THE FUNCTION OF HAEMOCYANIN IN THE RESPIRATORY PHYSIOLOGY OF THREE SPECIES OF SOUTH AUSTRALIAN ABALONE (GENUS HALIOTIS)

by

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A thesis submitted to the University of Adelaide in part fulfilment of the requirements for the degree of Doctor of Philosophy.

FEBRUARY 1977

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DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma at any University, and to the best of my knowledge contains no material previously published or written by another person, except where due reference is made in the test of the thesis.

Acknowledgements

I wish to thank my supervisor Dr. Michael Coates for his many suggestions, and patient help throughout this project, both in the laboratory, and in the field. I also wish to thank him for his helpful criticisms of the text.

The South Australian Department of Agriculture and Fisheries supplied me with SCUBA equipment, and Mr. S. Shepherd, and Mr. K. Brandon allowed me to accompany them on numerous field trips to West Island.

Mr. Brandon also took the underwater photographs of the abalone species in this thesis.

Dr. R. Henzell, and Mr. P. Leppard advised me on statistical procedures.

I thank Professor H.G. Andrewartha for allowing me to undertake this research in his department, Professor W. Williams for providing facilities during the period of the production of the thesis, and Professor C. Manwell for his interesting discussions.

I wish also to thank my wife Lindy and my parents for their patience and encouragement during this work.

CHAPTER I

GENERAL INTRODUCTION

1.1.1 Review of Problem

Haemocyanin is a copper containing protein which occurs dissolved in the haemolymph of some arthropod and molluscan groups. In the molluscs haemocyanin is found in the cephalopods (squid and octopi), the amphineurans (chitons) and in the prosobranch and pulmonate gastropods (snails). In the arthropods haemocyanins are found predominantly in the Malacostraca (including all larger forms of crustaceans - crabs, lobsters, woodlice etc.). It is also found in the Xiphosuran Limulus polyphemus (the horseshoe crab) and is recorded in the Arachnida (scorpions and spiders). (Redfield, 1934; Manwell, 1960; Prosser, 1966, 1973; Van Bruggen, 1968; Jones, 1972).

Although haemocyanin's role as a respiratory pigment has been demonstrated in some arthropods, notably decapod crustaceans and Limulus, in the cephalopod molluscs, and in at least one chiton, its role in the oxygen transport system of the gastropod mollusc has not been adequately described.

"Very little is known about the in vivo functioning of gastropod haemocyanins. Respiratory studies on these haemocyanins have been limited almost exclusively to the determination of oxygen equilibrium curves" (Redmond, 1968a).

In this study, as a step in closing this large gap in the understanding of the physiological role of haemocyanins in this group of haemocyanin bearing organisms, the function of haemocyanin in three species of marine archaeogastropods of the family Haliotidae is investigated.

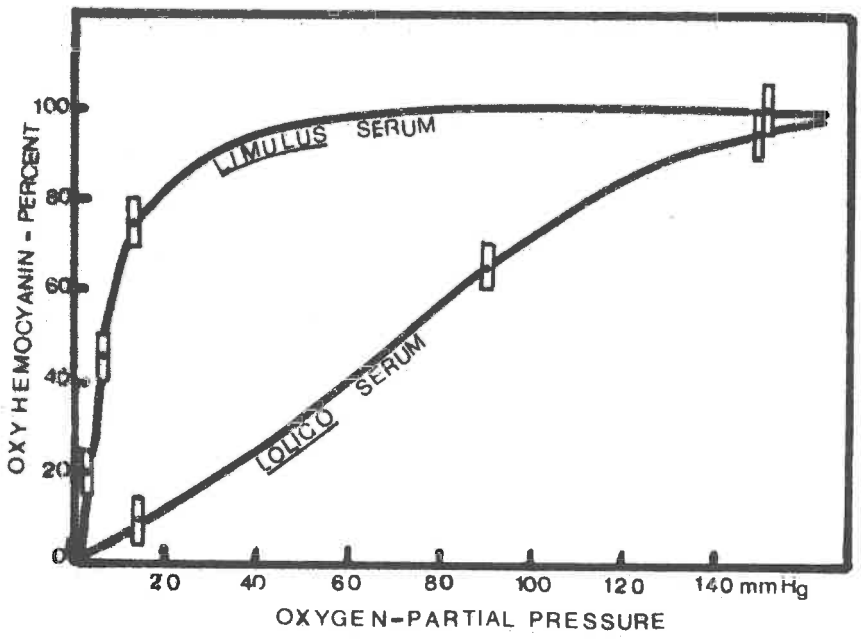
1.1.2 Oxygen equilibrium curves of haemocyanins

The oxygen equilibrium curve of a respiratory pigment is described by plotting the percent saturation of the haemocyanin (% sat) against the partial pressure of oxygen (P_{O_2}). Since the early determination of oxygen equilibrium curves for the haemocyanins of Limulus polyphemus, and the squid Loligo pealei by Redfield, et al. (1925) a large body of data describing such curves for a variety of haemocyanins has accumulated.

Figure 1.1 below shows the oxygen equilibrium curves determined by Redfield, et al. (1925) for the haemocyanins of Limulus and Loligo. It can be seen that the curve for Loligo haemocyanin lies far to the right of that for Limulus haemocyanin.

Figure 1.1

Oxygen equilibrium curves of the haemocyanins of Limulus polyphemus and Loligo pealei (after Redfield et al., 1925)



By convention the oxygen affinity of a given haemocyanin is normally expressed in terms of the P_{50}, the partial pressure of oxygen at which the haemocyanin solution is half saturated. This expression is used because the P_{50} is more accurately determined than the P_{O_2} of 100% saturation (Prosser, 1966). It is apparent that the P_{50} of Loligo haemocyanin - 70 mm Hg as determined by Redfield, et al. (1925) - is considerably greater than the P_{50} of Limulus haemocyanin - 7 mm Hg. Hence a much higher P_{O_2} is required to saturate the squid haemocyanin, than to saturate the haemocyanin of the horseshoe crab. (It should be noted that later determinations of the P_{50} of Loligo haemocyanin give somewhat lower values.)

It has been shown that such equilibrium curves are fairly closely described by the equation

$$Y = \frac{100\left(\frac{P}{P_{50}}\right)^n}{1 + \left(\frac{P}{P_{50}}\right)^n}$$

where Y = % haemocyanin combined and P = the P_{O_2} in mm Hg. This equation is known as the Hill approximation (Manwell, 1960; Prosser, 1966, 1973; Jones, 1972). The slope of $\log \frac{Y}{100-Y}$ plotted against log P, at the point where Y = 50% defines n, the sigmoid coefficient. n gives a measure of the degree of sigmoidicity of the oxygen equilibrium curve - as n increases so does the sigmoid nature of the curve (cf. Jones, 1972 figure 8.3). This sigmoidicity of a given oxygen equilibrium curve depends on the interactions between subunits of the respiratory pigment (Manwell, 1960; Jones, 1972; Prosser, 1973).

It can be seen then, that the position, and shape of an oxygen equilibrium curve is defined by two constants, P_{50} and n, respectively.

Factors which may modify the oxygen equilibrium curve of a given haemocyanin

There are two major factors which may modify the shape and position of the oxygen equilibrium curve of any haemocyanin.

Effect of CO_2 on oxygen affinity - the Bohr effect

In their early paper Redfield and Hurd (1929) described the effect of various partial pressures of CO_2 (P_{CO_2}) on the oxygen equilibrium curves of various haemocyanins.

They found in the case of Loligo blood that increasing the P_{CO_2} decreased the affinity of the haemocyanin for oxygen. This is analogous to the normal Bohr effect observed in human blood in the presence of CO_2. (This effect is also sometimes called the negative Bohr effect - as defined by Jones (1972)).

The Limulus haemocyanin demonstrated an entirely new characteristic when CO_2 was added to the gas mixture. The affinity of the pigment for oxygen was increased in the presence of CO_2. This phenomenon is now known as the reverse (or positive) Bohr effect. The Bohr effect or reverse Bohr effects are now known to be consequences of pH changes brought about by the varying P_{CO_2} and can be induced by other acidic metabolites (cf. Jones, 1972). The magnitudes and directions of the Bohr effects of the haemocyanins of the various haemocyanin bearing groups are described in more detail in section 2.2.11.

The role which the Bohr effect plays in the oxygen transport systems of specific organisms is discussed further later in the introduction.

Temperature

In early studies (e.g. Redfield and Ingalls, 1933) it was demonstrated that the temperature at which the oxygen equilibrium of a given haemocyanin was determined affected the relationship between % sat

and P_{O_2}. With increased temperature the oxygen equilibrium curves of haemocyanins were shifted to the right. Accumulated studies have shown that this is generally the case (Jones, 1962), although the degree to which temperature affects the P_{50} of a haemocyanin varies considerably between organisms (Redmond, 1955, 1968a; Young, 1972).

The possibility that temperature sensitivity of a haemocyanin, as recognised by the change in P_{50} for a given temperature change, may be the result of adaptation to a particular temperature environment is discussed in some detail by Redmond (1968a) and Young (1972) and is referred to again in section 2.3.11 of this thesis.

Other factors which may influence the oxygen equilibrium curve of haemocyanin

The form of the oxygen equilibrium curve of a haemocyanin can be considerably influenced by the salt environment of the blood (cf. Jones, 1972). This has lead to the fact that many oxygen equilibrium curves determined on haemocyanins in dialysed solutions bear little or no relationship to the oxygen equilibrium curves of unaltered haemolymph (section 2.3.13).

Oxygen equilibrium curves of haemocyanins must be determined under conditions which are controlled with respect to factors such as temperature, P_{CO_2}, and salt environment of the blood

1.1.3 Oxygen capacity of haemocyanin

The oxygen capacity is the amount of oxygen carried in the blood at saturation. Oxygen capacity is usually expressed in terms of volumes of oxygen per 100 volumes of blood (vol %). Using a modified Van Slyke apparatus Redfield et al. (1926) determined the oxygen capacities of the blood of Loligo, Limulus, Busycon and Cancer. They

also determined the amount of oxygen carried in solution in these haemocyanin bearing bloods, using a method devised by Stedman and Stedman (1925). This method was simply based on comparing the O_2 content (Vol %) of a blood sample at two P_{O_2} where the haemocyanin was 100% saturated - any increment in O_2 content at the higher P_{O_2} was therefore due to dissolved O_2 alone. They found that at a given P_{O_2} the amount of oxygen in solution in the haemolymph was about 90% of that dissolved in seawater under the same conditions. Knowing this, and knowing the total amount of oxygen carried by the blood Redfield *et al* were able to deduce the amount of oxygen actually bound to the haemocyanin. Their O_2 capacity data is summarised on Tables II and III of their paper. At high P_{O_2} (153-749 mm Hg) the bloods of all species yielded much more oxygen than could be accounted for by solubility alone. By far the greatest percentage of oxygen in the blood is therefore bound to the haemocyanin.

Redfield *et al.* (1926) also noted that although there were obvious differences in O_2 capacities between the species, *Loligo* blood having a higher O_2 capacity than *Limulus* blood, there was also considerable variation within a species, particularly in *Limulus* blood. They attributed this variation to observed variation in the haemocyanin contents of the blood.

More recent determinations of O_2 capacities of haemocyanin have yielded results which are generally similar to the early determinations of Redfield *et al.* (1926) for the various groups of haemocyanin bearing organisms, e.g. cephalopods (Wolvekamp, 1942; Lenfant, 1965; Johansen, 1965), decapod crustaceans (Redmond, 1955, 1962a, 1968b; Lenfant *et al.*, 1970), and gastropod and amphineuran molluscs (Redmond, 1962b). In Chapter 2, section 2.3.2, a

detailed comparison of the O_2 capacities of the various classes of molluscs is made, and the relationship between O_2 capacity and haemocyanin concentration is discussed in the light of results obtained in this study.

1.1.4 Function of haemocyanin in the living organism

For a haemocyanin to function effectively as a respiratory pigment its oxygen affinity must be such that it loads with oxygen at the respiratory surface and unloads at the metabolising tissues (cf. Redfield et al., 1925, 1926). Moreover, the oxygen delivered to the tissues by the haemocyanin must significantly contribute to the organism's overall oxygen needs.

The understanding of the in vivo functioning of haemocyanin involves relating the characteristics of the haemocyanin (oxygen equilibrium curves, O_2 capacities) to the gas tensions within the living animal.

For those groups of haemocyanin bearing animals for which the physiological role of haemocyanin as a respiratory pigment has been adequately described, the in vivo oxygen tensions of the organisms have been described. The internal gas tensions of gastropod molluscs are however largely unknown, and it is this fact which has resulted in the lag in the understanding of the physiological role of haemocyanins in these organisms compared to some other groups.

Before discussing in more detail the state of knowledge of haemocyanin function in gastropod molluscs, a brief summary of the in vivo respiratory role of haemocyanin in other organisms will be given.

1.1.41 Cephalopods

As early as 1878, Fredericq had shown that it was relatively easy to cannulate the blood vessels of the cephalopod molluscs. Redfield et al. (1929), took blood samples directly from the systemic

ventricle (arterial), and posterior sinuses (venous) of living specimens of the cephalopod Loligo pealei, and analysed them for O_2 and CO_2 content using Van Slyke apparatus. Redfield et al. (1926) had determined oxygen equilibrium curves and CO_2 equilibrium curves of squid blood. Combining these data, Redfield et al. (1929) found that for animals in which fresh seawater was washing the gills, the mean arterial O_2 content was 4.27 vol %, corresponding to a P_{O_2} of about 120 mm at the mean CO_2 content of 3.98 vol % (= 2 mm Hg P_{CO_2}). The mean venous O_2 content was .37 vol % corresponding to a P_{O_2} of about 48 mm Hg at the mean CO_2 content of 8.27 vol % (P_{CO_2} = 6 mm).

Knowing the O_2 capacity of the blood, Redfield et al therefore demonstrated that for a squid in aerated conditions

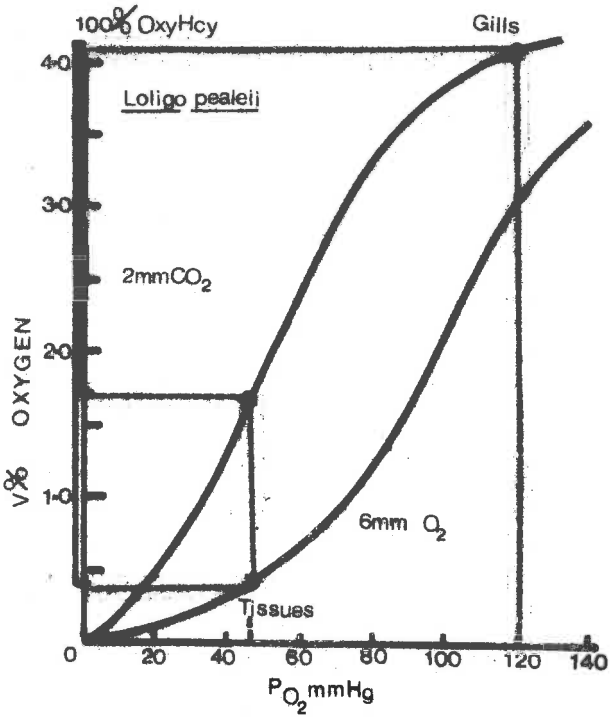
1. the blood is saturated with oxygen at the gills
2. the venous blood has given up nearly all its dissociable oxygen even though the P_{O_2} is still 48 mm Hg and
3. in increasing from 3.98 vol % to 8.27 vol % CO_2, the corresponding P_{CO_2} has only increased 4 mm.

