxygen demands are somewhat similar to those of crustaceans), probably relates to the rather low oxygen capacity of crustacean blood. Fish have oxygen capacities on the order of 10 vol. %, while for fed, intermolt crustaceans the figure is about 2 vol. %. Also, in crustaceans the hemocyanin concentration occasionally drops to very low levels, during periods of starvation or just after the molt (39), resulting in capacities of about 0.5 vol. %. With such low oxygen carrying ability, crustaceans could not take advantage of the prolonged exchange time at the tissues found in capillary systems, and are better served by an open system with fast circulation of the blood.

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1. Alexander, N.M. Spectrophotometric assay for sulfhydryl groups using N-ethylmaleimide. Anal. Chem. 30: 1292-1294, 1958.

2. Bang, F.B. Cellular aspect of blood clotting in the seastar and the hermit crab. J. Reticuloendo. Soc. 7: 161-172, 1970.

3. Belman, B.W. Some aspects of the circulatory physiology of the spiny lobster Panulirus interruptus (Randall). Mar. Biol. 29: 295-305, 1975.

4. Belman, B.W. New observations on blood pressure in marine Crustacea. J. Exp. Zool. 196: 71-78, 1976.

5. Brett, J.R. The metabolic demand for oxygen in fish, particularly salmonids, and a comparison with other vertebrates. Resp. Physiol. 14: 151-174, 1972.

6. Bryan, F.T., C.W. Robinson, C.F. Bilbert and R.D. Langdell. N-ethylmaleimide inhibition of horseshoe crab (Limulus polyphemus) hemocyte agglutination. Science. 144: 1147-1148, 1964.

7. Burggren, W.W., B.R. McMahon and J.W. Costerton. Branchial water-flow and blood-flow patterns and the structure of the gill of the crayfish Procambarus clarkii. Can. J. Zool. 52: 1511-1518, 1974.

8. Cameron, J.N. and T.A. Mecklenburg. Aerial gas exchange in the coconut crab Birgus Latro with some notes on Gecarcoidea lalandii. Resp. Physiol. 19: 245-261, 1973.

9. Dejours, P. and H. Beekenkamp. Crayfish respiration as a function of water oxygenation. Resp. Physiol. 30: 241-251, 1977.

10. Eddy, F.B. Blood gases of the tench (Tinca tinca) in well aerated and oxygen-deficient waters. J. Exp. Biol. 60: 71-83, 1974.

11. Florey, E. and M.E. Kriebel. The effects of temperature, anoxia and sensory stimulation on the heart rate of unrestrained crabs. Comp. Biochem. Physiol. 48A: 285-300, 1974.

12. Garey, W.F. Cardiac output of the carp (Cyprinus carpo). Comp. Biochem. Physiol. 33: 181-189, 1970.

13. Gregory, J.D. The stability of N-ethylmaleimide and its reaction with sulfhydryl groups. Anal. Chem. 77: 3922-3923, 1955.

14. Horstman, D.H., M. Gleser and J. Delehunt. Effects of altering O_2 delivery on $\dot{V}_{O_2}$ of isolated, working muscle. Am. J. Physiol. 230: 327-334, 1976.

15. Howell , B.J., H. Rahn, D. Goodfellow and C. Herreid. Acid-base regulation and temperature in selected invertebrates as a function of temperature. Amer. Zool. 13: 557-563, 1973.

16. Johansen, K., C. Lenfant and T.A. Mecklenburg. Respiration in the crab, Cancer magister. Z. Vergl. Physiol. 70: 1-19, 1970.

17. Jones, D.R. Theoretical analysis of factors which may limit the maximum oxygen uptake of fish: The oxygen cost of the cardiac and branchial pumps. J. Theor. Biol. 32: 341-349, 1971.

18. Jones, D.R., D.J. Randall and G.M. Jarman. A graphical analysis of oxygen transfer in fish. Resp. Physiol. 10: 285-298, 1970.

19. Kerley, D.E. and A.W. Pritchard. Osmotic regulation in the crayfish, Pacifastacus leniusculus, stepwise acclimated to dilutions of sea water. Comp. Biochem. Physiol. 20: 101-113, 1967.

