RESPIRATORY RESPONSES TO LONG-TERM HYPOXIC STRESS IN THE CRAYFISH *ORCONECTES VIRILIS*

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INTRODUCTION

Wolvekamp & Waterman (1960) have reviewed an extensive literature dealing with the responses of decapod crustacea to hypoxic stress. This literature is contradictory, with some authors finding oxygen consumption varying with oxygen availability over a wide range of external oxygen tensions as in *Homarus americanus* (Amberson, Mayerson & Scott, 1924), *H. vulgaris* (Thomas, 1954) and *Procambarus simulans* (Larimer & Gold, 1961), while others find a considerable ability to regulate oxygen consumption down to a low critical oxygen level in the external medium, as in *Callianassa* and *Upogebia* (Thompson & Pritchard, 1969), *Panulirus interuptus* (Winget, 1969) and *Cancer magister* (Johansen, Lenfant & Mecklenburg, 1970). Recently Mangum & Van Winkle (1973) have determined the relationship between oxygen consumption and oxygen availability in a large number of non-crustacean invertebrate species and described many degrees of oxygen regulation as well as complete dependency. Such variation can perhaps be expected over a wide range of animal groups but is surprising within the decapods where the presence of a complex branchial and cardiac pumping apparatus would suggest considerable regulatory potential. McMahon & Wilkens (in preparation) observe good regulatory ability in *Homarus americanus*, but suggest that the degree of regulation achieved may be dependent on both·the length of the hypoxic experience and the rate at which the external oxygen tension is changed. Consequently the present authors feel that much of the existing controversy might be explained by the brief nature of some previous experiments. This study utilizes animals which were acclimated to the experimental chamber for 2–3 days prior to a hypoxic experience of up to 12 days to differentiate clearly between hypoxic and other responses.

MATERIALS AND METHODS

Crayfish respiratory performance parameters were measured using an apparatus modified from that of van Dam (1938). It consisted of four identical chambers (Fig. 1) receiving water from a common source. In this manner the responses of four animals could be measured simultaneously.

Water circulating through these chambers was maintained at 18–21 °C (daily

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variation 0·5 °C) and was air-saturated (P_{O_2} = 130 mm Hg) at the start of each experiment. During the experiments the desired oxygen tension of the water entering the posterior chambers was maintained by exposure to a controlled nitrogen flow in a gas-exchange column. The crayfish were inserted through a thin rubber membrane which was mounted so as to divide the experimental chamber into two parts. A thread was passed through a small hole drilled in the rostrum and tied to the chamber to anchor the animal securely. The membrane formed a tight seal around the carapace without compressing the branchiostegite and thus assured that only water passing through the branchial chambers by the action of the scaphognathites entered the forward chamber. Fire-polished glass standpipes in each chamber were adjusted to minimize any differential hydrostatic pressure effects between the chambers. With the crayfish restrained as illustrated, exhalent branchial water passed down the forward standpipe from which it was collected and measured intermittently to determine the branchial irrigation volume (BIV). The oxygen tension of the inhalent water (P_{I,O_2}) was determined from samples of water taken into a glass syringe from the area just above the pereiopods. Exhalent oxygen tensions (P_{E,O_2}) were determined from water samples taken into a syringe via a plastic cannula placed approximately 1 mm from the excurrent canal. In both cases a volume greater than the dead space volume of the syringe and cannula was taken and discarded immediately prior to sampling. Oxygen tensions were measured with a Radiometer (BMS 3) blood-gas analyser. The oxygen electrode was calibrated frequently with air-equilibrated and deoxygenated water, and all measurements were carried out at the same temperature as that of the experimental chambers.

Pressure changes in the epibranchial cavity of the crayfish resulting from the rhythmic movements of the scaphognathite (Wilkens & McMahon, 1972) allowed measurement of branchial irrigation rate (BIR, scaphognathite beats/minute), branchial irrigation amplitude (BIA, force generated by each scaphognathite beat) and the mean negative pressure developed in the branchial cavity (BMNP, an indication of the force available to power the respiratory water flow). These parameters were determined using pressure-sensitive transducers (Hughes, Knights & Scammell, 1969; Wilkens & McMahon, 1972) connected to the epibranchial cavity by plastic cannulae inserted through the carapace posterior and dorsal to the scaphognathite channel. Heart rate (ECG) was monitored by a method essentially similar to that developed by Larimer (1962) from that of Dubuisson (1934) & Ozawa et al. (1955). In the present study an insulated unipolar silver spade electrode was inserted through a small hole drilled through the carapace at a level midway down the areola and to one side of the heart. The electrode was inserted carefully through the hole until it lay alongside the pericardial membrane. Electrical signals between this and an external reference electrode were amplified and filtered by a Grass P15 amplifier prior to recording on an oscillograph.