Jones (1965), Figure 3, Redmond (1968a), Figure 1, and Jones (1972), Figure 9.14 are diagrammatic representations of the O_2 transport by the blood of the squid Loligo pealei, constructed from the data of Redfield et al., (1929). Figure 2 below shows this O_2 turnover, as given by Redmond (1968a). It can be seen that the difference in position of the oxygen equilibrium curve between the arterial, and venous blood due to the Bohr effect associated with a 4 mm Hg increase in P_{CO_2}, causes the amount of oxygen to be given off to the tissues to be considerably increased compared to what it would have been in the absence of such an affect.

Figure 1.2

Oxygen transport by the blood of Loligo pealei

(after Redmond 1968a)



In fact, quoting the figures of Redfield et al. (1929), in the course of a respiratory cycle 3.9 vol % oxygen is given up to the tissues. If CO_2 were not produced during the course of the cycle, and the position of the oxygen equilibrium curve had not changed, only 2.3 vol % O_2 would have been given up.

> "The differences between this value and the 3.9 vol % of the normal cycle, or 1.6 volumes per cent, represents the oxygen exchange due to the influence of carbon dioxide on the system".

Redfield et al. then demonstrate that this oxygen supplied by the haemocyanin is necessary to sustain life in Loligo. They conducted a series of experiments in which animals were placed in oil sealed seawater aquaria enriched with various concentrations of CO_2. They found that with increased concentrations of CO_2, higher concentrations of oxygen are

required for survival of the squid. Because of the Bohr effect, increased concentration of CO_2 in the seawater caused the O_2 affinity of the haemocyanin at the gills to be decreased so that the haemocyanin could not be sufficiently saturated to supply the animals oxygen needs. Death of the squid occurred when the P_{O_2} and P_{CO_2} in the environment were such that the O_2 content of the blood was limited to between 1.5 and .5 vol %. As will be seen, for the relatively inactive gastropod and amphineuran molluscs, and for some crustaceans, this is not necessarily a very low value; however in the extremely active squid this is only a fraction of the oxygen normally bound by the haemocyanin.

Jones (1963) summarised data from two early studies on the in vivo function of haemocyanin in another cephalopod Octopus vulgaris (Fredericq, 1911; Winterstein, 1909), and concluded from the limited data available that the respiratory cycle of Octopus vulgaris was similar to that of Loligo "except that it works at a relatively low arterial oxygen tension and the Bohr effect must be much less important". The more recent series of papers describing the oxygen equilibrium characteristics and in vivo conditions of Octopus dofleini (Johansen, 1965; Lenfant and Johansen, 1965; Johansen and Lenfant, 1966) has supported the early determinations with Octopus vulgaris, and verified Jones's deductions from them. Table I, presents a summary of the parameters describing the role of haemocyanin in the delivery of oxygen in Octopus vulgaris (Jones, 1963) and in Octopus dofleini (Johansen, 1965; Johansen and Lenfant, 1966), with the data of Redfield and Goodhind, (1929) for Loligo as a comparison.

In both species of Octopus it is apparent that the arterial P_{O_2} is considerably lower than in Loligo. However the arterial haemocyanin of Octopus dofleini is still highly saturated, although not always approaching 100% saturation as proposed for Loligo.

Table 1.1

Oxygen delivery by haemocyanin in cephalopods

Species	T°C	Arterial P_{O_2} mm Hg	Venous P_{O_2} mm Hg	Arterial haemocyanin % sat	Venous haemocyanin % sat	Arterial O_2 content vol %	Venous O_2 content vol %	O_2 delivery A-V	Arterial P_{CO_2} mm Hg	Venous P_{CO_2} mm Hg	Arterial CO_2 vol %	Venous CO_2 vol %	Venous P_{50} mm Hg	Arterial P_{50} mm Hg	Source
Loligo pealei		120	48.0	100*	7*	4.27	.37	3.9	2	6	3.98	8.27	90*	55*	Redfield & Goodkind 1929
Octopus vulgaris		55-85				4.6	.4	4.2			3.9	6.6			Jones 1963
Octopus dofleini	11	67.9	9.4	89.2	18.8	3.43	.72	2.71							Johansen 1965
Octopus dofleini	11	77.5	9.7	82.4	9.6	3.3	.4	2.9	3.1	4.5	4.2	7.6	+55	+37	Johansen & Lenfant 1966

* Approximate values from Fig. 1.2

+ Approximate values from Fig. 5 Johansen & Lenfant 1966

The venous P_{O_2} for Octopus dofleini is considerably lower than that of Loligo. However, although there is a considerable arterio-venous gradient in CO_2 content resulting in an increase in P_{CO_2} from 3.1 mm Hg in arterial blood to 4.5 mm Hg in venous blood, the Bohr effect in Octopus haemocyanin is not nearly as pronounced as in Loligo haemocyanin. This results in the fact that, despite the higher venous P_{O_2} in the latter species, the % saturations of the venous blood of Octopus and Loligo are quite similar (Table 1.1).

Taking into account species differences in such factors as oxygen capacities of the haemocyanins it would appear that the general form of O_2 delivery by haemocyanin is fairly similar in all cephalopods described, and closely approaches that proposed in Redfield and Goodkind's (1929) classical study on Loligo haemocyanin.

Cardiac output in Cephalopods

Johansen (1965) measured the oxygen consumption of his Octopus dofleini specimens. He then related the oxygen delivered by the blood in a single circulation (A-V difference vol % O_2) to the oxygen consumption using the Fick principle.

This principle, discussed in more detail in Chapter 3 is expressed by the equation

$$\text{Cardiac output ml/min} = \frac{O_2 \text{ consumption ml/hr} \times 100}{\text{A-V difference vol \%} \times 60}$$

Johansen determined that the average cardiac output necessary for Octopus dofleini to maintain its oxygen consumption, given the measured A-V difference, was 10 ml/kgm/min.

It is interesting to note that this calculated value compares closely to that measured directly by Redfield et al. (1929) by cannulation of the ventricle of a Loligo pealei heart (11 ml/kgm/min).

Johansen (1965) also determined the heart rates of the individual Octopus specimens, and hence deduced the stroke volumes necessary to maintain the calculated cardiac outputs. These data are discussed more fully in Chapter 3, in the light of results obtained in this study.

1.1.42 Decapod crustaceans

Redmond (1955), described in detail the function of haemocyanin in the decapod crustaceans Panulirus interruptus, Loxorhynchus grandis, and Homarus americanus.

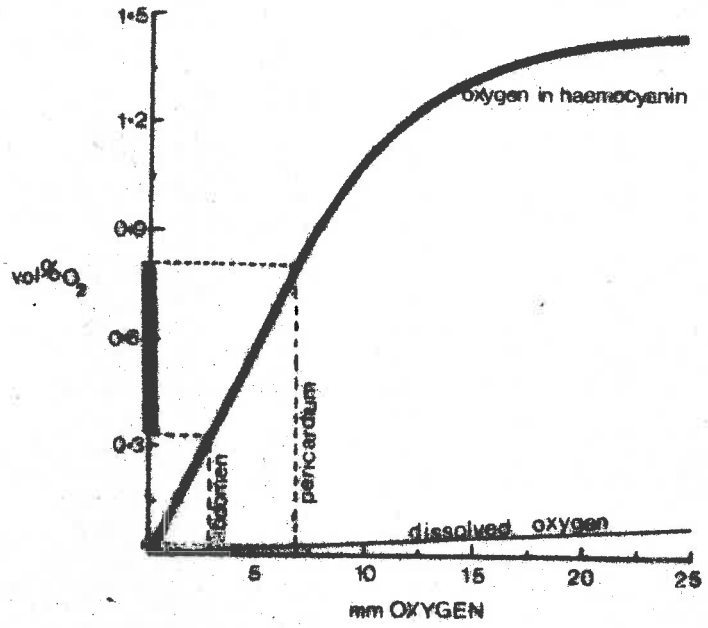
The detailed examination of the role of haemocyanin in Panulirus is representative of his findings.

Redmond finds that Panulirus haemocyanin demonstrated a normal Bohr effect in response to increased P_{CO_2}. However, as this effect was much less pronounced than in the cephalopods, and as the pH difference between freshly drawn arterial and venous bloods was very slight, he felt justified in concluding that the Bohr effect would have little role in the off-loading of O_2 to the tissues.

From the position of the oxygen equilibrium curve, the arterial and venous O_2 contents, the oxygen capacity of the haemolymph, and the amount of O_2 in solution (Redfield et al. 1926) Redmond deduced the % sat of arterial and venous blood samples of the Panulirus. Redmond found that even immediately post branchial blood was only about 50% saturated; from this it can be deduced that the arterial P_{O_2} is only about 7 mm Hg, although the P_{O_2} of the surrounding water is well over 100 mm Hg. The venous blood was slightly more than 20% saturated, with the venous P_{O_2} being about 3 mm Hg (see Figure 1.3). For the average Panulirus, oxygen capacity about 1.5 vol %, it can be seen that despite its unsaturated condition the haemocyanin delivers about .45 vol% O_2 to the tissues, about 26 times as much O_2 as that delivered in solution over the same range of P_{O_2} values.

Figure 1.3

Oxygen delivery by the blood of Panulirus interruptus (after Redmond, 1955)



Cardiac Output of theoretical Panulirus, based on Redmonds A-V vol % data

Using theoretical values for oxygen consumption derived from the data of Weymouth et al. (1944) for a series of other decapod crustaceans, Redmond calculated the cardiac output of a 750 gm Panulirus to be 60 ml/min (80 ml/kgm/min) according to the Fick principle.

He then used a theoretical heartrate for Panulirus, derived from Burger and Smythe's (1953) study on the circulation of another decapod Homarus americanus, to calculate a theoretical stroke volume for Panulirus. As this calculated stroke volume was of the same order as that roughly measured by Burger and Smythe, Redmond concluded that the premises of his calculation were correct.

Following Redmond's (1955) description of haemocyanin function in three species of decapod crustacean, a series of studies supporting his findings of low arterial P_{O_2}, and unsaturated condition of arterial

haemocyanins in these animals were carried out. A summary of this data is given by Jones (1972) pg 104 Table 9.2. This Table is reproduced below on Table 1.2.

Table 1.2

Summary of earlier work on oxygen exchange in decapod crustaceans (after Jones, 1972)

		P_{50} mm	Oxygen capacity vols % Bound	Soln.	T °C	*in vivo* O_2 content Total O_2 vols%	Hcy %satn.	P_{O_2} mm	% O_2 as O_2Hcy	*in vivo* CO_2 P_{CO_2} mm	A-V pH	Author
Panulirus interruptus	A	6	1.53	0.46	15	0.82	54	7.0	96	5.0	.02	Redmond 1955
	V					0.35	22	3.0	97	5.3		
Loxorhynchus grandis	A	5	0.58	0.45	14	0.41	68	8.0	90	18.0	.01	"
	V					0.17	30	3.0	94	18.5		
Homarus americanus	A	5	0.86	0.45	14	0.44	49	5.3	95	2.3	.03	"
	V					0.18	20	2.3	95	2.5		
Cardisoma guanhumi	A	4	2.43	0.40	29	1.66	68	5.7	99			Redmond 1962a
	V					0.90	33	3.2	99			
Gecarcinus lateralis	A	18	1.72	0.45	26	1.45	81	29.0	94		.06	Redmond 1968b
	V					0.61	36	14.0	94			
Maia squinado	A	19	0.74		21	0.54	61	24.4	84	1.3		Spoek 1962
	V					0.33	33	18.3	81	3.1		
Homarus gammarus	A	10	0.94		20	0.61	67	14.9	91	3.3		Spoek 1962
	V					0.31	29	8.2	89	3.7		

Despite this accumulation of evidence as to the low *in vivo* O_2 tensions persisting in decapod crustacean, recent studies have raised serious doubts as to the accuracy of the above determinations.

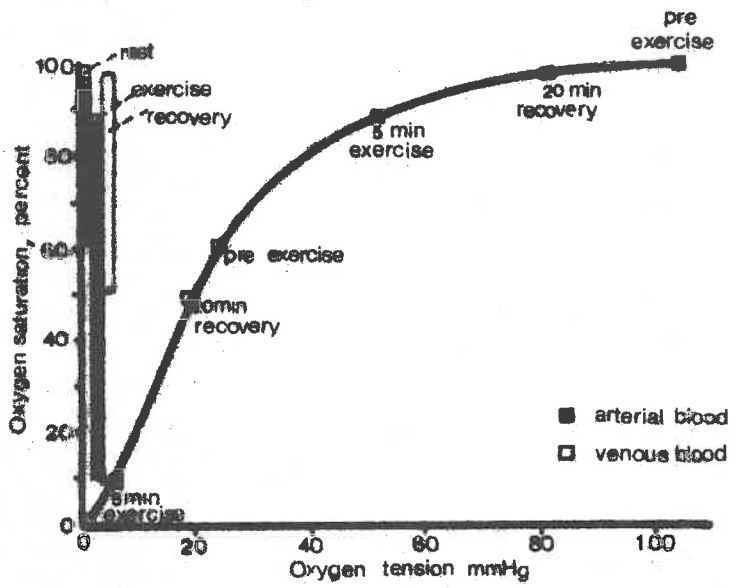
Johansen *et al.* (1970) sampled pre and post branchial blood of the large crab *Cancer magister*, using indwelling catheters in free moving animals. Their analyses indicated that the prevailing arterial P_{O_2} values in these crabs ranged from 65 to 107 mm Hg. This means that the arterial blood is always 90 to 100 % saturated. The venous blood, however, in the resting animals had a much greater % saturation than in previous studies on decapod crustaceans, in some cases up to 70% saturated. When the crabs became active, although the arterial P_{O_2} fell, this made little difference

to the % saturation of the arterial haemocyanin (see Figure 1.4), whereas the drop in venous P_{O_2} lowered the % sat. of the venous haemocyanin quite drastically to 17%. After a recovery period the arterial and venous P_{O_2} once again approached the resting values. Johansen et al. regard this capacity of the venous P_{O_2} to vary, causing a large drop in venous % saturation, as a potential O_2 store for use during activity.

Figure 1.4

Oxygen delivery by the haemocyanin of Cancer magister

(after Johansen et al., 1970; Figure 9)



Cardiac Output of Cancer magister

Johansen et al. (1970) use their A-V difference, and a measurement of O_2 consumption of Cancer magister to calculate the cardiac output of this crab according to the Fick principle.

They obtained a value of 29.5 ml/kgm/min. This value is considerably smaller than that obtained by Redmond (1955) for Panulirus,

and Johansen et al. (1970) are critical of Redmond's value as being excessively large for an invertebrate.

Johansen et al's (1970) brief examination of two other crab species, and the recent work on other decapod crustaceans (McMahon and Wilkens, 1972; Taylor et al., 1973) indicate that many decapod crustaceans may have high arterial P_{O_2} and indicate that the earlier studies (Table 1.2) may have quite markedly underestimated arterial P_{O_2}.

However recently Mangum and Weiland (1975) have presented evidence that, contrary to the suggestion of Johansen et al. (1970) not all decapod crustaceans have high arterial P_{O_2}. In the small active crab Callinectes sapidus arterial P_{O_2} was found to be 35 mm Hg, and venous P_{O_2} 14 mm Hg in the inactive state, with corresponding % saturations of arterial and venous blood of 100, and 52.5 respectively. In the active animals, the arterial P_{O_2} fell to 21.7 mm Hg and the venous P_{O_2} to 8.5 mm Hg. At these oxygen tensions the arterial haemocyanin was only 35% saturated, while the venous haemocyanin was found to be completely unsaturated.

Mangum and Weiland (1975) used a mean O_2 capacity value for Callinectes blood to estimate the A-V difference in O_2 content to be .00595 ml/ml (.595 vol %).