20. Larimer, J.L. and A.F. Riggs. Properties of hemocyanins. I. The effects of calcium ions on the oxygen equilibrium of crayfish hemocyanin. Comp. Biochem. Physiol. 13: 35-46, 1964.

21. Mangum, C.P. and A.L. Weiland. The function of hemocyanin in respiration of the blue crab Callinectes sapidus. J. Exp. Zool. 193: 257-263, 1975.

22. McMahon, B.R. and J.L. Wilkens. Simultaneous apnoea and bradycardia in the lobster Homarus americanus. Can. J. Zool. 50: 165-170, 1972.

23. McMahon, B.R. and J.L. Wilkens. Respiratory and circulatory responses to hypoxia in the lobster Homarus americanus. J. Exp. Biol. 62: 637-655, 1975.

24. McMahon, B.R., W.W. Burggren and J.L. Wilkens. Respiratory responses to long-term hypoxic stress in the crayfish Orconectes virilis. J. Exp. Biol. 60: 195-206, 1974.

25. Piiper, J., M. Meyer, H. Worth and H. Willmer. Respiration and circulation during swimming activity in the dogfish Scyliorhinus stellaris. Resp. Physiol. 30: 221-239, 1977.

26. Prosser, C.L. Comparative Animal Physiology. Philadelphia: Saunders, 1973. 966 pp.

27. Rahn, H. Aquatic gas exchange: Theory. Resp. Physiol. 1: 1-12, 1966.

28. Randall, D.J., G.F. Holeton and E.D. Stevens. The exchange of oxygen and carbon dioxide across the gills of rainbow trout. J. Exp. Biol. 46: 339-348, 1967.

29. Redmond, J.R. The respiratory function of hemocyanin in Crustacea. J. Cell. Comp. Physiol. 46: 209-248, 1955.

30. Rutledge, P.S. Temperature acclimation response of crayfish hemocyanin. In review to Am. J. Physiol.: Regulatory Integrative Comp. Physiol., 1978.

31. Rutledge, P.S. and A.W. Pritchard. Scope for activity in the crayfish Pacifastacus leniusculus. In review to Am. J. Physiol.: Regulatory Integrative Comp. Physiol., 1978.

32. Spaargaren, D.H. On stroke volume of the heart and cardiac output in aquatic animals. Neth. J. Sea Res. 10: 131-139, 1976.

33. Spoek, G.L. The relationship between blood haemocyanin level, oxygen uptake, and the heart-beat and scaphognathite-beat frequencies in the lobster Homarus gammarus. Neth. J. Sea Res. 8: 1-26, 1974.

34. Steen, J.B. and A. Kruysse. The respiratory function of teleostean gills. Comp. Biochem. Physiol. 12: 127-142, 1964.

35. Stevens, E.D. and D.J. Randall. Changes of gas concentrations in blood and water during moderate swimming activity in rainbow trout. J. Exp. Biol. 46: 329-337, 1967.

36. Taylor, E.W. and P.J. Butler. The behavior and physilogical responses of the shore crab Carcinus maenas during changes in environmental oxygen tension. Neth. J. Sea Res. 7: 496-505, 1973.

37. Wood, P.J. and P.P. Karpawich. Studies on the coagulation process in the crayfish: Attempts to identify a fibrinogen- like factor. Comp. Biochem. Physiol. 42B: 41-48, 1972.

38. Zuckerkandl, E. La teneur en oxygène de l'hemolymphe arterielle de Maia squinado aux divers stades d'intermue. C. R. Soc. Biol. 151: 524-528, 1957.

39. Zuckerkandl, E. Hémocyanin et cuivre chez un crustacé décapode, dans leurs rapports avec le cycle d'intermue. Ann. Inst. Ocean. 38: 1-122, 1960.

Table 1. Blood parameters during routine and forced activity.