In order to establish baseline normoxic performance rates prior to hypoxic stress, animals were left undisturbed in the experimental chambers for 3 days or until consistent minimum respiratory rates were observed over a 24-hour period. Thus experiments were performed only on animals which were well acclimated to the experimental conditions. Animals were not fed during the experiment.

The crayfish used were collected in Wisconsin and obtained from commercial
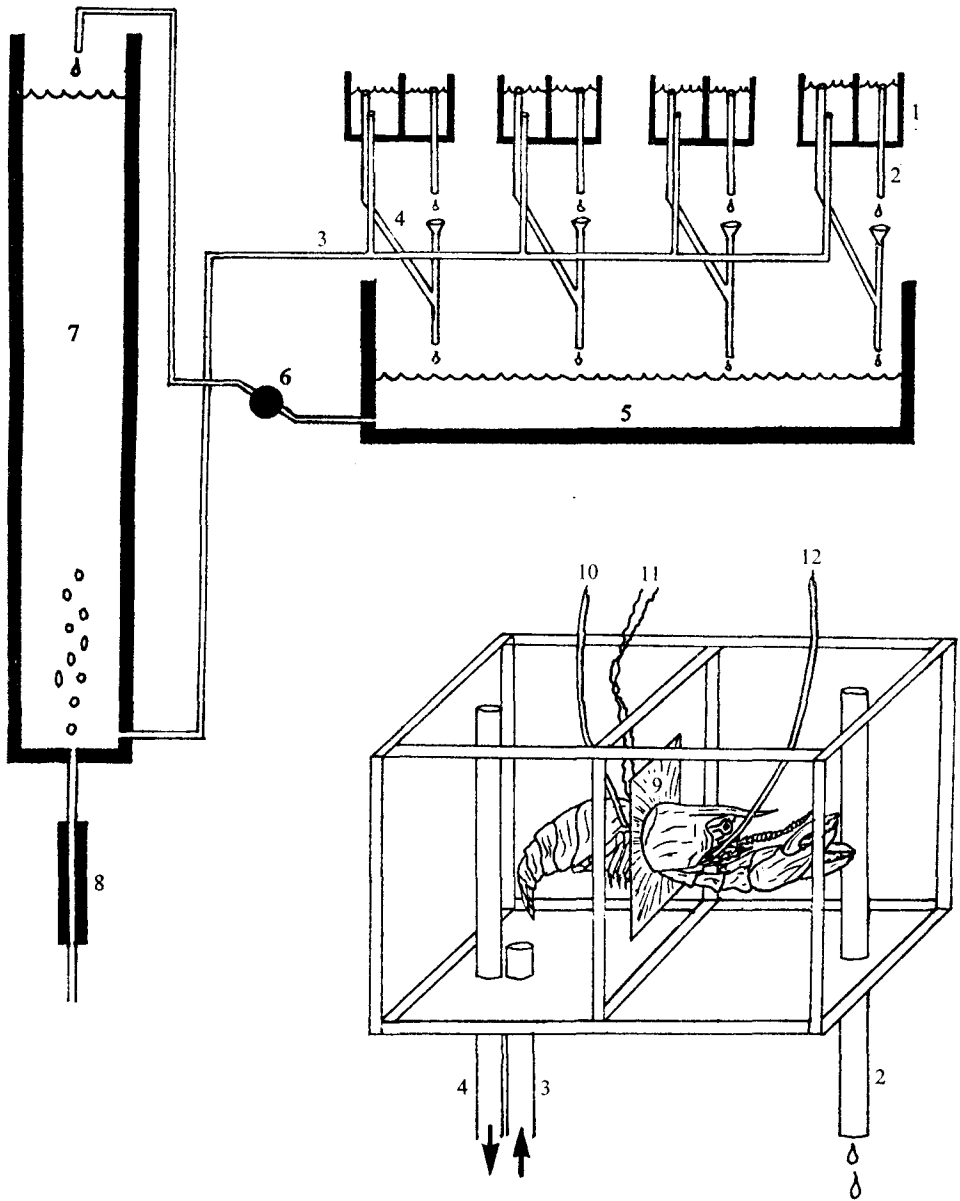
Fig. 1. A diagram of the experimental apparatus. The closed system is based on the circulation of water from a reservoir to the gas exchange column through which the oxygen content of the water is regulated. From the column, water flows into the experimental chambers. That volume of water which has been pumped by the crayfish over its gills and into the forward chamber is returned by the forward standpipe through a measuring station to the reservoir. The remainder of the water in the rear chamber is returned directly to the reservoir by the rear standpipe.