These authors then used this value to calculate the Cardiac Output necessary to sustain a measured oxygen consumption of Callinectes according to the Fick principle and obtained a value of 207-238 ml/kgm/min. From estimations of heart rates, and measurements of stroke volumes by syringe sampling Mangum and Weiland propose that contrary to the claims of Johansen et al., such a cardiac output is within the capabilities of the circulatory system of such an organism. They point out that the relatively low cardiac output found by Johansen et al. (1970) for Cancer magister results largely from the unusually high O_2 capacity of the haemocyanin of this crab species, and is not the result of a large A-V difference.

To summarise, the respiratory function of haemocyanin in decapod crustaceans has been well demonstrated, and although there is still some controversy about the details of the *in vivo* conditions in these animals, the weight of evidence suggests that the arterial P_{O_2} values are somewhat higher than those reported in earlier studies, although not necessarily as high as those found by Johansen *et al.* (1970) for *Cancer magister*.

The role of the circulatory system in the supply of O_2 in the decapod crustaceans is still open to some conjecture, and the reasonableness of various estimations of cardiac-output "cannot be assessed without more knowledge of heart function in intact animals". (Mangum and Weiland, 1975).

1.1.43 *Limulus polyphemus*

The respiratory function of haemocyanin in this sluggish arthropod has also recently been described through studies of the *in vivo* gas tensions.

However, as with the crustaceans, some controversy exists about the details of the *in vivo* function of haemocyanin in the horseshoe crab.

Using implanted oxygen electrodes in submerged animals Falkowski (1973) obtained the values presented on Table 1.5 for the *in vivo* gas tensions of this animal.

He then related the mean pre and postbranchial O_2 tensions to the oxygen equilibrium curves (Fig. 1.5) of the haemocyanin of *Limulus* at the appropriate temperatures to deduce the % saturation of the haemocyanins at any measured P_{O_2} values. In both cases the arterial haemocyanin was in the region of 60% saturated, while the venous haemocyanin was 34% saturated at 24°C and 22% saturated at 8°C.

Taking the O_2 capacity of Limulus haemocyanin to be 2 vol % (Prosser, 1961) Falkowski estimated that the haemocyanin of the horseshoe crab delivered .36 vol % O_2, and .28 vol % O_2 at 8°C and 24°C respectively.

Table 1.5

In vivo gas tensions of *Limulus polyphemus*

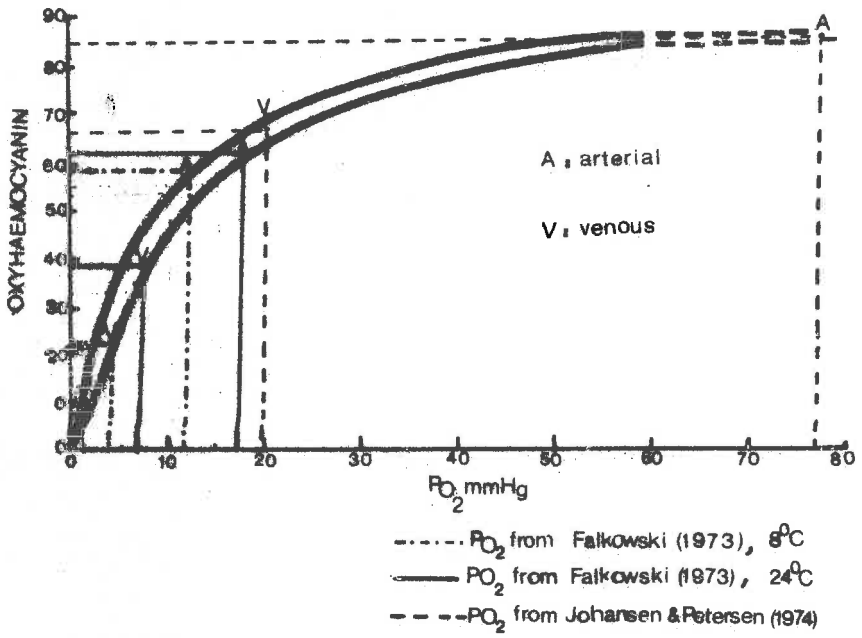
(after Falkowski, 1973, Table 1)

Measured at 24°C		Measured at 8°C	
Epi-pericardial region (A)-postbranchial	Esophageal region (V)-prebranchial	Epi-pericardial region (A)-postbranchial	Esophageal region (V)-prebranchial
mm Hg O_2	mm Hg O_2	mm Hg O_2	mm Hg O_2
16.95	7.25	11.65	2.40
15.65	6.90	10.40	2.85
16.50	7.35	10.35	3.20
16.75	7.30	11.00	2.15
mean: 16.5±0.49	7.2±0.18	10.8±0.53	2.65±0.25

Figure 1.5

Oxygen delivery by haemocyanin of *Limulus polyphemus*

(Oxygen equilibrium curves taken from Falkowski, 1973, Figure 2)



Falkowski presented oxygen consumption data for a few specimens, but made no calculations of cardiac output from his data.

However, as is shown later in this thesis (Chapter 3) such a calculation for Limulus based on Falkowski's data gives a cardiac output value considerably greater than any reported for decapod crustaceans, including Callinectes sapidus.

Mangum and Weiland (1975) present data of Johansen and Petersen (1974) on in vivo gas tension measurements in Limulus. These data indicate that at 16°C the arterial P_{O_2} of Limulus blood is 77 mm Hg, while the venous P_{O_2} is 20 mm Hg. These data differ greatly from those of Falkowski (1973) (Table 1.3).

As Falkowski (1973) shows, the position of the oxygen equilibrium curve of Limulus haemocyanin does not change greatly with temperature (Figure 1.5). Relating the P_{O_2} values of Johansen and Petersen (1974) approximately to the oxygen equilibrium curves for Limulus haemocyanin (Figure 1.5) indicates that only the "top" end of the curve would be used in O_2 transport in this organism. Hence, although both recent studies indicate a similar arterio-venous difference, there remains a difference of opinion about the in vivo conditions which determine the O_2 delivery by this respiratory pigment.

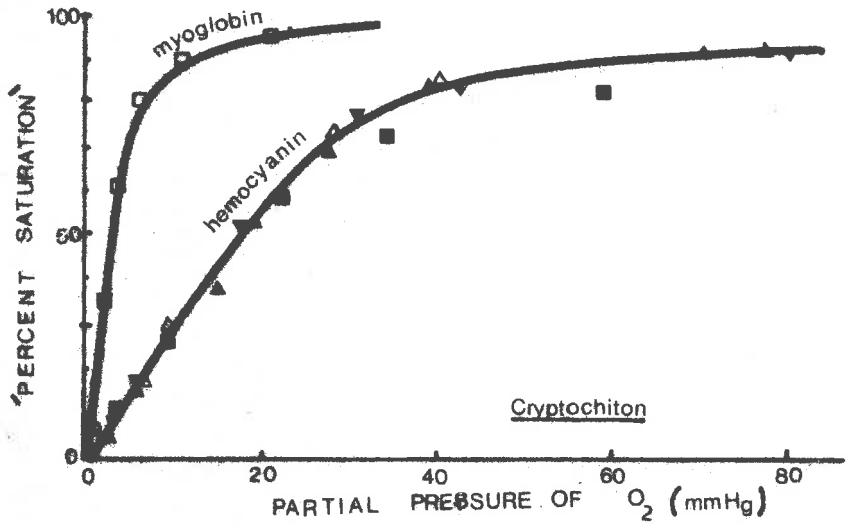
1.1.44 Amphineuran molluscs (Chitons)

Until relatively recently the difficulty in obtaining reasonable measurements of in vivo gas tensions in the chitons had severely limited the understanding of the respiratory function of haemocyanin in these organisms.

Manwell (1958) considered the juxtaposition of different "respiratory pigments" in the amphineurans. He demonstrated that the oxygen equilibrium curve for Cryptochiton stelleri radular myoglobin lies well to the left of the oxygen equilibrium curve of the haemocyanin (Figure 1.6). This means that the myoglobin is saturated at much lower

Figure 1.6

Oxygen equilibrium curves of Cryptochiton myoglobin and haemocyanin (after Manwell, 1958)



oxygen tensions than the haemocyanin, suggesting that at low P_{O_2} there will be a transfer of oxygen from the haemocyanin to the radular muscle myoglobin. Manwell suggests that this oxygen transfer system is analogous to those involving haemoglobin in blood and muscle myoglobin, or maternal and foetal haemoglobins in various vertebrates; he therefore implies that the haemocyanin is effectively functioning as a respiratory pigment in these primitive molluscs.

Redmond (1962) also examined the haemocyanins of several amphineuran molluscs, Chiton tuberculatus, Acanthopleura granulata, Katherina tunicata and Hopalia muscosa. He found that the haemocyanins of two of these species, C. tuberculatus and A. granulata show a reverse Bohr effect, while the other two show a normal Bohr effect. Manwell (1960) reported that the haemocyanin of another chiton Amicula (Cryptochiton) stelleri demonstrates no Bohr effect over a considerable pH range (6.4 7.5). Therefore the haemocyanins of the chitons exhibit all possible responses to change in pH. Redmond was not able to correlate these

different responses with any differences in the habitat or way of life of these animals, and concludes that the Bohr effect has no physiological significance in the respiratory exchange of these animals.

Using the Van Slyke gas analysing apparatus Redmond (1962b) determined the oxygen capacity of the whole blood of these chitons, and from this he calculated the O_2 capacities of the haemocyanins. The oxygen capacities were found to be quite low; the highest for an individual of any species was 1.57 vol % and in the case of A. stelleri less than 1 vol %. In the latter species in some cases more oxygen is carried in solution than bound to the haemocyanin.

Redmond (1962b) was only able to sample blood from the heart and pericardial region for direct oxygen analysis by Van Slyke apparatus. The oxygen content of the blood sampled was always low. Relating the oxygen contents to the O_2 capacity, the oxygen equilibrium curve, and the O_2 solubility curve reveals that there is considerable variation in P_{O_2} of the blood, and hence in percent saturation of the haemocyanins. For example, the pericardial samples from K. tunicata show P_{O_2} values ranging from 22 mm Hg to 5 mm Hg, with a corresponding range in haemocyanin saturation from 89% to 37%. Redmond concludes that the fact that the haemocyanin is to a greater or lesser degree unsaturated in these samples means that "any movement of oxygen into or out of the blood must necessarily include oxygen combination or release by the haemocyanin. The pigment therefore may definitely be stated to serve as a transporter of oxygen (in these chitons)".

Redmond (1968a) reports that he has obtained direct measurements of the *in vivo* P_{O_2} in Cryptochiton (= Amicula) and Katherina using O_2 electrodes. No detailed data is given in this review paper. However he found P_{O_2} values which indicate in each case the oxygenated blood in the heart was about 90% saturated while blood from the haemocoel was only

45% saturated. Redmond points out however that the haemocoel samples should not be considered immediately prebranchial, and that more oxygen could be lost to the tissues before the blood returns to the gills of the animals.

Petersen and Johansen (1973) sampled blood from the heart and dorsal aorta (arterial) and perivisceral sinus (venous) of this same chiton species, and determined the P_{O_2} and P_{CO_2} of their samples using a Radiometer PH M71 gas analyser. As their determinations were made at 10°C, they related their gas tension data to the oxygen equilibrium curve for Cryptochiton haemocyanin determined at this temperature by Manwell (1958) (See Figure 1.6).

In these Chitons, at 10°C, in constantly submerged conditions, the mean arterial P_{O_2} was 98.5 mm Hg, corresponding to a mean arterial % saturation of the haemocyanin of 92.8%, which closely agrees with that proposed by Redmond (1968a). However, Petersen and Johansen (1973) found a mean venous P_{O_2} of 31.7 mm Hg, which corresponds to a mean venous % saturation of about 74.2%, markedly higher than that reported by Redmond (1968a).

As details of the temperature and other conditions under which Redmond measured *in vivo* P_{O_2} are unavailable, it is not possible to speculate on the reasons for the different venous % saturations found in these two recent studies of *in vivo* function of chiton haemocyanin.

Neither Redmond (1968a), nor Petersen and Johansen (1973) relate their *in vivo* findings to the oxygen capacity of Cryptochiton haemocyanin, and hence the A-V differences in O_2 content are not given in these studies.

To summarise, in the chitons, due to recent *in vivo* gas determinations, there is good evidence that haemocyanin contributes significantly to the O_2 supply to the tissues. However, as with some other groups of haemocyanin bearing organisms discussed in this introduction, the details of the *in vivo* function of chiton haemocyanins have still to be resolved.

1.1.45 Gastropod Molluscs

It is this significant group of haemocyanin bearing organisms with which this project is primarily concerned. As the quotation from Redmond (1968a) in section 1.1.1 suggests, the understanding of the role of haemocyanin in the physiology of these animals is less complete than in any of the other groups discussed in this introduction. I believe that this is largely because technical difficulties have prevented the adequate measurement of *in vivo* gas tensions, essential for such an understanding.

To my knowledge the most complete, and perhaps still the only reasonable description of the *in vivo* function of gastropod haemocyanin is that calculated by Florkin (1939) using data from Redfield *et al.* (1926), and Henderson (1931), for the conch *Busycon canaliculatum*. Redmond (1955, 1968a), and Jones (1963, 1972) also use this example as the best available description of *in vivo* function of gastropod haemocyanin.

Table 1.4 below, gives the essential data describing O_2 exchange in the blood of *Busycon*.

Table 1.4

Data describing oxygen exchange in the blood of *Busycon* (after Redmond, 1968a)

	Arterial blood	Venous blood	Art.-Ven. difference
O_2 vol %	2.6	0.9	1.7
Hcy, % sat.	95.0	33.0	62.0
P_{O_2}, mm Hg	36.0	6.0	30.0
pH	7.96	7.79	0.17

(based on the Henderson-Hasselbalch equation)

Total oxygen capacity of blood: approx. 3.0 vol %.

Temperature: 22°C.

Busycon haemocyanin, like many gastropod haemocyanins shows a reverse Bohr effect; however it is not a marked effect, and with the relatively small change in pH from arterial to venous blood calculated by Florkin, can probably be ignored in describing O_2 delivery under normal oxygenated conditions.

It can be seen that the haemocyanin of Busycon is about 95% saturated at the gills, and in the absence of any marked effect of increased P_{CO_2}, only about 33% saturated at the tissues. As is shown on Table 1.4 a haemocyanin of 3 vol % O_2 capacity therefore delivers about 1.7 vol % oxygen per circulation to the tissues of this gastropod. Over the same change in P_{O_2}, only a fraction of this oxygen will be carried in solution. It appears, as Jones (1963) points out that the pigment is "well adapted to work at moderately low internal oxygen tensions which result from the very substantial gradient across the respiratory surface". Largely on the basis of the understanding of haemocyanin function in Busycon there has been a great deal of speculation on the possible role of the reverse Bohr effect in gastropod haemocyanins. Discussion of this aspect of haemocyanin function in gastropods is left until Chapter 2.

There have been numerous other examples of circumstantial evidence suggesting a respiratory role of haemocyanin in gastropod molluscs.

For example, Spoek et al. (1964), having described the oxygen equilibrium characteristics of the haemocyanin of the edible snail Helix pomatia, demonstrated that puncturing the vena magna which collects blood from the intestine yields colourless, hence unsaturated blood, while puncturing the heart, which is immediately postbranchial in gastropods, yields blue, hence O_2 saturated, blood. These crude observations suggest that the heart pumps out oxygenated blood which is deoxygenated before returning to the gills. These authors however reiterate that "experiments on the function of snail Hcy are absent".