T(°C)	n	Hcy (mg/ml)	Sample Site	P_{O_2} (torr)	pH	%Sat Hcy	Total V_{O_2} (ul/ml)	A-V (ul/ml)	$\dot{V}_{O_2}$ (ml/kg·hr)	$\dot{Q}$ (ml/kg·min)
ROUTINE ACTIVITY										
10°	7	66.2 ±10.0	A	8.8 ±1.6	7.95 ±0.04	74% ±6	13.61 ±1.66	7.02	31.5 ±3.0	94 ±20
			V	4.4 ±0.7	7.93 ±0.04	35% ±8	6.59 ±1.71			
20°	6	57.1 ±13.5	A	8.9 ±2.1	7.83 ±0.07	55% ±7	8.66 ±1.75	3.75	43.6 ±5.9	236 ±47
			V	5.0 ±0.7	7.80 ±0.06	32% ±5	4.91 ±1.23			
25°	6	40.1 ±7.7	A	12.1 ±2.8	7.63 ±0.01	64% ±8	7.32 ±0.72	1.71	48.3 ±5.8	496 ±66
			V	7.6 ±1.5	7.60 ±0.01	50% ±7	5.61 ±0.69			
FORCED ACTIVITY										
10°	4	62.2 ±4.3	A	6.2 ±1.1	7.66 ±0.03	36% ±9	5.86 ±0.39	3.42	100.9 ±2.1	496 ±24
			V	3.6 ±0.8	7.62 ±0.04	15% ±5	2.44 ±0.29			
20°	6	45.9 ±6.8	A	11.2 ±0.7	7.44 ±0.06	49% ±3	6.83 ±0.66	2.97	171.3 ±7.0	969 ±44
			V	6.8 ±0.5	7.42 ±0.06	27% ±3	3.91 ±0.55			
25°	4	55.9 ±5.6	A	6.6 ±0.5	7.36 ±0.02	37% ±2	6.15 ±0.59	2.22	159.5 ±4.7	1202 ±67
			V	4.0 ±0.4	7.33 ±0.03	23% ±2	3.92 ±0.63			

Values in columns 3 - 11 are means ± SE. "A" and "V" refer to pericardial sinus (or arterial) and prebranchial (or venous), respectively. "% Sat Hcy" is percent saturation of hemocyanin. "A-V" corresponds to arteriovenous difference, given as ul O_2/ml blood. $\dot{V}_{O_2}$ is oxygen consumption rate; and $\dot{Q}$ is cardiac output.

Table 2. Respiratory and circulatory parameters during routine and forced activity.

Condition	T (°C)	Ventilation Rate (bpm)	Heart Rate (bpm)	Stroke Vol. (ul/beat)
Routine	10°	54.5 ±8.0	41.8 ±4.7	56
	20°	87.8 ±20.0	101.2 ±7.9	59
	25°	72.3 ±16.3	119.3 ±11.4	104
Active	10°	157.0 ±6.2	73.3 ±1.9	169
	20°	273.7 ±14.9	154.7 ±2.3	157
	25°	282.0 ±14.5	191.5 ±3.0	157

Values shown for ventilation and heart rates are means ± SE. 6 crayfish were tested at each acclimation temperature. Stroke volume is calculated for a 25 gm crayfish using heart rates from this table and cardiac output values from Table 1.

Figure 1. Relation between cardiac output ($\dot{Q}$) and $\dot{V}_{O_2}$ during routine activity (R) and forced activity (A) in crayfish acclimated to different temperatures. Animals were run only at their own temperature of acclimation: 10°C (○), 20°C (●), or 25°C (△). Lines indicate ± SE.