The enlargement of a single experimental chamber illustrates the positioning of the crayfish in the rubber membrane partition as well as the placement of cannulae and ECG leads. The direction of water flow in the standpipes is indicated. (1) experimental chamber, (2) forward standpipe, (3) rear water inlet, (4) rear standpipe, (5) reservoir, (6) water pump, (7) gas exchange column, (8) nitrogen flow meter, (9) rubber membrane, (10) branchial water pressure cannula, (11) ECG leads, (12) P_{E,O_2} cannula.
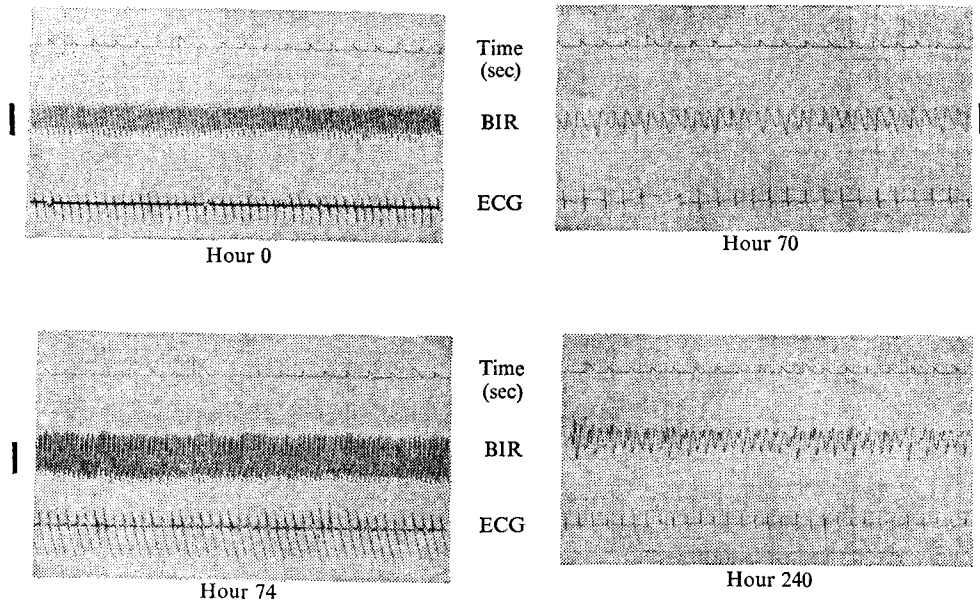
Fig. 2. Records of the ECG and the pressure waveform produced by scaphognathite as recorded from the epibranchial space. At hour o the animal had just been placed in the experimental chamber. At hour 70 the crayfish had been undisturbed for 70 hours. At hour 74 the crayfish had been exposed to an environmental oxygen tension of 60 mm/Hg for 2 h. The animal had been undisturbed in this low oxygen environment for 168 h at hour 240. The vertical calibrations indicate the pressure generated by a 1 cm high water column.

suppliers. The animals were held in air-equilibrated running water at 20 °C and under constant light conditions for a period of at least one month before being transferred to the experimental chambers. Only animals which were apparently healthy and in the intermoult condition were used in the experiments. Equal numbers of males and females were used.

RESULTS

Respiratory performance under normoxic conditions

The pressure waveforms recorded from the epibranchial cavities of *Orconectes* (Fig. 2) were essentially similar to those recorded during forward scaphognathite beating in other crustacean species studied. Reversed scaphognathite beating as described by Hughes *et al.* (1969) for *Carcinus* and by Wilkens & McMahon (1972) for *Homarus* was never seen in recordings from *Orconectes*.