Some authors have virtually disclaimed a respiratory function for gastropod haemocyanin. Pilson (1965) found enormous variation in the concentration of haemocyanin in the blood of four species of California Haliotis. He concluded that the large, apparently random variation of haemocyanin concentration in these gastropods was not compatible with the idea that haemocyanin acts either as a respiratory pigment or as a storage molecule. Similarly, Petzer and Pilson (1974) found large variation in the haemocyanin concentration of Busycon canaliculatum. Although they found some evidence of seasonal variation in the haemocyanin concentrations in this species they maintained that considerable doubt must exist as to its primary physiological function.

It is evident from this introduction that there are still many gaps in the understanding of the physiological role of haemocyanin in the gastropod molluscs. The major aim of this study is to assess the importance of haemocyanin in the supply of the oxygen needed to maintain aerobic respiration of these animals under various conditions. Other possible physiological roles of haemocyanin in the gastropods are also examined.

Before discussing in detail the experimental approaches used in this study the animals used in the investigation will be introduced.

GENERAL INTRODUCTION PART 2

1.2.1 Experimental Animals

The gastropods of the family Haliotidae are commonly known as abalone. The systematics of Australian abalone is somewhat confused, however in accordance with the opinions of Ino (1952), Cox (1962), Wilson and Gillette (1972), and the recommendation of Shepherd (1973), the generic name Haliotis is adopted for all species used in this study.

The three species of abalone in which the function of haemocyanin is investigated in this project are Haliotis ruber, Haliotis laevigata, and Haliotis roei.

These three species have different, though overlapping distributions about the southern coast of Australia (see Map 1 after Shepherd, 1973).

Haliotis roei ranges from as far north as Shark Bay in Western Australia to Wilson's promentory in Victoria. At Shark Bay the species experiences maximum annual temperatures as high as 26°C, while at its eastern-most limit the maximum is only 18°C. The corresponding minimums at these localities are 18°C and 12°C respectively.

Haliotis ruber ranges from Rottnest Island in the west to as far north as Coffs Harbour on the east coast of Australia. It therefore occurs in regions where the maximums range from 25°C to 17°C with corresponding minimums from 18°C to 11°C.

H. laevigata has a distribution which overlaps both of the above species, but only goes as far as Rottnest Island on the west coast of Australia, and as far east as Tasmania. This species therefore occurs over a more restricted temperature range than the other two species, maximum temperatures ranging from 23°C to 18°C with corresponding minimums from 16°C to 12°C.

The habitats and behaviour of these three species have been well described by Shepherd (1973).

H. roei is the smallest of the three species, easily recognised by the distinctive concentrix spiral sculpture of the robust shell (Plate I).

H. roei shelters in narrow crevices in shallow sublittoral water. It is commonly found on calcareous rock platforms, and on granitic coasts if suitable crevices are present.

It is by far the most active of the South Australian abalone, being the only grazing species studied during this project. Although

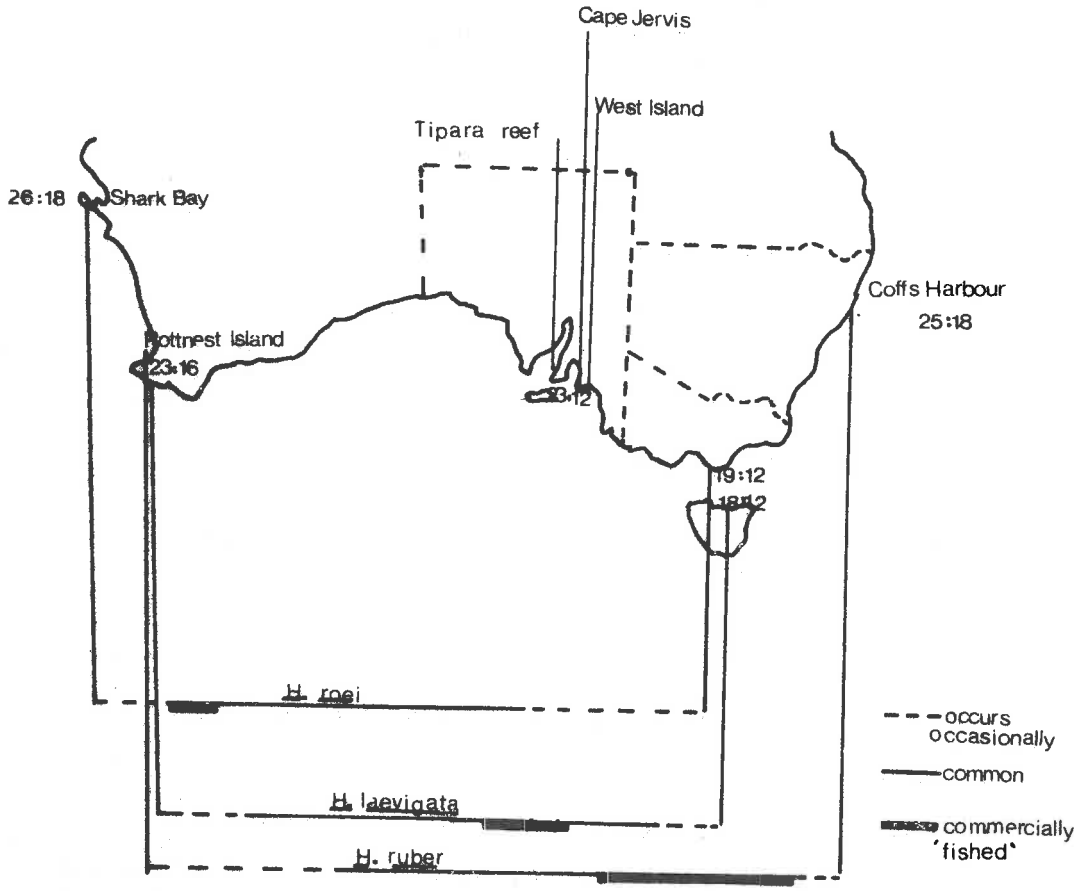
Map 1

Showing the distribution of the three species of Haliotis studied (after Shepherd, 1973).
The numbers are maximum and minimum sea temperatures at the various localities.

Map 2

Showing the detail of the study area.
Unless otherwise mentioned all abalone used in this study were taken from the region between Restless Point, and Penguin Rock.

MAP 1


Cape Jervis
West Island
Tipara reef
26:18
Shark Bay
Coffs Harbour
25:18
Rottnest Island
23:16
23:12
19:12
18:12
H. roei
H. laevigata
H. ruber
occurs occasionally
common
commercially fished

MAP 2

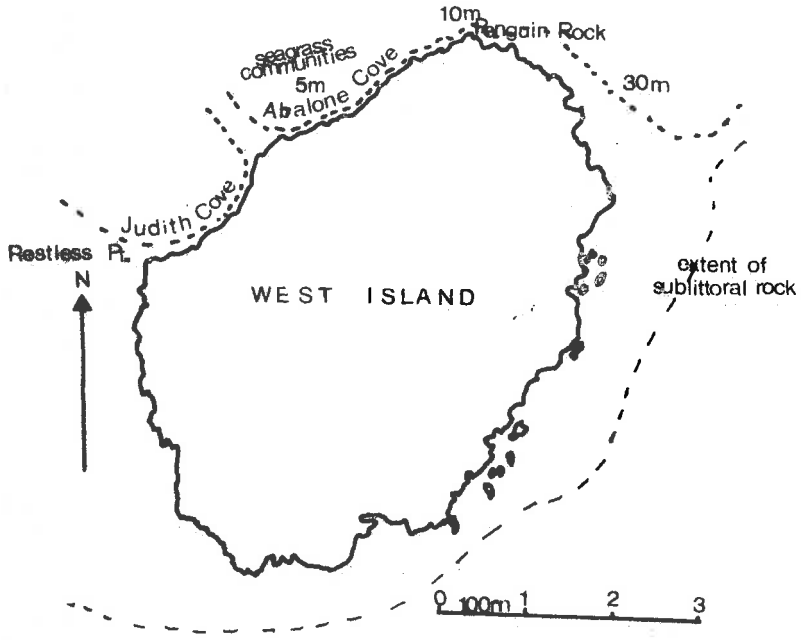

10m
Penguin Rock
30m
seagrass communities
5m
Abalone Cove
Judith Cove
Restless Pt.
N
WEST ISLAND
extent of sublittoral rock
0 100m 1 2 3

Plate 1

Typical Haliotis roei specimen, showing distinctive concentric spiral of the robust shell. This specimen, grazing in the open when photographed has a dymotape tag applied to the shell with Vepox underwater putty.

Plate 2

Two typical H. ruber specimens, showing shells encrusted with bryozoans, hydrozoans, and algae. Four bonnet limpets are also attached to the anterior end of the shell of the specimen in the top photograph. The broad epipodium of this species can be clearly seen in the lower photograph.

(a)

(b)

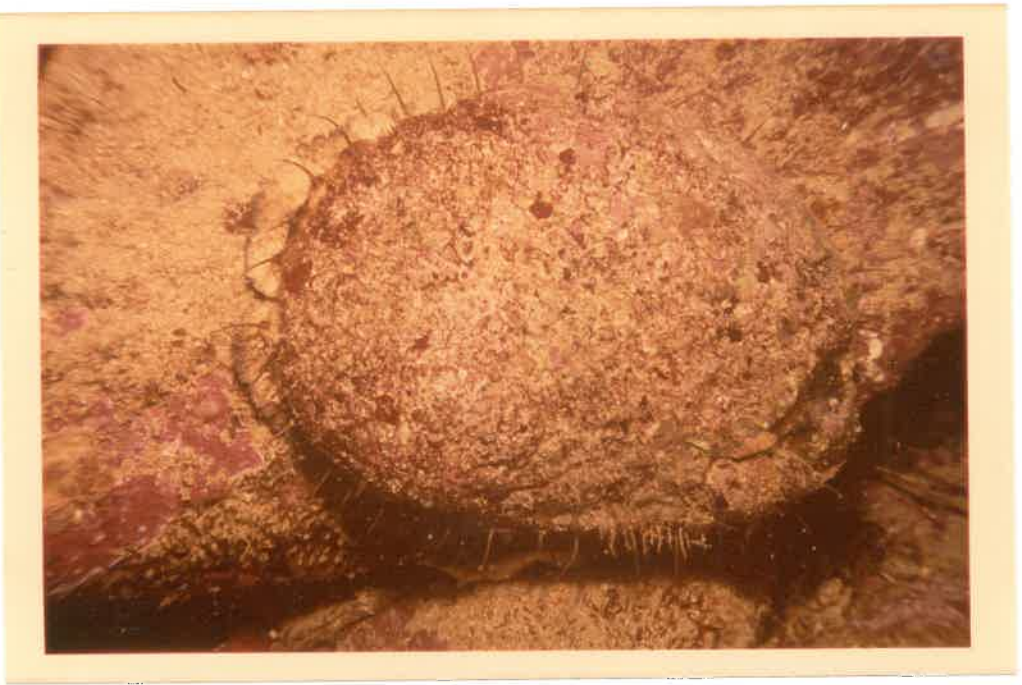


Plate 3

Two photographs of H. laevigata specimens. The top photograph clearly shows the normal exposed habitat of this species.

The specimen in the lower photograph is demonstrating a typical escape response to predator attack (in this case the large starfish Coscinasterias)

(a)

(b)

cryptic during the day the animal emerges after dark to actively graze on the open rock surfaces (Plate 1). If disturbed (e.g. by exposure to artificial light) this species can often be observed to move extremely rapidly to a less exposed position.

H. ruber occurs in slightly deeper water than H. roei, at depths from about 1-2 m to 10 m, and rarely to 25 m. H. ruber is also a relatively cryptic species, usually occurring in larger caves, under ledges, or on verticle rock faces. Because of its cave-type habitat the shells of this species are often encrusted with bryozoans, hydrozoans, and algae (Plate 2, Figs. a and b).

The epipodium of this species is large and distinctive, and is exposed when the animal is clamped to the substrate (Plate 2, Fig. b).

H. ruber normally feeds on drift algae, rarely moving from its home site. As a consequence, when animals of this species are removed from the substrate an obvious scar can often be seen on the rock surface. Shepherd (1973) has shown that feeding of this species (and H. laevigata) is largely dependent on water movement. There must be enough water movement to transport algae along the bottom so that it can be caught by abalone; however in rough weather the movement of the drift algae is too vigorous for abalone to catch and hold food.

On night dives during the course of this study H. ruber specimens were occasionally observed to be moving actively across the rock substrate.

H. laevigata occurs in deeper water, usually from 10-30 m. However in more sheltered areas it may occur in water of lesser depth.

This large, smooth shelled abalone, easily recognised by the lettuce green colour of the sides of the foot, lives on the open rock faces (Plate 3). Probably because of its open habitat the shell is usually relatively free of encrusting organisms. H. laevigata is the most inactive of the species studied. It is almost entirely sedentary, and can most often be observed clamped tightly to the substrate, with

the narrow epipodium withdrawn under the shell. Like H. ruber, H. laevigata feeds almost entirely on drift algae, and is dependent on water movement for its food supply.

West Island, South Australia, was chosen as the main study area for the three species of abalone (Map 2). West Island occurs on the open coast of South Australia, and is subject to a prevailing swell from the Southern Ocean. However the north coast of the small island is facing toward the mainland shore, and is relatively protected.

West Island is one of the few places in South Australia where the habitat diversity is such that a large number of all three species considered above occur together.

Nearly all animals used in experiments during this study were taken from Abalone Cove, West Island.

The typical seasonal water temperature fluctuation at West Island is shown in Figure 3.8 (Chapter 3) drawn from data of Shepherd and Laws (1974).

It can be seen from this figure that all three species of abalone experience considerable seasonal temperature fluctuation ($> 10^{\circ}C$).

However, on the sheltered north shore of West Island during 'spells' of hot calm weather, the water of the shallow sublittoral habitat of H. roei becomes appreciably warmer than that of the deeper water of the habitats of H. ruber and particularly H. laevigata.

A typical example of such a temperature gradient between the shallow water over the rock platform, and deeper water near the sand line was recorded during a period of hot weather from January 28th-February 4th, 1975: maximum air temperature $35.5^{\circ}C$, surface water temperature $21.5^{\circ}C$, temperature on the sand line (5-10 metres) $19^{\circ}C$. It is highly probable that even larger temperature gradients occur during hotter weather. When the weather "breaks" and water movement increases, subsequent mixing of deeper water with the shallow surface water

eliminates the temperature gradient.

During low tides in summer, H. roei specimens were occasionally observed completely emerged at West Island.

For some purposes (Chapter 6) animals from other areas were compared to those from West Island. The localities referred to in these cases are given on Map 1.

Choice of abalone as experimental animals

There are numerous reasons why these abalone make an ideal choice for this study of the role of gastropod haemocyanin.

1. They are relatively abundant, easy to collect, and to maintain in the laboratory.
2. The ecology, and some aspects of the physiology of the South Australian species are well documented by Shepherd (1973).
3. They have large volumes of haemocyanin bearing blood.
4. Crofts (1929) has made a classical study of the morphology of the abalone, including a detailed description of the circulatory system.
5. Finally, it was anticipated a priori that the differences in the habitats and behaviour of the three species might make a comparative study of the function of their haemocyanins more rewarding.

GENERAL INTRODUCTION PART 3

1.3.1 Experimental Approach

It is the primary aim of this project to investigate the role of haemocyanin in the oxygen transport systems of the three species of abalone H. roei, H. ruber and H. laevigata.

The questions which are of prime importance are therefore:

1. Is the haemocyanin in the haemolymphs of the abalone involved in the transport of oxygen?
2. If so, how important is haemocyanin in the delivery of oxygen to the tissues of the abalone. Is it likely to be essential for survival of the organism, or does it just supplement oxygen delivered in solution by the haemolymph?