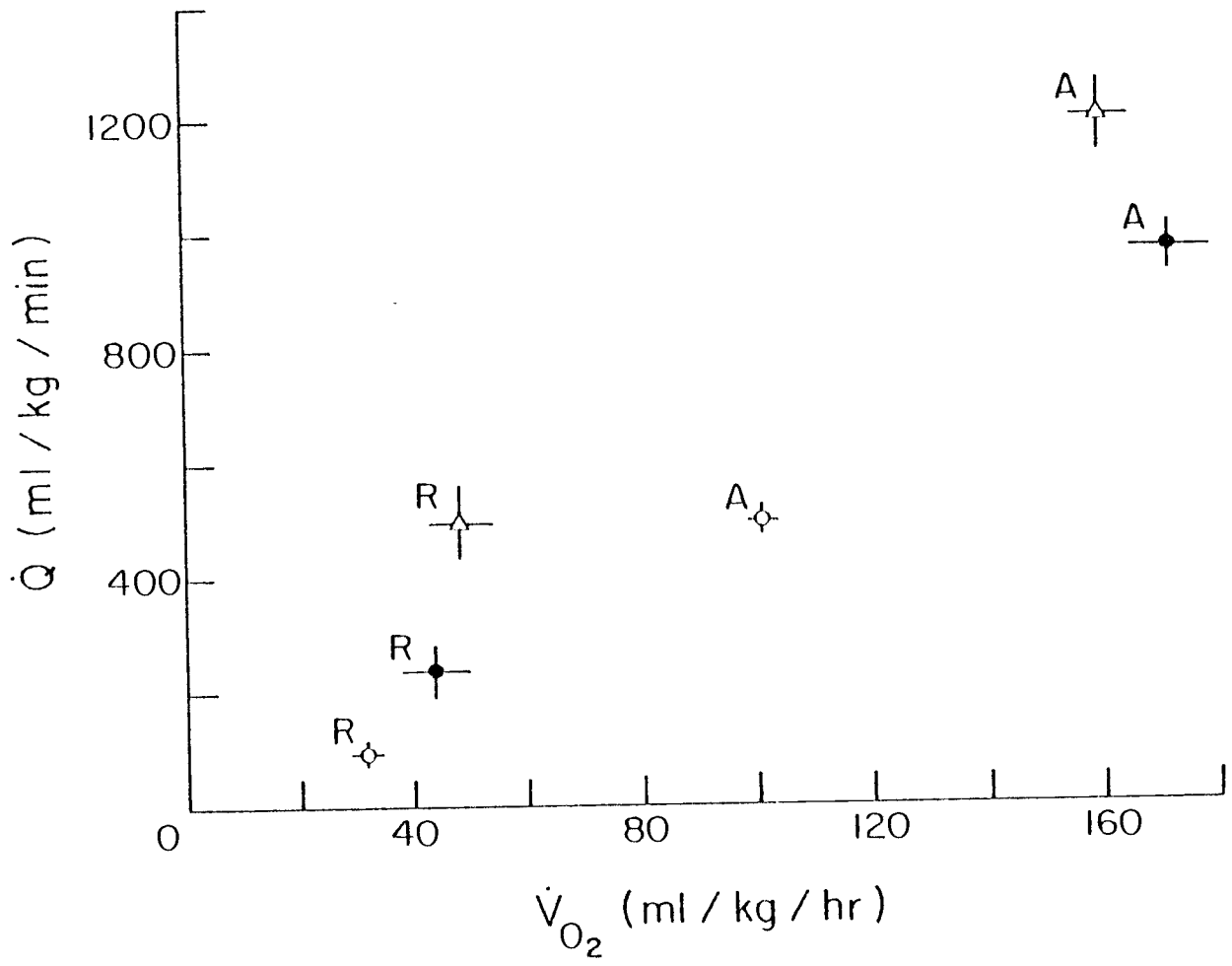
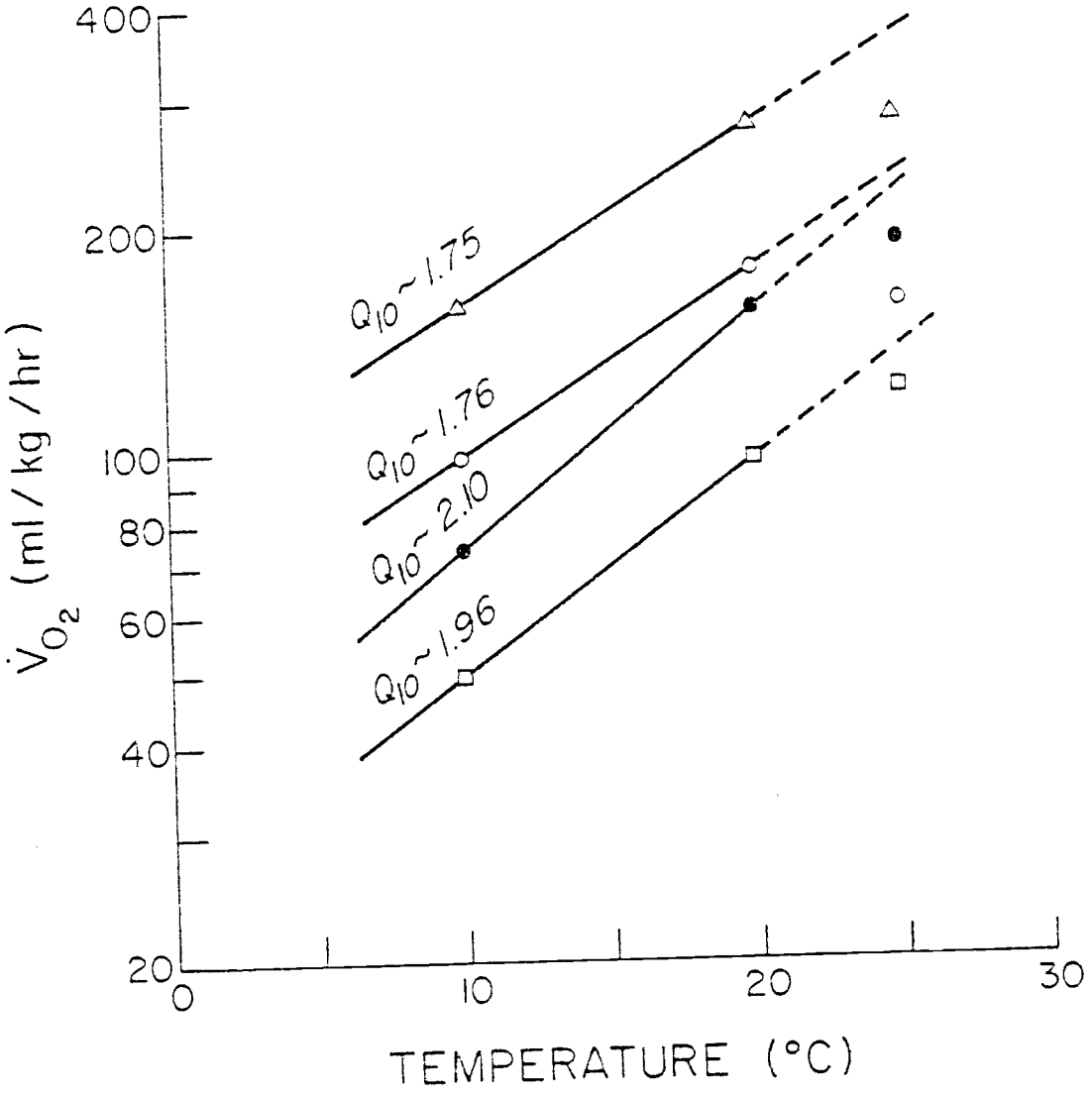

1200
800
400
0
40
80
120
160
$\dot{Q}$ (ml / kg / min)
$\dot{V}_{O_2}$ (ml / kg / hr)
A
A
A
R
R
R

Figure 2. Effect of temperature on respiratory and circulatory parameters during forced activity. Animals were acclimated for one month to the test temperatures indicated. The four parameters are $\dot{V}_{O_2}$ (O), ventilation rate (Δ), heart rate (●), and cardiac output ($\dot{Q}$) (□). Dashed lines are to emphasize the lower than expected values at 25°C. The scale for ventilation and heart rates is the same as shown for $\dot{V}_{O_2}$, with units being beats/minute. For $\dot{Q}$, the scale is 10 X that shown; units being ml/kg·min.


400
200
100
80
60
40
20
0
10
20
30
$\dot{V}_{O_2}$ (ml / kg / hr)
TEMPERATURE (°C)
Q_{10} ~ 1.75
Q_{10} ~ 1.76
Q_{10} ~ 2.10
Q_{10} ~ 1.96