Immediately after the animals had been installed in the experimental chambers branchial and cardiac pumping rates were very high. These values declined sharply during the first 24 h and more slowly for another 48 h so that consistent measurements of respiratory parameters (over a 24 h period) were rarely obtained until 2–3 days had elapsed. Fig. 3 presents the averaged data for 15 experiments while the range and mean levels of raw data are presented in Table 1. During this initial acclimation period the rates declined considerably (BIR to 55%, BIA to 35%, BMNP to 71% and the resulting BIV to 18% of the initial values). Concomitant with
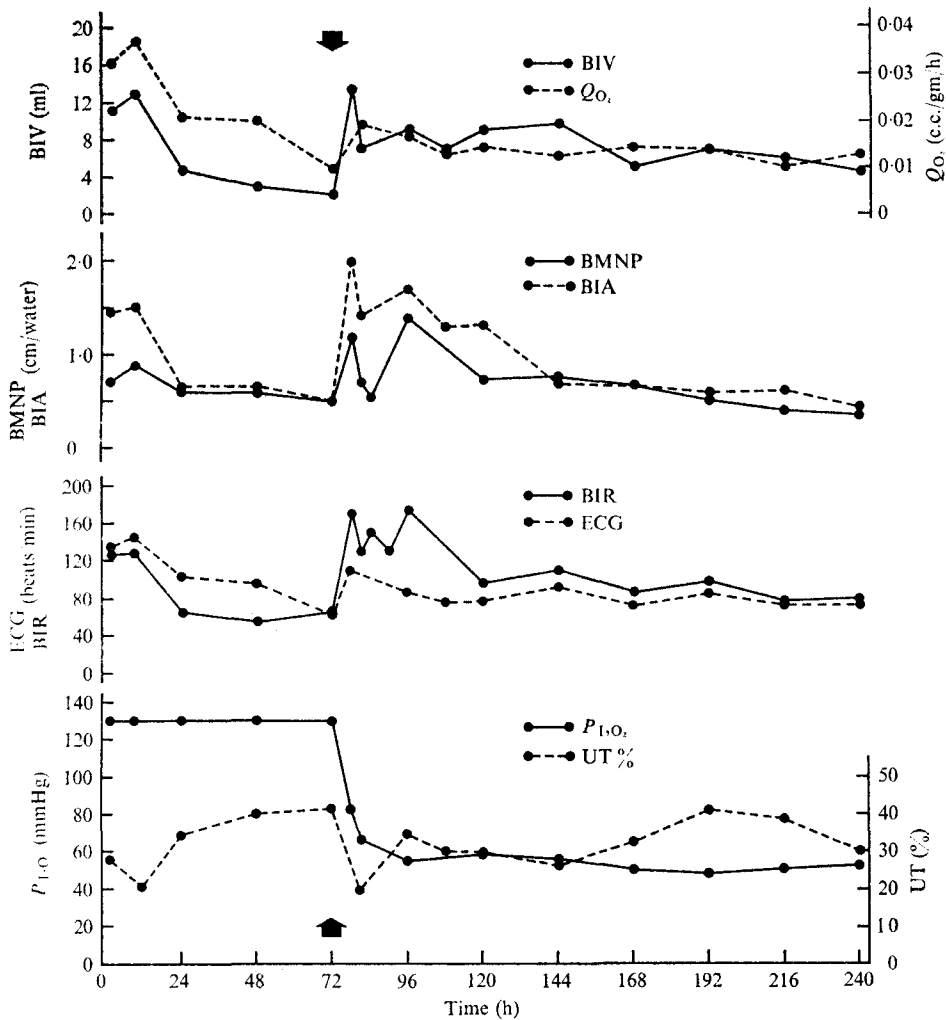
Fig. 3. Respiratory responses of the crayfish during the course of the experiment. All parameters measured are plotted against time. The arrows indicate the onset of hypoxia. The mean values of 15 experimental animals are represented. Branchial Irrigation Volume (BIV), Oxygen Consumption (Q_{O_2}), Branchial Mean Negative Pressure (BMNP), Branchial Irrigation Amplitude (BIA), Branchial Irrigation Rate (BIR), heart beats per minute (ECG), Inhalent Oxygen Tension (P_{I,O_2}), and the Percent Utilization (% UT).

this decrease in branchial pumping the percentage of oxygen extracted from the branchial water flow increased from 20 to 40 % (i.e. a 100 % increase), but oxygen consumption nonetheless decreased to 42 % of its initial value. Heart rate also decreased during this period of normoxic acclimation falling to 51 % of the initial value.