(Obviously a priori it is possible that haemocyanin's primary function is not as a respiratory pigment but in some other role, for example as a storage molecule (cf. Zucherkandle, 1960). This alternative is considered in this thesis, but for the moment I will discuss only the experimental approach taken in answering the first two questions.)

1.3.11 Oxygen equilibrium curves, and O_2 capacities of abalone haemocyanins

The approach taken to investigate these questions closely parallels that used in the investigations of haemocyanin function in other groups of organisms (e.g. Redmond, 1955, 1962, 1968; Johansen et al. 1970; Falkowski, 1973). The first step in the study is the description of the oxygen equilibrium and O_2 capacity, characteristics of the haemocyanins of the three abalone species.

1.3.12 In vivo gas tensions

The above first step is the extent to which previous studies on gastropod haemocyanins have proceeded. However in this study a method is developed whereby reasonable measurements of gas tensions at arterial and venous "sites" within the living animal can be made. With this additional information on in vivo gas tensions the oxygen delivered to the tissues by haemocyanin in a respiratory cycle can be calculated. Moreover, the O_2 delivered as dissolved oxygen can also be estimated. Therefore, the total O_2 delivered, and the relative contribution of haemocyanin to the oxygen supplied to the tissues of the abalone per

respiratory cycle can be ascertained for each species.

1.3.13 Response of in vivo haemocyanin function to abrupt temperature change

This study of the O_2 delivered to the tissues by the haemocyanin of the abalone (the A-V difference in volumes %) is then taken a step further.

From the brief introduction to the ecology of the abalone (General Introduction Part 2) it can be seen that each of the abalone species are likely to encounter temperature fluctuations.

Little is known about the effect of temperature change on the in vivo function of haemocyanin (Redmond 1968a). In this study measurement of in vivo gas tensions allows an investigation of the response of the in vivo delivery of oxygen by abalone haemocyanin to an abrupt temperature change from an acclimation temperature to a temperature approaching the upper limit experienced by abalone in the field. In considering the results of experiments investigating the response of in vivo haemocyanin function to abrupt temperature increase and also in all other temperature change experiments, comparisons are drawn between the three abalone species with different ecologies.

1.3.14 Relationship between oxygen delivered by haemolymph, heart function and overall oxygen consumption of the abalone

As values for the amount of oxygen delivered to the tissues by the blood in a single respiratory cycle are obtained in this study, further hitherto impossible investigations of the relationship between the respiratory systems, the circulatory systems and the overall oxygen consumptions of the abalone can be made (cf. Redmond, 1968a, pp. 21).

(a) The oxygen consumptions of each of the abalone species are compared over a range of temperature conditions, taking into account numerous other factors which may modify this parameter.

From the oxygen consumption data, and the A-V difference data, the Fick principle can be used to estimate the cardiac output of the three abalone species, both at acclimation temperatures, and after abrupt temperature increase. The interactions of these factors described by the Fick principle are compared for each of the three species, in the light of their differing ecologies.

(b) As a further step in a complete understanding of the O_2 delivery system in the abalone, an investigation is made of the parameters of heart function which determine cardiac output, namely heart rate, and stroke volume. In this study emphasis is placed on the determinations of heart rates of abalone subjected to various temperature conditions. Cardiac output, and heart rate data are then used to calculate stroke volumes of abalone at acclimation temperature, and after an abrupt temperature increase.
The overall picture of the O_2 delivery system obtained for the three abalone species is compared to that of the few other invertebrates in which the O_2 delivery system, or part of the system, has been adequately described.

1.3.2 Possibility of anaerobic respiration supplementing aerobic respiration during times of stress in abalone

It appeared possible that after an abrupt temperature change the respiratory and circulatory factors which determine the O_2 supply of the abalone might respond in such a way that O_2 supplied to the organism is not sufficient to maintain aerobic metabolism. Under such circumstances it was hypothesised *a priori* that anaerobic metabolism might to some extent supplement aerobic metabolism in maintaining the overall metabolic requirements of the abalone. This hypothesis was formulated with the knowledge that it has been proposed that anaerobic metabolism might supplement aerobic metabolism in other groups of haemocyanin bearing organisms where the whole animals or organs of the animals are for some

reason oxygen stressed (Johansen et al., 1970; Falkowski, 1973).

In view of the above facts it was decided that some measurement of the abalone species' ability to respire anaerobically should be made.

1.5.5 Summary of the factors investigated to define the role of haemocyanin in the overall O_2 supply system of the abalone

Figure 1.7 diagrammatically summarises the various interacting components of the abalone respiratory and circulatory system which are investigated in this study. In the central column 2 of the figure (headed Animals) the figure shows how the components which determine the A-V difference, (haemocyanin characteristics, in vivo gas tensions, dissolved O_2) interact with the components of heart function which determine circulation (heart rate, stroke volume) to determine the oxygen consumption of the organisms. In column 1 headed Modifying Factors are listed such factors which are known in any way to influence the values of the corresponding parameters in column 2. Where possible, during this study these modifying factors are kept constant, or otherwise accounted for. However one important modifying factor (temperature) is experimentally varied, and the influence of such variation on each of the components which are involved in the Fick principle (Column 2, Fig. 1.7), and hence on the interaction of these factors is determined.

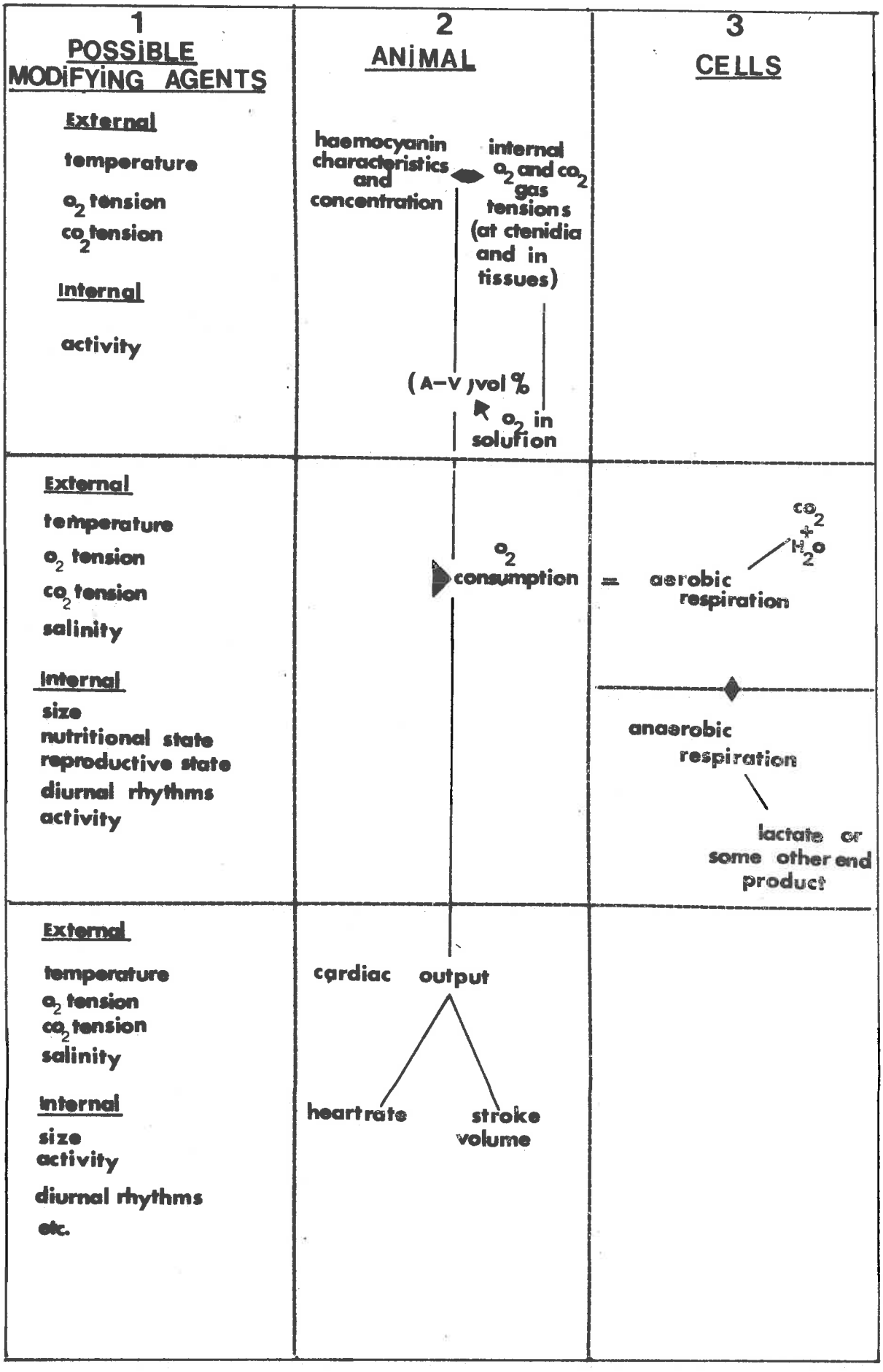
At some temperatures the oxygen delivery system may fail to supply sufficient oxygen to maintain cellular metabolism. It is hypothesised that at such times the aerobic respiration of the abalone maintained by the components considered in Column 2, Fig. 1.7, may be supplemented to a greater or lesser extent by anaerobic respiration, Column 3, Fig. 1.7. This possibility is investigated.

At all stages in this study, comparisons are drawn between the three abalone species, and the general patterns obtained are compared to information on other haemocyanin bearing invertebrates in the literature.

Figure 1.7

Diagrammatic summary of interacting components of the abalone respiratory and circulatory system

DIAGRAMMATIC REPRESENTATION OF VARIOUS INTERACTING COMPONENTS OF ABALONE RESPIRATORY AND CIRCULATORY SYSTEMS



1.3.4 Possible non respiratory roles of abalone haemocyanin

As mentioned at the beginning of this discussion of the experimental approach used in this study, it is possible that the primary function of abalone haemocyanin might be quite unrelated to a function in the O_2 delivery system. This possibility may still hold even if a respiratory function can be demonstrated for the haemocyanin.

In order to check whether there were any probable alternative functions of haemocyanin in abalone the concentrations of haemocyanin in the haemolymphs of abalone populations and individuals in the field were monitered over a long time period. Each experimental species was examined.

It was anticipated that such monitering would detect any pattern of change in the haemocyanin concentration which could be associated with other known physiological patterns in abalone (e.g. breeding seasons) or with recognisable changes in the environment of the animals (e.g. seasonal temperature changes, food availability).

Any such recognisable correlations might be expected to lead to a better understanding of an alternative role for haemocyanin in these animals.

CHAPTER 2

Respiratory function of haemocyanin in abalone

2.1.1 Introduction

In this chapter the following factors are investigated:

1. The oxygen equilibrium curves of the haemocyanins of the three abalone species Haliotis roei, Haliotis ruber, and Haliotis laevigata, including
   (a) the effect of temperature on the position and shape of the oxygen equilibrium curves, and
   (b) the effect of varying CO_2 tensions and pH's on the position and shape of the oxygen equilibrium curves.
2. The oxygen capacities of the haemocyanins of H. roei, H. ruber and H. laevigata, and
3. The P_{O_2} and P_{CO_2} of pre and postbranchial bloods of living abalone subjected to various temperature regimes.

The above factors are then related to determine the arterial venous difference in O_2 content and an estimation of the contribution by haemocyanin to the oxygen delivered to the tissues is made.

2.2.11 Oxygen equilibrium curves

(a) Effect of temperature on oxygen equilibrium curves

At the main study area, West Island, the temperature undergoes considerable seasonal and short term fluctuation, particularly in the shallow water where summer calms alternate with periods of rougher weather.

Temperature has been shown to have quite a marked effect on the position and shape of the oxygen equilibrium curves of the haemocyanins of some animals. Furthermore, some evidence suggests that animals adapted to warmer conditions possess haemocyanins which are less sensitive to temperature. For example, Redmond (1955) proposed that this was the

explanation for the difference in response to temperature of the haemocyanins of two species of crustacean, the crayfish Panulirus interruptus, which occurs off the coast of California, and the sheep crab Loxorhynchus grandis, which occurs in cooler areas. Panulirus haemocyanin is much less temperature sensitive in terms of the relative change in P_{50} for a given temperature change, than the haemocyanin of Loxorhynchus.

Redmond (1968) also proposes that the P_{50} values of related species from different environmental temperatures may correspond at these temperatures as a result of adaptation to maintain the "same internal oxygen tensions in the face of differing temperatures". For example, the P_{50} of Panulirus interruptus haemocyanin at 15°C, the average field temperature for this species, corresponds closely to the P_{50} of H. argus haemocyanin at 25°C, which is approximately the average field temperature of the latter species. There is less evidence of such adaptation to temperature in the haemocyanins of other groups of haemocyanin bearing organisms.

In view of the considerable seasonal temperature fluctuation to which the abalone are subjected, the effect of varying temperatures on the oxygen equilibrium curves of their haemocyanins is determined in this study. The different habitats, and geographical distributions suggest a priori that interspecific differences in response to temperature could be found. For example, H. roei haemocyanin might be expected to be less temperature sensitive than the haemocyanins of the other two species (cf. species introduction).

(b) Effect of P_{CO_2} changes on the oxygen equilibrium curves of abalone haemocyanins - The Bohr Effect

A change in the P_{CO_2} can have a considerable effect on the shape and position of the oxygen equilibrium curve of a haemocyanin, just as has been demonstrated with haemoglobins. This influence of P_{CO_2} known as the Bohr effect, is now known to be at least partly a consequence of changes

in pH and can also be demonstrated by resuspension of haemocyanin in various buffers, or by dialysis of the haemolymph against them (e.g. Redfield et al., 1932; Redmond, 1955; Djangmah, 1971). However, it has often been shown that such treatment may considerably change the properties of the haemocyanin from those of undiluted haemolymph (Redmond, 1955; Djangmah, 1971). Those studies which most accurately duplicate in vivo conditions are those carried out using fresh haemolymph, and directly varying the P_{CO_2} (Young, 1972).

To avoid confusion, the terminology as defined by Jones (1972) describing the direction of the shift of the oxygen equilibrium curves in the presence of increases P_{CO_2} is adopted in this thesis. A haemocyanin demonstrates a normal, or negative Bohr effect when an increase in P_{CO_2} causes a shift to the right in the oxygen equilibrium curve. If an increase in P_{CO_2} induces a shift to the left in the oxygen equilibrium curve, the haemocyanin demonstrates a reverse or positive Bohr effect.

Crustacean haemocyanins, like most haemoglobins, have a normal Bohr effect (Redmond, 1955; Manwell, 1960); at least one arthropod, Limulus polyphemus, the horseshoe crab, has a haemocyanin which demonstrates a reverse Bohr effect (Redfield et al., 1926). Cephalopod mollusc haemocyanins demonstrate a pronounced normal Bohr effect (Redfield et al., 1926, 1929; Manwell, 1960; Jones, 1963; Lenfant et al., 1965; Johansen et al., 1966). The response of other molluscan haemocyanins to changes in P_{CO_2} is however much more variable. Some, for example the gastropod molluscs Helix aspersa, and Cepaea nemoralis, exhibit both normal and reverse Bohr effects depending on the P_{CO_2} (Spoek et al., 1964).

Commonly gastropod mollusc haemocyanins exhibit a reverse Bohr effect (Manwell, 1960; Redmond, 1968a; Jones, 1965, 1972), but at least one gastropod haemocyanin, that of the conch Pleuroploca, has been reported to demonstrate a normal Bohr effect (Manwell, 1964).