Respiratory performance under hypoxic conditions

Below an ambient oxygen tension of 30 mm Hg both circulatory and respiratory pumping rates decreased rapidly and death resulted from prolonged exposure. Hypoxic experiments were thus usually conducted at 40–60 mm Hg P_{I,O_2}. At these

levels marked increases in both cardiac and branchial pumping parameters were routinely seen as soon as the ambient oxygen tension was reduced (Fig. 3 and Table 1). In acclimated animals these increases were dramatic. Branchial irrigation rate and amplitude increased by 150 and 300 % respectively causing a 130 % increase in the level of negative pressure in the branchial cavity and resulting in an almost 600 % increase in branchial irrigation volume. During the initial hyperirrigation,

Table 1. *The respiratory responses of the crayfish* Orconectes virilis *to normoxic and hypoxic exposure. The means and ranges of 15 experimental animals are represented. Branchial Irrigation Volume* (*BIV*), *Oxygen Consumption* (Q_{O_2}), *Branchial Mean Negative Pressure* (*BMNP*), *Branchial Irrigation Amplitude* (*BIA*), *Branchial Irrigation Rate* (*BIR*), *heart beats per minute* (*ECG*), *Inhalent Oxygen Partial Pressure* (P_{I,O_2}), *and the Percent Utilization* (% *UT*).

	Normoxia				
Hour ...	0	9	24	48	72
P_{I,O_2}	130* (130–130)†	130 (130–130)	130 (130–130)	130 (130–130)	130 (130–130)
% UT	28 (6–47)	21 (8–29)	34 (9–49)	40 (27–55)	41 (30–61)
Q_{O_2}	0·032 (0·026–0·039)	0·037 (0·032–0·045)	0·021 (0·012–0·030)	0·020 (0·012–0·027)	0·010 (0·008–0·020)
ECG	135 (120–153)	145 (134–150)	103 (84–116)	96 (78–108)	72 (55–120)
BIR	127 (54–192)	128 (80–175)	65 (45–84)	56 (42–68)	67 (51–78)
BMNP	0·71 (0·50–1·48)	0·90 (0·41–2·56)	0·60 (0·20–1·01)	0·60 (0·30–0·91)	0·51 (0·44–0·54)
BIA	1·46 (0·75–1·98)	1·50 (0·60–1·99)	0·67 (0·75–1·71)	0·67 (0·44–1·22)	0·50 (0·40–1·60)
BIV	11·0 (4·7–20·0)	13·0 (8·3–18·5)	4·8 (2·2–5·8)	3·0 (1·9–3·2)	2·0 (1·0–2·5)

	Hypoxia				
Hour ...	78	81	96	108	120
P_{I,O_2}	85 (43–89)	66 (45–71)	55 (54–70)	56 (49–66)	59 (40–69)
% UT	25 (21–58)	20 (16–48)	20 (28–57)	30 (21–51)	30 (22–55)
Q_{O_2}	0·017 (0·009–0·020)	0·015 (0·008–0·025)	0·016 (0·011–0·035)	0·013 (0·013–0·024)	0·015 (0·009–0·020)
ECG	110 (72–118)	97 (71–130)	86 (54–119)	78 (66–110)	78 (75–110)
BIR	170 (78–327)	130 (102–270)	175 (84–210)	140 (81–202)	96 (73–150)
BMNP	1·20 (0·77–2·51)	0·70 (0·66–1·63)	1·41 (0·50–2·20)	1·45 (0·67–1·91)	0·70 (0·41–1·01)
BIA	2·04 (0·20–3·05)	1·43 (0·95–1·77)	1·75 (0·10–1·76)	1·30 (0·10–1·43)	1·30 (0·85–1·62)
BIV	13·3 (3·9–20·8)	7·0 (5·1–17·7)	9·1 (2·6–16·0)	7·0 (3·3–18·0)	9·0 (3·7–16·0)

Table 1 *(cont.)*

Hour ...	Hypoxia				
	144	168	192	216	240
P_{I,O_2}	56 (43–85)	50 (39–76)	48 (47–59)	50 (32–65)	53 (32–84)
% UT	27 (17–58)	33 (21–57)	41 (22–58)	38 (22–63)	30 (21–79)
Q_{O_2}	0·012 (0·006–0·011)	0·014 (0·010–0·016)	0·014 (0·012–0·016)	0·010 (0·009–0·014)	0·012 (0·010–0·015)
ECG	86 (75–114)	72 (50–79)	85 (49–120)	72 (49–120)	72 (70–96)
BIR	110 (93–132)	87 (75–96)	98 (86–120)	78 (66–84)	80 (78–144)
BMNP	0·82 (0·40–1·20)	0·69 (0·49–3·02)	0·50 (0·20–1·56)	0·41 (0·20–1·00)	0·45 (0·17–1·68)
BIA	0·72 (0·31–1·55)	0·72 (0·22–1·96)	0·61 (0·10–1·03)	0·60 (0·18–1·08)	0·35 (0·12–0·93)
BIV	9·6 (2·4–11·5)	5·0 (2·4–8·0)	6·7 (4·7–7·0)	6·0 (4·7–10·1)	4·5 (4·3–5·3)

* Mean.   † Range.

percentage oxygen utilization from the branchial water decreased sharply to 20 % (a 50 % decrease) but oxygen consumption, nonetheless, increased by 70 % concomitant with the increased branchial water flow. Heart rate also was increased by 50 % at this time. This rise may have occurred directly in response to hypoxia or indirectly from the increased muscular effort involved in the increased branchial pumping. All the above figures are expressed as percentage gain over the 2- to 3-day acclimated baseline levels. In a few experiments animals struggled intermittently during the initial hypoxic period but most animals generally rested quietly in the chambers.

In the great majority of the animals studied the above responses were not maintained in constant hypoxic conditions but gradually returned towards the pre-hypoxic acclimated levels over a period of 8 days. The time course of this response is shown in Fig. 3. After an 8-day (h 240 in Fig. 3) hypoxic experience the amplitude of the pressure waveform, the mean negative pressure in the branchial cavities, the heart rate and the percentage oxygen extracted from the branchial water had all reached their pre-hypoxic values. However, both the rate of scaphognathite beating and the branchial irrigation volume remained significantly higher. In experiments of 12 days duration these rates had not reached the pre-hypoxic levels. In two animals the rates remained high throughout the hypoxic stress period. Neither of these animals survived the experiment and may have been weakened by operative procedures.

In one preliminary experiment the ambient oxygen tension was reduced progressively ($130 \rightarrow 90 \rightarrow 60$ mm Hg P_{O_2}) at 3- to 6-day intervals (Fig. 4). In this case initial hyperirrigation and some recovery were observed in response to each P_{O_2} reduction.
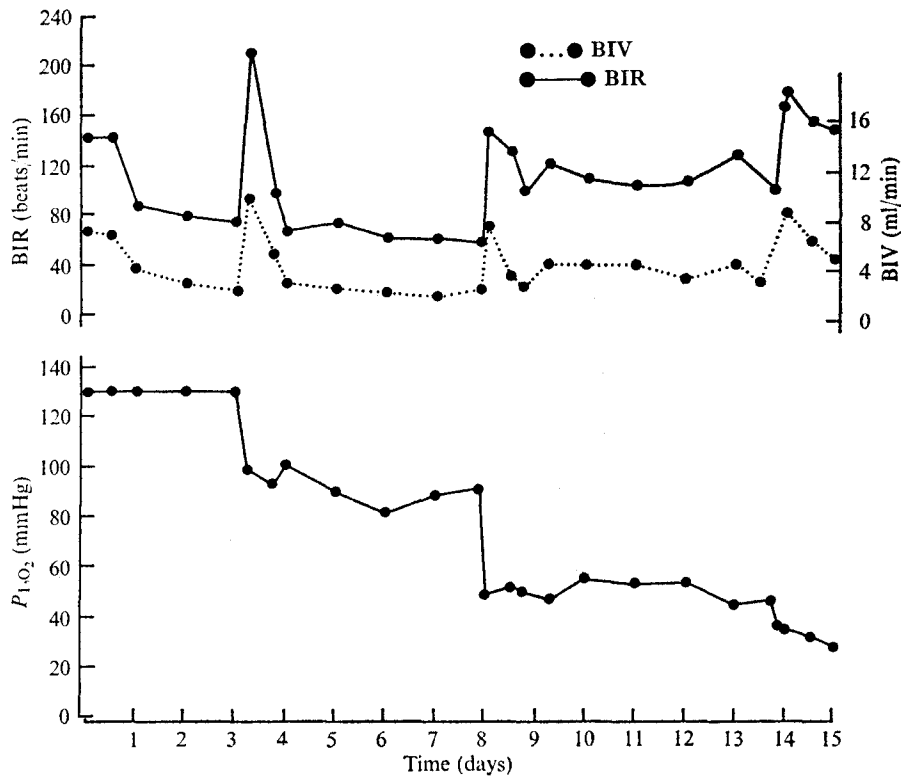
Fig. 4. Respiratory responses of a single crayfish exposed to progressive step-wise decreases in oxygen tension. ●...●, Branchial Irrigation Volume (BIV): ●——●, Branchial Irrigation Rate (BIR). Inhalent Oxygen Tension (P_{I,O_2}).