The haemocyanin of the keyhole limpet Diodora aspersa apparently shows no response to change in pH over a considerable pH range (Redmond, 1963). The amphineuran molluscs (chitons) also have haemocyanins which demonstrate both normal and reverse Bohr effects (Redmond, 1962b), and in at least one species Amicula stelleri, (Manwell, 1958) has reported an absence of a Bohr effect in the pH range from 6.4-7.5.

In some organisms, notably the decapod crustaceans it appears that the change in pH from arterial to venous blood is so small that the effect on the position of the oxygen equilibrium curves of the haemocyanins (which in these organisms are relatively insensitive to pH) is likely to be negligable (Redmond, 1955, 1968 b; Johansen, 1970). Similarly the haemocyanin of Limulus is relatively pH insensitive, and the effect of the reverse Bohr effect of this haemocyanin was ignored by Falkowski (1973) when estimating oxygen exchange in this species.

In their classic early work on the cephalopod Loligo pealei Redfield et al. (1929) demonstrated that CO_2 played an important part in the offloading of oxygen from haemocyanin in the tissues. This appears to be generally true for cephalopods which all demonstrate a quite significant arterio-venous gradient in P_{CO_2} (Johansen et al., 1966).

As little information is available about in vivo gas tensions of gastropod molluscs, there remain questions about the role which CO_2 plays in haemocyanin function in these animals. As a first step towards answering these questions the effect of varying P_{CO_2} on the oxygen equilibrium curves of the haemocyanins of the three abalone species is investigated.

2.2.12 Methods and Materials

(a) Preparation of haemolymph sample

The haemolymph was extracted from the abalone using a disposable plastic 10 ml syringe fitted with a 21 gauge needle. A sample of about 6 mls of blood could be removed from the foot muscle by

penetrating to the region of the pedal sinus, just posterior to the head, and on the median line of the foot. When the needle struck this region the blood flowed easily into the syringe. After sampling the animals were often returned to the aquarium where they survived without any apparent ill effects. A second sample was sometimes taken from the same animal, usually after 2-3 days. Determination of haemocyanin concentration (see section 5.2.1) showed no difference between the two samples.

After extraction, the fresh haemolymph samples were centrifuged for six minutes at 4800 r.p.m. using a B.H.C. bench centrifuge. This was found to be sufficient to remove amoebocytes and other cells from the haemolymph of these molluscs. Unless otherwise mentioned the haemolymph was not further altered when used for oxygen equilibria determinations.

(b)

The oxygen equilibrium curves of the abalone haemocyanin were obtained using the spectrophotometric technique similar to that used by previous workers on both haemoglobins and haemocyanins (e.g. Riggs, 1951; Redmond, 1955, 1962; Manwell, 1958; Rossi et al., 1958; Young, 1972; Coates, 1975).

The type of tonometer used in the experiment is shown in Figure 2.1.

All experiments were carried out in constant temperature rooms adjusted to the required experimental temperatures ($\pm$ 0.5°C).

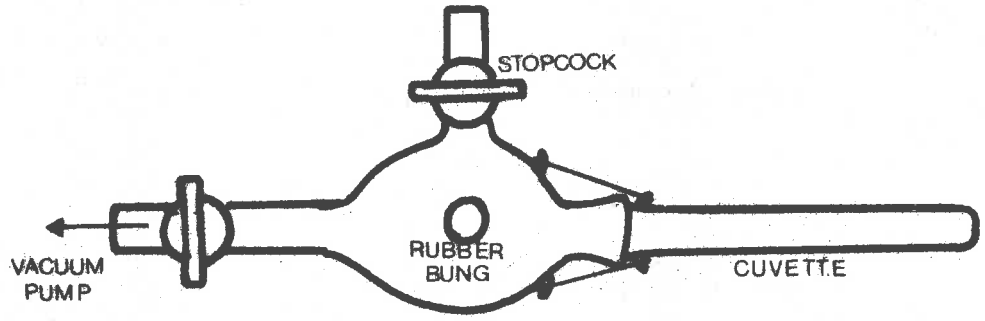
The procedure was as follows:

(1) 6 ml of fresh, centrifuged haemolymph was placed in each tonometer.

(2) Pure nitrogen was blown over the haemocyanin at a pressure of 2 lb/sq. inch for two minutes, partly deoxygenating the haemocyanin to prevent excessive bubbling when the tonometer was evacuated. (Excessive bubbling caused precipitation of protein fibres).

Figure 2.1

Tonometer used in oxygen equilibrium curve determinations



(3) the tonometer was then evacuated using a DYNAVAC MODEL TD60 high vacuum pump for three minutes.

(4) Steps (2) and (3) were repeated with the second evacuation lasting four minutes. This treatment was found through experience to completely deoxygenate the haemocyanin. The evacuation times were kept as low as possible because excess evacuation resulted in aggregation and considerable light scattering by the haemocyanin.

(5) The evacuated tonometer was equilibrated to the temperature at which the oxygen equilibrium curve was to be determined.

(6) The absorbance of the haemocyanin was measured using an Erma Photoelectric colourimeter Model AE-11 fitted with a 620 μm filter. This initial absorbance of the completely evacuated haemocyanin is Ko.

(7) A known amount of air was injected into the tonometer through the rubber bung. The tonometer was equilibrated with gentle shaking for eight (8) minutes before a further reading (Ka) was taken.

Preliminary experiments had shown that at all experimental temperatures 8 minutes allowed abalone haemocyanin to reach equilibrium with the injected air.

(8) Further amounts of air were injected and equilibrated and the absorbances of the solutions were read (Ka's).

(9) When no further increase in the absorbance was obtained, the tonometer was opened, and equilibrated at atmospheric pressure. This last reading when the haemocyanin is completely saturated is termed Kt. Obviously Kt was often the same as the previous reading if the haemocyanin was already saturated. At very high temperatures (38°C) the haemocyanin would not saturate at atmospheric pressure. In these conditions the solution was cooled to obtain Kt.

(10) The percent saturation of the haemocyanin is calculated by:

$$\frac{Ka - Ko}{Kt - Ko} \times \frac{100}{1}$$

The oxygen partial pressure P_{O_2} in mm Hg is calculated by the formula

$$P_{O_2} = \frac{.21\ (B - H \times P) \times Vo}{\text{Vol. tonometer} - \text{Vol. haemocyanin soln.}}$$

where P_{O_2} = partial pressure of oxygen in the tonometer

Vo = total injected amount of air

B = barometric pressure mm Hg

H = relative humidity measured by wet and dry bulb thermometers

P = vapour pressure of water at the experimental temperature

(c) Bohr Effect

(1) For the determination of the Bohr effect, known volumes of CO_2 were added to the tonometers, before commencement of the oxygen equilibrium measurements.

A tonometer was sealed, and evacuated. It was then filled with pure CO_2 at a pressure of about 4 lb/sq. inch. A sample of this CO_2 was then taken with a sealed glass syringe through the rubber bung. When the needle of the CO_2 filled syringe was removed from the bung, the plunger was depressed to the required volume and quickly injected into the experimental tonometer. The partial pressure of CO_2 in the tonometer could then be calculated by

$$P_{CO_2} = \frac{1 \times B \times VCO_2}{\text{Vol.ton} - \text{Vol.soln}}$$

where B = barometric pressure

and VCO_2 = volume of CO_2 injected

(2) As a comparison to the above Bohr effect determinations after varying P_{CO_2} one series of determinations were made after adjusting the pH of the haemolymph using the Tris-HCl buffers recommended by Redmond (1962b). These buffers were designed to adjust the pH of the blood without appreciably changing the concentration of the blood. The two buffers were made up by adjusting the pH of 0.83M Tris in seawater to 8.5 and 6.7 using 1N HCl.

Redmond (1962b) found that "at least at normal blood pH" this buffer did not introduce abnormalities into the position and shape of the oxygen equilibrium curves of chiton haemocyanins.

The "normal" pH's of freshly drawn abalone haemolymphs were determined using a Radiometer model 26 pH meter. When the pH electrodes were inserted into the sample, the pH remained stable for a short period, followed by a slow, but continuous increase. The haemolymph pH values were taken as those of the initial stable period.

The abalone haemolymphs were adjusted to different pH values using a few drops of one or the other of the above buffers, the pH being determined with the Radiometer Model 26 pH meter. The haemolymph with

even a small amount of one of these buffers added did not show the pH instability recorded for pure haemolymph.

2.2.21 Oxygen capacity determinations of the haemocyanins of the three abalone species H. roei, H. laevigata and H. ruber

The oxygen capacity of the blood is the amount of oxygen carried by the blood when it is fully saturated (e.g. Prosser, 1973). Two factors determine the oxygen capacity of whole blood (Prosser, 1973), the amount of oxygen carried by the respiratory pigment, and the amount of oxygen carried in solution in the blood.

In order to determine how much oxygen the haemocyanin is supplying to the tissues it is necessary to know at least the total oxygen capacity of the haemocyanin in terms of oxygen carried by the haemocyanin in 100 volumes of blood (vol %).

In this study then, the oxygen capacities of the haemocyanins of the three experimental abalone species were measured and this data was related to the oxygen equilibrium curve and internal gas tension data of the animals to estimate the actual amount of oxygen delivered to the tissues by the haemocyanins.

The oxygen capacities of the haemocyanins were determined using the method of Maegraith et al., (1950). This method does not measure the amount of oxygen carried in solution in the blood.

2.2.22 Method and materials

Warburg flasks were calibrated using the ferri cyanide-hydrazine method (Umbreit et al., 1972). For oxygen analysis a 5 ml sample of blood was freshly drawn from individuals of each species. It was centrifuged to remove cell mater and then shaken gently for 10 minutes in air to ensure complete oxygenation. Four ml of oxygenated blood were pipetted into the calibrated flask under a 1 ml layer of oxygen free borate buffer (Maegraith et al., 1950). 1 ml 6M NaCN was carefully pipetted into

the sidearm of the flask. The flask was then seated on the manometer arm and placed in a constant temperature bath at 20°C. After 10 minutes equilibration the manometer fluid heights were adjusted on both experimental manometers and on the thermobarometer. The stopcocks were closed and the NaCN was tipped into the haemocyanin solution (thus diluting the 6M NaCN to 1M). The agitator was then started. Readings in change of height (h) of the open manometer arm were taken at intervals of 10, 20, and 30 minutes. From the final value, adjusted to change in the thermobarometer, and the flask constant (k), the volume of oxygen released by the haemocyanin in 4 ml of blood was calculated:

μl O_2 carried by haemocyanin in 4 ml = h (change in height) x k

Therefore O_2 capacity of haemocyanin in 100 ml of blood

$$\left(\frac{\text{ml } O_2}{\text{100ml blood}} \quad \text{or vol \%} \right)$$

$$= \frac{h \times k}{40}$$

To check if any oxygenated haemocyanin remained at the conclusion of the O_2 capacity determinations, the protein precipitates were centrifuged off, and an absorbtion spectrum was run on the oxygenated supernatant. As the results will show, in no case was there evidence of oxygenated haemocyanin remaining in the supernatant.

As a further check on the effect of haemocyanin concentration on the O_2 capacity, serial dilutions of blood samples of several individuals of each species were made with 0.025M Tris - HCl 0.5M NaCl pH 7.55 buffer. O_2 capacity determinations were made on the fresh blood samples, and on the diluted samples.

2.2.31 Internal gas tensions of abalone

One of the major factors which has lead to the dearth of knowledge about the in vivo functioning of haemocyanin in the gastropod mollusc is the difficulty in obtaining blood samples from suitable pre

and postbranchial sites of the animal for oxygen analysis. This is partly due to the difficulty in approaching the sampling areas through the hard shell protection and the extremely delicate nature of sampling areas such as the gastropod heart. The non clotting haemolymph poses further difficulties to sampling techniques, as sampling results in unrepairable injury to the organism. Further, the circulatory systems of the gastropod molluscs are less well described and defined than those of the more highly active cephalopod molluscs and crustaceans. Since the last century (cf. general introduction) workers have successfully cannulated the large vessels of the cephalopods, and more recently cannulae have been successfully used in sampling from crustaceans (Redmond, 1968b; Johansen et al., 1970). The clotting blood of these latter organisms probably helps prevent too much disturbance to the animals undergoing such treatment.

Falkowski (1973) demonstrated how micro-oxygen electrodes could be moved through the tissues of the horseshoe crab Limulus polyphemus to obtain readings of the internal oxygen tensions of these animals. Preliminary experiments on abalone with the same electrodes indicated that this method has definite limitations with gastropod molluscs. Insertion of even a fine electrode into most soft tissues caused considerable loss of the non clotting haemolymph in the abalone.

In this project a method was devised by which it is believed good measurements of internal gas tensions in the Haliotis species were obtained. This was possible because the circulatory system of Haliotis has been clearly described by Crofts (1929).

Her work shows that the oxygenated blood comes straight from the ctenidia into the heart where it is pumped from the large ventrical through the arteries to the tissues. Immediately before returning to the gills the deoxygenated blood collects in the renal sinus, a relatively well defined area, in close proximity to the abalone heart (see Figure 2.2).

Figure 2.2 a

Diagram showing major sampling points for *in vivo* P_{O_2} and P_{CO_2} determinations. (Figure after Crofts, 1929).

1. ventricle of heart
2. renal sinus

The third sample was taken directly from sinuses in the foot muscle.

Figure 2.2 b

Haliotis laevigata shell, showing the region clipped for blood sampling. The heart region is defined by two sets of sutures in the photograph.

The exposed ventricle (round bladder-like organ) is clearly defined in this photograph.

Fig. 2.2 a

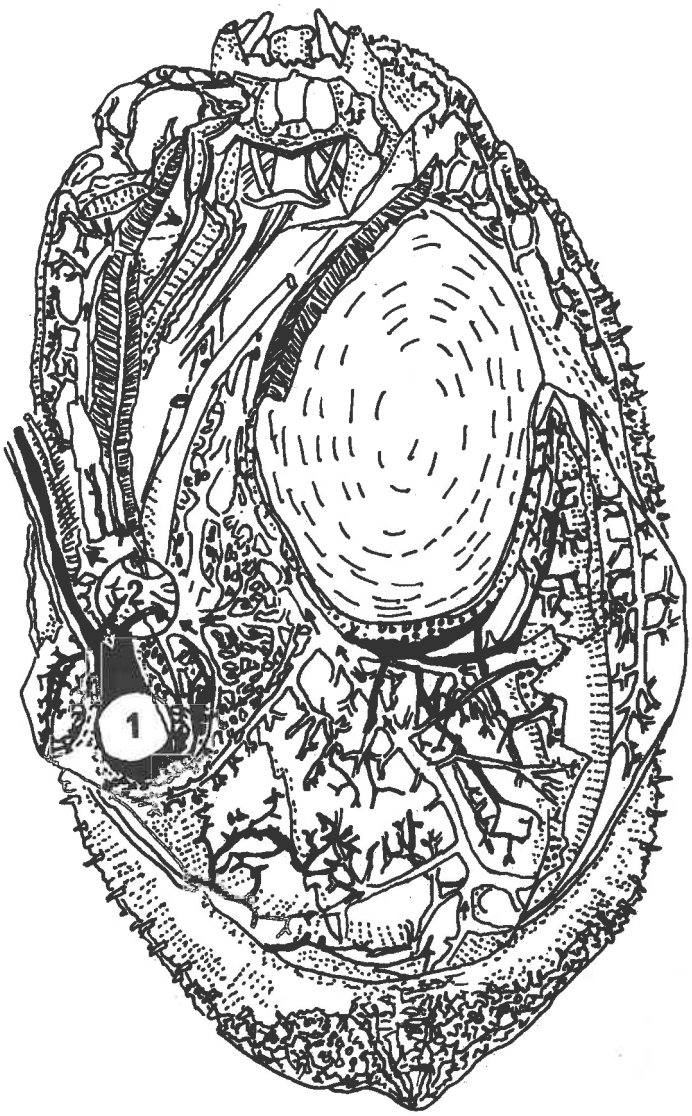


Fig 2.2 b

Samples of haemolymph for oxygen and carbondioxide analysis were therefore taken from the ventricle and from the renal sinus, representing immediate post and prebranchial blood samples. Further samples were taken from the foot muscle of the abalone in the region of the pedal sinus. These samples were taken to estimate how much oxygen the haemocyanin had given up at various stages during its circulation through the abalone.