DISCUSSION

In *Orconectes virilis* the response to chronic hypoxia can be regarded as having two components: initial hyperirrigation followed by a slow recovery to near pre-hypoxic levels (Fig. 3). The initial hyperirrigation and tachycardia increased gill perfusion and irrigation and allowed maintained oxygen consumption from the depleted water, but in themselves consumed much additional oxygen as evidenced by the initial rise in oxygen consumption (Fig. 3, Table 1). In these acclimated animals the branchial irrigation volume was increased 600 % on exposure to low oxygen and it is interesting to speculate on how this response was mediated.

Clearly, changes in external oxygen tension are detected quickly by the animal, and the pacemaker and motor systems responsible for scaphognathite action (Mendelsohn, 1971) increase their output, resulting in hyperirrigation. Both hypoxia and hypercarbia are known to increase the bursting rate of pacemaker neurons in *Aplysia* (Chalazonitis, 1963), and the hyperirrigation response in *Orconectes* may be caused directly by a reduction of oxygen tension acting on the pacemaker cells. Alternatively, preliminary studies in our laboratory (Burggren *et al.* in preparation) indicate the presence of oxygen sensors on the gills of *Orconectes* and these may 'stimulate' the respiratory pacemakers to cause hyperirrigation. Possibly both of these systems participate. At either level the initial response of the sensing cells would be to stimulate

increased scaphognathite activity as soon as the oxygen fell below a certain critical value. In prolonged hypoxic exposure adaptation might occur within the sensing cells so that a new lower set point occurs which allows scaphognathite rate and irrigation volume to decrease. Some substantiation of this hypothesis is seen in Fig. 4 where hyperirrigation and some recovery are seen repeatedly in response to progressive step changes in hypoxia imposed on the animal at 3–6-day intervals.

After 7–8 days of hypoxic exposure respiratory and circulatory pumping rates had all decreased and only branchial irrigatory rate (19 %) and irrigation volume (125 %) remained above pre-hypoxic acclimated levels. The percent ultilization of oxygen from the branchial water also reached pre-hypoxic levels (around 40 %) but as 60 % less oxygen was available, the 120 % increase in branchial irrigatory volume was needed to maintain sufficient oxygen exchange. In essence at this stage the animals pump twice as much water and extract half the oxygen when compared with normoxic adapted levels. However, the substantially reduced oxygen gradient across the gills must impose restrictions on gas transport and we must postulate adaptive changes in other parameters which would increase the efficiency of gaseous exchange and contribute to the observed reduction of branchial pumping. The present study can offer little conclusive evidence as to the nature of these changes but serves as a basis for discussion.

Increased efficiency of the branchial pumping mechanism may occur during prolonged hypoxic exposure. After 7–8 days branchial water flow was increased by over 100 %, but the rate of scaphognathite beating and the mean negative pressure developed were much closer (plus 19 % and 0 % respectively) to pre-hypoxic levels. Possibly the water flow resistance of the branchial system was decreased by realignment of the appendages and other structures forming the incurrent and excurrent apertures. This could reduce oxygen demand by reducing the load on the scaphognathite.

Acclimative changes could also occur in the circulatory system. Increased perfusion of the gills with blood or the opening of previously poorly perfused regions of the gill system coupled with increased branchial water flow could serve to promote gas exchange under hypoxic exposure. Fox (1949) showed increased hemoglobin production in *Daphnia* exposed to hypoxia. Such changes could also be expected to occur in *Orconectes*. Here increased hemocyanin concentration could increase the carrying capacity of the blood or changes could occur in the structure of the hemocyanin molecule. Gruber (1966) in *Helix* and Johnston *et al.* (1967) in *Homarus* have examined the quaternary structure of the hemocyanin molecule and demonstrate at least *in vitro* that hemocyanin can be dissociated into α and β subunits which have significantly different oxygen binding characteristics. If proportions of these dissociated molecules could be changed *in vivo* in response to hypoxia in the crayfish, a change in their relative contribution to the hemocyanin molecule could change oxygen-binding characteristics and increase oxygen uptake at low environmental levels.