2.2.32 Methods and materials

(a) Sampling

In the animals to be used for the internal gas tension determinations, the region of the shell immediately covering the heart, and renal sinus area, was carefully clipped off (Fig. 2.2.b). Care was taken not to injure the animal in any way, as even a small cut in the soft tissues surrounding the heart (gonad, digestive gland) caused considerable haemoraging and lead to the animal's death. Injured animals were not used in the experiments.

Further, care was taken to keep the exposed region as small as possible so that the effective circulation of water through the enclosed respiratory chamber was not disturbed. Animals treated in this way survived as well as "non-shell clipped" animals in the laboratory, and exhibited no discernable ill-effects.

When the animal had been prepared it was left for two hours in the water at the temperature at which it was to be sampled. Two series of experiments were conducted. In the first, animals taken from the field at about 20°C, and maintained in the laboratory for at least 1 week at this temperature were sampled at 20°C. These animals were considered to be 20°C acclimated. In the second series of experiments the effect of an abrupt temperature increase on the respiratory system was investigated and 20°C acclimated animals were transferred to water at 25°C two hours before sampling.

The animals to be sampled were placed in well aerated constant temperature aquaria, in a constant temperature room. For sampling, the animals were quickly removed from the substrate in the large aquaria, and placed in a shallow plastic aquarium containing water dipped from the larger one. The heart and renal sinus region of the animal were not submerged. To sample the foot muscle, the animal was removed entirely from the container.

The blood samples were taken using 125 mm, 140-160 μl radiometer capillary tubes (type D551/12.5/140) which had been previously sharpened at one end.

The pericardial cavity in the abalone, as in most molluscs, is closely connected with the kidneys, and the reproductive system (Harrison, 1962). This is probably a result of the development of these structures as derivatives of the coelom (Goodrich, 1945). Care was taken when obtaining the heart sample to avoid pericardial contamination of the sample. The pericardium was quickly opened with the sharp end of the capillary tube, and the tube was then inserted into the ventricle. Blood from the ventricle was then allowed to flow briefly through the tube to get rid of pericardial contamination before the sample was removed for oxygen analysis. In sampling the heart care had to be taken not to penetrate the gut which is surrounded by the heart (Crofts, 1929). In experiments where the gut was penetrated the results were discarded. The whole sampling operation only took a few seconds, and did not appear to immediately harm the animal; the heart could be observed to continue beating after such sampling, although where submerged, "puffs" of blood could be seen being forced through the sampling hole.

Immediately after the heart sample had been taken, another sample was taken in the region of the renal sinus (Fig. 2.2.a). Again care was taken in inserting the capillary tube into the vessels of the

renal sinus. This region obviously is closely connected with the kidney of the animal; in these animals the kidney filters the blood to produce a clear urine (Harrison, 1962). Attempts were made to insure that minimum contamination of the renal sinus samples by urine occurred. In some cases when sampling from the renal sinus, gonad or digestive gland contaminants were observed in the samples. Badly contaminated samples were discarded. Possible consequences of such contamination will be discussed further in the results section of this Chapter.

In many cases the samples taken from the heart showed a much bluer colour than the samples from the renal sinus (cf. Spoek et al., 1964). However this depended on the haemocyanin concentration - in some cases both heart and renal sinus samples appeared colourless. Oxygenated samples from the heart and renal sinus were compared spectrophotometrically and found to have haemocyanin at the same concentration. This was used as a check to show that no significant amounts of any other contaminating fluid (e.g. urine) was entering the system due to the sampling technique. After sampling the renal sinus, a further sample was obtained from the pedal sinus by inserting a capillary tube into the foot muscle of the abalone.

In most cases, only a single sample was taken from each of the sampling points of a given specimen. Once the heart of an animal had been sampled, there was a considerable loss of haemolymph through the ventricle. It was considered necessary that the renal sinus sample should be taken within a few seconds of the heart sample so that as little disturbance as possible resulted from the effects of penetrating the ventricle. As the results will indicate, where duplicate samples were taken, there was close agreement between the gas tensions in the samples. Although as far as possible the animals were held motionless during the samplings it was not possible to prevent all activity; the sampling

procedures sometimes stimulated the animals into movement: the result of this variable amount of activity will be discussed later.

The samples were analysed for P_{O_2} and P_{CO_2} within a minute of the commencement of the operation. During this short wait the samples were kept in the long capillary tubes fitted with rubber capillary stoppers at the temperature (20°C or 25°C) at which they were taken.

Finally, a sample of the aquarium water was taken for analysis of P_{CO_2} and P_{O_2}.

(b) Analysis of samples for P_{O_2} and P_{CO_2}

The measurements of the partial pressures of oxygen and carbondioxide in the blood samples were made using a modified Radiometer BMS3Mk2 blood microsystem fitted with a Radiometer E5047 oxygen electrode and an E5037 CO_2 electrode connected to oxygen and CO_2 modules in the Radiometer PHM71 Mk 2 Acid Base Analyser. In order to use this system at 20-25°C the heating circuit was disconnected and a Churchill water circulating heat exchanger was externally connected to the water bath. In the constant temperature room this system allowed extremely accurate maintenance of temperature ($\pm$ .1°C).

The O_2 and CO_2 electrodes were calibrated as described in the Radiometer Operating Instructions. The O_2 electrode was zeroed using both Radiometer P_{O_2} zero solution 10-S4150, and pure nitrogen, and calibrated with air equilibrated thermostat water. *A priori* it was anticipated that abalone would have reasonably low internal CO_2 tensions as have been observed in other marine and aquatic animals (Ramsay, 1968); the CO_2 electrode was therefore zeroed using pure nitrogen, and calibrated with 2% CO_2 in nitrogen and 5% CO_2 in nitrogen.

Calibration was completed immediately before blood sampling commenced.

Samples of haemolymph were drawn as previously described and analysed immediately.

The P_{O_2} of the sample was determined first. For a correctly introduced sample the P_{O_2} reading would drop steadily until reaching a point of stability. Further introduction of blood did not cause a change in this stable point, although if the blood was allowed to sit in the measuring chamber for some time a slow decline in oxygen tension was observed. This can probably be attributed to use of oxygen by the electrode in its function; the electrode is a Clark-type oxygen electrode involving a reduction process of diffused oxygen on a platinum electrode,

$$O_2 + 2H^+ + 2e \rightarrow H_2O_2$$

In such a small sample volume this process might appreciably lower the oxygen tension over a reasonable period.

The stable point was taken as the correct P_{O_2}. If the sample was not introduced correctly, and air bubbles entered the system, the normally observed downward trend in P_{O_2} reading was rapidly reversed. Results of experiments in which the samples were incorrectly introduced into the measuring chamber were discarded.

The P_{CO_2} of each sample was determined immediately after a stable P_{O_2} had been obtained.

After these internal gas tension experiments had been completed a paper was published in which similar sampling techniques were used to obtain a .5 ml and 1 ml blood samples from the heart of the blue crab Callinectes sapidus in heparinized syringes. As in this project the samples were analysed for P_{O_2} using a Radiometer blood gas analyser, in this case Model BMS1 (Mangum and Weiland, 1975). This recent publication, and other work, both on haemocyanin and haemoglobin bearing organisms (e.g. Gatten, 1975), support the contention that blood samples rapidly taken from undisturbed animals and analysed immediately, reflect accurately the in vivo blood gas tensions of the animal.

2.3 Results and Discussion

2.3.1 Oxygen equilibrium curves

2.3.11 Effect of temperature

The oxygen equilibrium curves for undiluted haemolymph of the three species, H. roei, H. ruber, and H. laevigata, in the absence of any CO_2 except that in injected air, are shown in appendix 1 a. The curves for all three species were obtained at 7°C, 15°C, 20°C, and 25°C; oxygen equilibrium curves for H. roei haemocyanin at the extreme temperature of 38°C are also given. Attempts to obtain similar high temperature curves for other haemocyanins failed due to excessive denaturation of haemocyanin. The temperatures 15°C and 20°C fall within the range of temperatures all three species experience in the field at West Island, and also within the geographical temperature range of the three species. No species ever experiences temperatures as low as 7°C in the field at any locality. 25°C is a higher temperature than is ever experienced in the field at West Island, except perhaps by H. roei exposed by low summer tides. Again, over the geographical range of the three species only H. roei is likely to experience temperatures above 25°C, although the maximum temperatures experienced by the other two species in some localities will approach this temperature.

In table 2.1 the P_{50} values obtained at the various temperatures for undiluted haemolymph in the absence of CO_2 are summarised for each species. These P_{50} values are plotted against temperature in Figure 2.3.

It can be seen that over the 18°C temperature range from 7°C to 25°C the P_{50} values of all species increase from 2.5 to 3 times. This sensitivity to temperature of the abalone haemocyanin is comparable to that observed in decapod crustacean Panulirus interruptus (Redmond, 1968a). In this temperate marine species the P_{50} more than doubles over a 10°C temperature rise from 15-25°C.

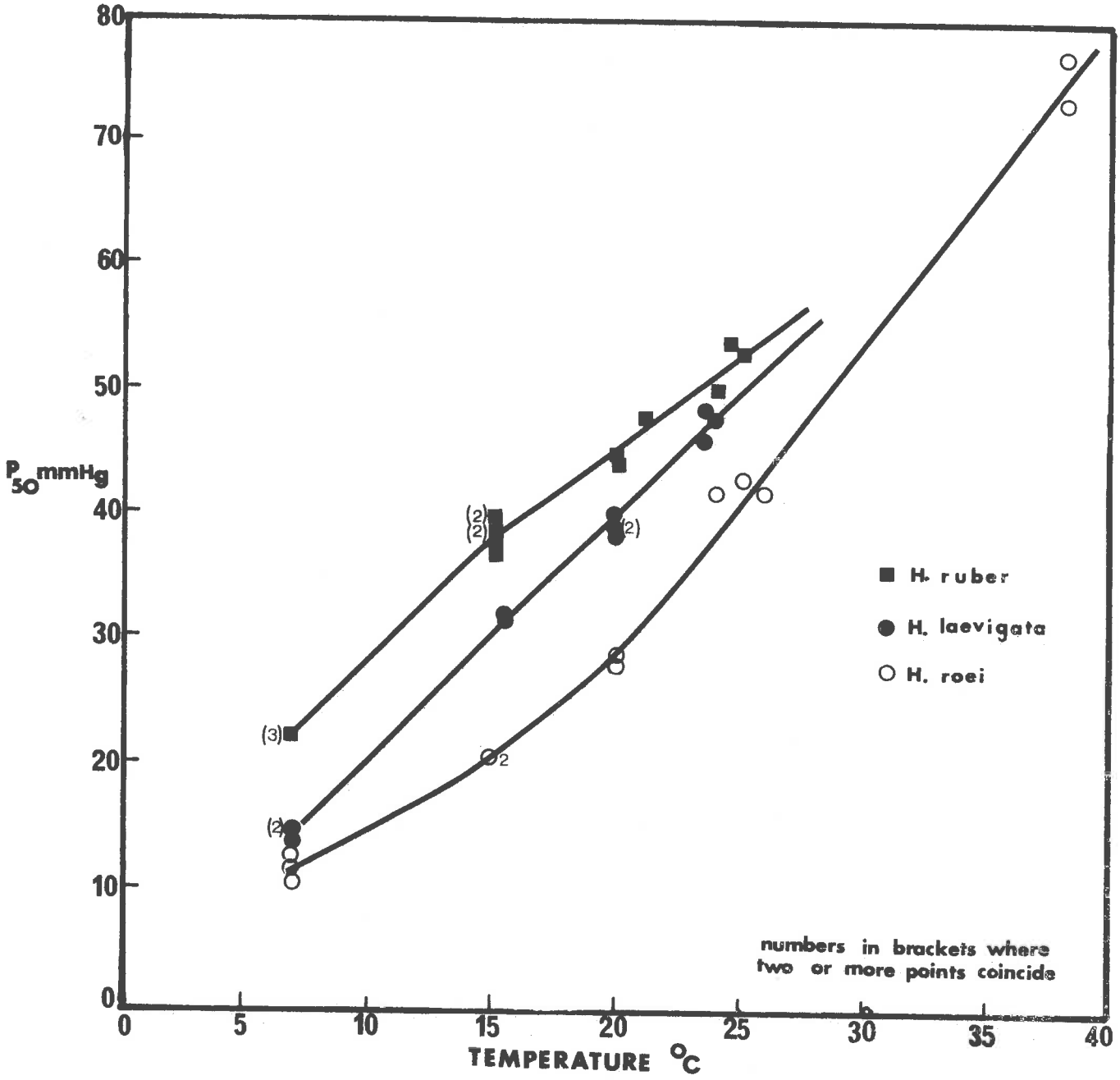
Table 2.1

P_{50} values of undiluted abalone blood at various temperatures in the absence of CO_2

Species	Animal Number	Temperature °C	P_{50} mm Hg
H. roei	1	38	74.0
H. roei	2	38	77.5
H. roei	1	26	42.0
H. roei	2	25	43.0
H. roei	3	24	42.0
H. roei	1	20	29.0
H. roei	2	20	29.0
H. roei	1	15	20.5
H. roei	2	15	20.5
H. roei	1	7	10.5
H. roei	2	7	12.0
H. roei	3	7	13.0
H. ruber	1	24.0	50
H. ruber	2	25	53
H. ruber	3	24.5	54
H. ruber	1	21	48
H. ruber	2	20	45
H. ruber	3	20	45
H. ruber	4	20	44
H. ruber	1	15	39
H. ruber	2	15	40
H. ruber	3	15	40
H. ruber	4	15	37
H. ruber	5	15	38
H. ruber	6	15	39
H. ruber	1	7	22
H. ruber	2	7	22
H. ruber	3	7	22
H. laevigata	1	23.5	49
H. laevigata	2	24.0	48
H. laevigata	3	23.5	46
H. laevigata	1	20	38.5
H. laevigata	2	20	40.0
H. laevigata	3	20	38.0
H. laevigata	4	20	38.5
H. laevigata	1	15.5	31.5
H. laevigata	2	15.0	32
H. laevigata	1	7	14.5
H. laevigata	2	7	14.5
H. laevigata	3	7	13.5

Figure 2.3

Plots of P_{50} values against temperature


80
70
60
50
40
30
20
10
0
P_{50} mmHg
0
5
10
15
20
25
30
35
40
TEMPERATURE °C
■ H. ruber
● H. laevigata
○ H. roei
numbers in brackets where two or more points coincide
(3)
(2)
(2)
(2)
(2)
2

From figure 2.3 it can be seen that although H. roei haemocyanin is evidently more resistant to heat denaturation than the haemocyanins of the other two species, there is no evidence to suggest that it is any less sensitive to temperature increase in terms of the effect of such an increase on the P_{50} over the whole temperature range from 7-25°C. However, it is perhaps worth noting that between 10°C and 20°C, the "normal" range of field temperatures abalone are likely to experience, the effect of temperature on the P_{50} of H. roei haemocyanin does seem slightly less pronounced than the effect on the P_{50}'s of the other two species.