Exposure to oxygen tensions below 30 mm Hg P_{O_2} caused serious reduction in branchial and heart pumping rates and eventually death. Above 40 mm Hg P_{O_2} hyperirrigation probably followed by some or all of the adaptive mechanisms discussed above serve to maintain oxygen consumption at pre-hypoxic levels (at least in this restricted experimental environment). These findings are in conflict with those of

Larimer & Gold (1961) for *Procambarus simulans* and of Wiens & Armitage (1961) for *Orconectes immunis* and *O. nais*; they report a decrease in oxygen consumption with decreasing oxygen concentration in these species. The present demonstration that at least 72 h are needed to establish a non-stressed level of respiratory and circulatory performance (routine metabolism) in *Orconectes* may explain how these contradictory results have arisen. Following operative procedures and introduction to the experimental chambers abnormally high levels of respiratory activity are observed. These rates, however, decline rapidly over the first 24 h. Thus, had hypoxic exposure commenced in the first day the initial hyperirrigation response could have been partially or completely masked by the initial stress responses. In other words, the capacity to perform the hypoxic response could be seriously reduced in stressed animals. Also, the reduction in oxygen consumption which occurs naturally as the animals acclimate to the experimental chamber could, if superimposed on a short-term hypoxic exposure, lead investigators to conclude erroneously that the observed reduction was due entirely to the reduced oxygen availability. This might be a logical explanation for the results of Larimer & Gold (1961) who showed reduced oxygen consumption during hypoxia in *Procambarus* despite evident hyperirrigation of the gills. The present study suggests that the results obtained from short-term hypoxic exposures on imperfectly acclimated animals can be misleading and that this may explain the contradictory results recorded for apparently similar species in the literature. Similar reasoning may explain why many previously reported estimations of oxygen consumption (see Wolvekamp & Waterman, 1960 for review) are considerably higher than the levels measured from acclimated animals in the present study.

One other factor could have contributed to the slow reduction in respiratory performance noted in our experiments. Our animals were not fed while in the experimental chamber, and starvation has been shown to be associated with decrease in metabolic rate in many aquatic species (Newell, 1973 for review). Roberts (1957) reports a 60 % reduction in the initial rate of oxygen consumption in 23 days of starvation in *Pachygrapsus crassipes* with the major reduction occurring in the first 7 days. Similar data are reported by Vernberg (1959) for *Uca* and by Thompson & Bayne (1972) for the mollusc *Mytilus*. The magnitude of these responses is rather less than that recorded in our experiments (see Fig. 3, Table 1), and although progressive starvation could have contributed to the gradual fall in oxygen consumption in hypoxia and may have reduced the scope for activity it probably made only minor contribution to the observed response. Starvation did not seem to affect the animals' ability to carry out at least the initial hyperirrigation response as one animal exposed to progressive decreases in oxygen tension at 3-day intervals gave typical hyperirrigation responses and some evidence of acclimation at each step (Fig. 4). In another animal (which may have been weakened by the experimental procedures) branchial irrigation continued at very high levels throughout 12 days of hypoxic exposure. The response to hypoxia described here is similar to the response generally observed in many poikilotherms subjected to sudden temperature change in that an initial overshoot in metabolic activity occurs followed by gradual compensation as acclimation occurs.

Previous studies on the responses of animals to prolonged hypoxic exposure are

essentially limited to work on the high-altitude responses of mammals and in particular man. Thus effective comparison cannot be made with other aquatic animals. However, some comparison can be made with the mammalian responses. In both cases hyperirrigation (hyperventilation) is seen initially, followed by a slow return towards pre-hypoxic levels as acclimation occurs. In mammals the initial hyperventilation is quickly limited by the resultant hypocarbia (Lenfant, 1973). Similar effects could well occur in *Orconectes*, but the technical difficulties of P_{CO_2} measurement in these relatively small animals precluded experimental work on this parameter in the present study. Slow adaption to the hypoxic state is known to occur in mammals exposed to high altitude (Lenfant & Sullivan, 1971), and although this process nears completion only after several years of exposure in man, adaptive changes in anatomical, physiological and biochemical parameters are similar to those suggested here for *Orconectes*.

SUMMARY

1. Changes in the rate and force of scaphognathite beating, irrigation volume, oxygen utilization, oxygen consumption and heart rate during acclimation in response to the experimental conditions and in response to long-term hypoxic exposure have been measured in the crayfish *Orconectes virilis*.

2. Immediately following placement in the experimental chamber the animals exhibited very high levels of respiratory and circulatory performance. These levels decreased slowly and stable minimal performance levels could be measured only after 2–3 days. A 3-day acclimation period under normoxic conditions thus routinely preceded hypoxic experiments to ensure measurement of unmasked hypoxic responses.

3. Two responses to hypoxia were routinely observed: an initial hyperirrigation response maintained oxygen consumption by increased branchial water flow. This response was not maintained, but oxygen consumption remained at pre-hypoxic levels while pumping rates decreased.

4. Possible mechanisms of acclimation to hypoxia are discussed.

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