From figure 2.3 it can be seen that the plots of P_{50} versus temperature for the three abalone species fall on three roughly parallel curves. At all temperatures the P_{50} of H. roei haemocyanin is considerably less than that of H. laevigata which is in turn considerably less than that of H. ruber. It is possible that the greater overall oxygen affinity of H. roei haemocyanin may be an adaptation which enables this species to "maintain about the same internal oxygen tensions" as the other two species at the higher temperatures which H. roei normally encounters (cf. Redmond, 1968a, p. 18).

From figure 2.3 it can be seen that there is a certain amount of individual variation in the P_{50} values. Although this variation does not prevent the observation of trends in P_{50} with temperature as discussed above, some possible reasons for it will be briefly considered. These experiments were all conducted on fresh haemolymph in the absence of CO_2. Nevertheless there are slight variations in the pH of freshly drawn haemolymph which may have contributed to individual variation in the P_{50} values (see section 2.3.13). Another possible source of variation in P_{50} between individuals may be the difference in concentration of the haemocyanin in the haemolymphs. Spoek et al., (1964) hypothesised that

this factor may have been the source of the variation they observed in the P_{50} values of Helix pomatia haemolymph. They state in the introduction of their paper that "In the course of our experiments it became clear that knowledge of influence of variations in the concentration of blood pigment and the salts would be of importance. Accordingly the copper content was determined in some of our samples". However they fail to point out in their results just how copper content (and hence haemocyanin concentration) influences the oxygen equilibrium curve. Extracting such information from their data is difficult because of the varying conditions of temperature and P_{CO_2} at which their experiments were conducted. In this study the concentration of haemocyanin in the various haemolymphs varied considerably. It was possible to see this simply by the varying blueness and varying maximum absorbances of the oxygenated haemolymphs. In some cases (Table 2.2) actual determinations of haemocyanin concentrations were made using methods described later in Chapter 5 (section 5.2.1). From this table it is apparent that for a given species, a considerable difference in haemocyanin concentration

Table 2.2

Effect of concentration of haemocyanin on P_{50} of oxygen equilibrium curve

Species and animal number (Appendix 1a)	Temperature °C	Haemocyanin concentration mg/ml	P_{50} mm Hg
H. roei 1	20.0	12.5	29.5
H. roei 2	20.0	8.5	28.5
H. ruber 2	24.5	8.8	43.0
H. ruber 3	25.0	3.6	44.5
H. laevigata 1	23.5	6.25	48.0
H. laevigata 5	23.5	4.25	46.0

does not appear to greatly influence the position of the oxygen equilibrium curve of the haemocyanin. Further, considering the results of all species, no constant relationship between the magnitude of the P_{50}, and concentration of the haemocyanin can be established.

Variation in the magnitude of the P_{50} values between individuals may be to some extent due to unavoidable experimental error, largely as a result of slight fluctuations in room temperature during the prolonged oxygen equilibrium determinations.

2.3.12 Oxygen affinities of Haliotis haemocyanins compared to oxygen affinities of other haemocyanins reported in the literature

The mean P_{50} values of H. roei, H. laevigata, and H. ruber haemolymphs at 20°C are 28.5 mm Hg, 38.4 mm Hg and 44.7 mm Hg. At first consideration, these might appear to be very high P_{50} values particularly in view of the fact that the P_{50} of at least one other marine gastropod (Busycon canaliculatum) has been shown to be only 12.2 mm Hg at the comparable temperature of 22°C (Redfield, 1926).

However, on examination of the literature reveals that many of the chitons (the other group of sluggish molluscs which bear haemocyanins) have haemocyanins with P_{50} values closely resembling those of the abalone in this study. Redmond (1962) determined the P_{50} values of the haemocyanins of four species of chiton at 25°C, finding values ranging from 20-26 mm Hg. Manwell (1958) found that the haemocyanin of a northern Pacific chiton Amicula stelleri had a P_{50} of 17 mm Hg at 10°C. Redmond (1962) extrapolates from this figure, on the basis of unpublished observations, and concludes that at 25°C the P_{50} of this species would be between 40 and 60 mm Hg. Thus the P_{50} values of this chiton would compare closely to those observed at similar temperatures for the three abalone species in this study. It seems then that these archaeo-gastropods share with the chitons a low oxygen affinity of the haemocyanin which has in the past been generally associated with highly

active animals such as the cephalopods (e.g. Loligo pealei P_{50} = 56 mm Hg at 23°C, Redfield et al., 1926).

2.3.13 Effect of P_{CO_2} and pH

(a) Effect of P_{CO_2}

The Figures in Appendix 1b are the oxygen equilibrium curves obtained for the haemocyanins of each of the species H. ruber, H. laevigata and H. roei over a range of temperatures (7°C, 15°C, 20°C and 25°C) at varying partial pressures of CO_2.

The P_{50} values of the oxygen equilibrium curves are summarised in Table 2.3. In Figure 2.4 the P_{50} values are plotted against P_{CO_2} for each species at each temperature.

An important fact to be gained from these data is that the haemocyanins of all three species of abalone demonstrate a strong reverse Bohr effect.

At low P_{CO_2} the increase in affinity of abalone haemocyanins is particularly marked. However, at increased CO_2 tensions the change in P_{50} becomes progressively less, and at least at 20°C, 15°C and 7°C it appears that a lower limit in the P_{50} of the haemocyanin is reached beyond which further increase in P_{CO_2} does not change the P_{50}. This lower limit is very similar for all three species, at each of the above mentioned temperatures.

It should be noted that the reverse Bohr effect in H. ruber haemocyanin appears to be slightly greater than that of H. laevigata which in turn is slightly greater than that of H. roei at all temperatures. CO_2 then appears to abolish the differences in the P_{50} values of the haemocyanins of the three species observed in the absence of CO_2.

At the lower temperatures of 7°C, and 15°C little difference can be seen in the P_{50} values of the three species when the P_{CO_2} is over about 4 mm Hg. Generally, however at low concentrations of CO_2,

Table 2.3

P_{50} values of oxygen equilibrium curves of undiluted abalone haemolymph, at 25°C, 20°C, 15°C and 7°C and with varying P_{CO_2}

H. laevigata

TEMPERATURE °C	P_{CO_2} mm Hg	P_{50} mm Hg
25	0	49.0
	2.8	27.0
	4.9	20.0
	11.1	13.5
20	0	55.0
	2.3	20.0
	14.5	8.0
	21.6	8.0
15	0	31.0
	2.4	15.5
	6.5	8.0
	11.1	7.0
7	0	15.0
	2.3	11.0
	9.5	6.0

H. ruber

25	0	54.0
	2.4	34.0
	5.0	24.0
	9.6	11.0
20	0	44.0
	2.7	19.0
	4.6	15.0
	9.2	10.0
15	0	41.0
	2.4	15.0
	5.0	11.0
	9.6	8.0
7	0	22.0
	2.6	9.0
	9.9	6.5

H. roei

25	0	46.0
	2.3	31.0
	5.1	22.5
	10.0	10.0
20	0	31.5
	2.7	17.0
	4.8	13.0
	11.0	8.0
	15.7	8.0

Table 2.3 (contd)

H. roei

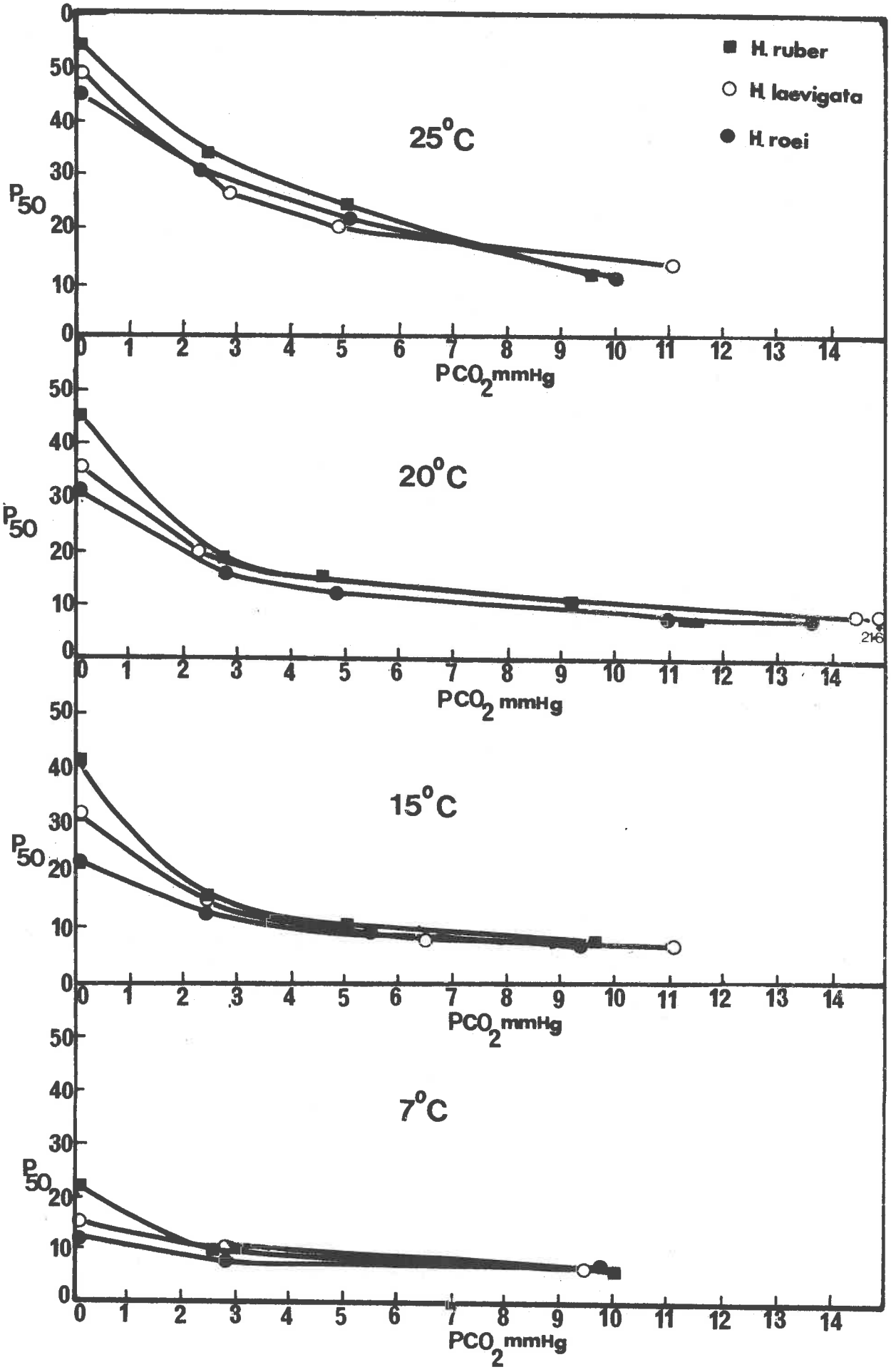
TEMPERATURE °C	P_{CO_2} mm Hg	P_{50} mm Hg
15	0	23.0
	2.4	15.0
	5.5	10.0
	9.4	7.0
7	0	12.0
	2.8	8.0
	4.9	8.0
	9.8	7.5

comparable to likely *in vivo* P_{CO_2}, H. roei haemocyanin has a greater oxygen affinity than H. laevigata which in turn is greater than H. ruber, as observed in the absence of CO_2.

Increasing P_{CO_2} not only affects the oxygen affinity but also the shape of the oxygen equilibrium curves of the haemocyanins of all three species. The Hill plots of the data from the P_{CO_2} and temperature experiments are given in Appendix 1 d. The hill plot is a plot of log $\frac{Y}{100-Y}$ against the log of the partial pressure of O_2, where Y is the percent haemocyanin combined with oxygen. The slope "n" of the curves thus obtained, where Y = 50%, is an approximate measure of the sigmoidicity of the oxygen equilibrium curves and hence of the degree of interaction between the sites of oxygen binding of the haemocyanin (see General Introduction; Manwell, 1960; Prosser, 1973). It can be seen from the values of "n" calculated from the Figures in Appendix 1 d, for all three species, that "n" is always greater than 1.6, indicating that there is always positive interaction between the oxygen combining sites of the haemocyanin. In all three species, at all temperatures, there is a trend towards decreasing values of "n" with increase in P_{CO_2}. Thus with increasing P_{CO_2} there is less interaction between oxygen combining sites on the haemocyanin.

Figure 2.4

Plots of P_{50} values against P_{CO_2} for *H. laevigata*, *H. roei* and *H. ruber* at 25°C, 20°C, 15°C and 7°C.


H. ruber
H. laevigata
H. roei
25°C
20°C
15°C
7°C
P50
PCO2 mmHg
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
0 10 20 30 40 50

Similarly in all three species there is a trend towards decrease in "n" with decrease in temperature, in the absence of CO_2.

(b) Effect of pH

As was pointed out in the introduction to this chapter, for comparison to the above results obtained by varying P_{CO_2} in undiluted haemolymph, a series of curves were determined at 20°C after adjusting the pH of the blood with the tris HCl buffers recommended by Redmond (1962).

The oxygen equilibrium curves obtained for the haemocyanins of the three species of abalone using these buffers to adjust pH are shown in Appendix 1 c.

A summary of the P_{50} values of the oxygen equilibrium curves at various pH's is given in Table 3.4.

In Figure 2.5 the P_{50} values are plotted against pH, for the haemocyanins of each species.

It can be seen that adjusting the pH using this recommended buffer again causes the haemocyanins to show a large reverse Bohr effect. The decrease observed in P_{50} is in this case directly related to pH; for each species the decrease in P_{50} is virtually linear over a wide pH range (8.4 - 6.8). As when the P_{CO_2} was varied, the reverse Bohr effect is greatest with H. ruber haemocyanin and slightly greater with H. laevigata than with H. roei haemocyanin. The "size" of the reverse Bohr effect can be expressed by ϕ values (e.g. Redmond, 1962) where $\phi = \log P_{50} / \text{pH}$. From the data in Figure 2.5, the following ϕ values are calculated for abalone haemocyanins.

Haliotis ruber ..... $\phi = +.45$

Haliotis laevigata ..... $\phi = +.32$

Haliotis roei ..... $\phi = +.30$

Table 2.4

Effect of pH on P_{50} values of oxygen equilibrium curves of buffered abalone haemolymph at 20°C

Species	pH	P_{50} mm Hg
H. ruber	6.78	9.0
H. ruber	6.85	7.0
H. ruber	7.25	22.0
H. ruber	7.65	34.0
H. ruber	8.30	52.0
H. ruber	8.40	51.0
H. roei	6.70	11.5
H. roei	6.78	12.5
H. roei	7.25	21.5
H. roei	7.80	32.0
H. roei	8.38	43.5
H. roei	8.42	40.0
H. laevigata	6.80	13.0
H. laevigata	6.85	12.0
H. laevigata	6.90	13.5
H. laevigata	7.25	20.0
H. laevigata	7.80	31.0
H. laevigata	8.20	35.0
H. laevigata	8.30	54.5
H. laevigata	8.30	40.0

These values can be compared with other ϕ values in the literature. For example Redmond (1962b) measured the Bohr effect of Chiton haemocyanins by adjusting the pH of the haemolymph with the Tris-HCl buffer used in this investigation. Chiton tuberculatus and Acanthopleura granulata haemocyanins both have reverse Bohr effects. The ϕ values for these two species, + .11 and + .02 respectively, are much smaller than those calculated for the abalone in this study. However, Redmond (1968a) reports ϕ values for two genera of marine snail, Fusitriton and Fasciolaria, to be + 2.12, and + 1.26 respectively. As Redmond states, these are both very large values, especially that for Fusitriton. It is evident that the ϕ values for abalone haemocyanins are well within the range of values observed for other molluscan haemocyanins which have a reverse Bohr effect.

Figure 2.5

P_{50} values plotted against pH

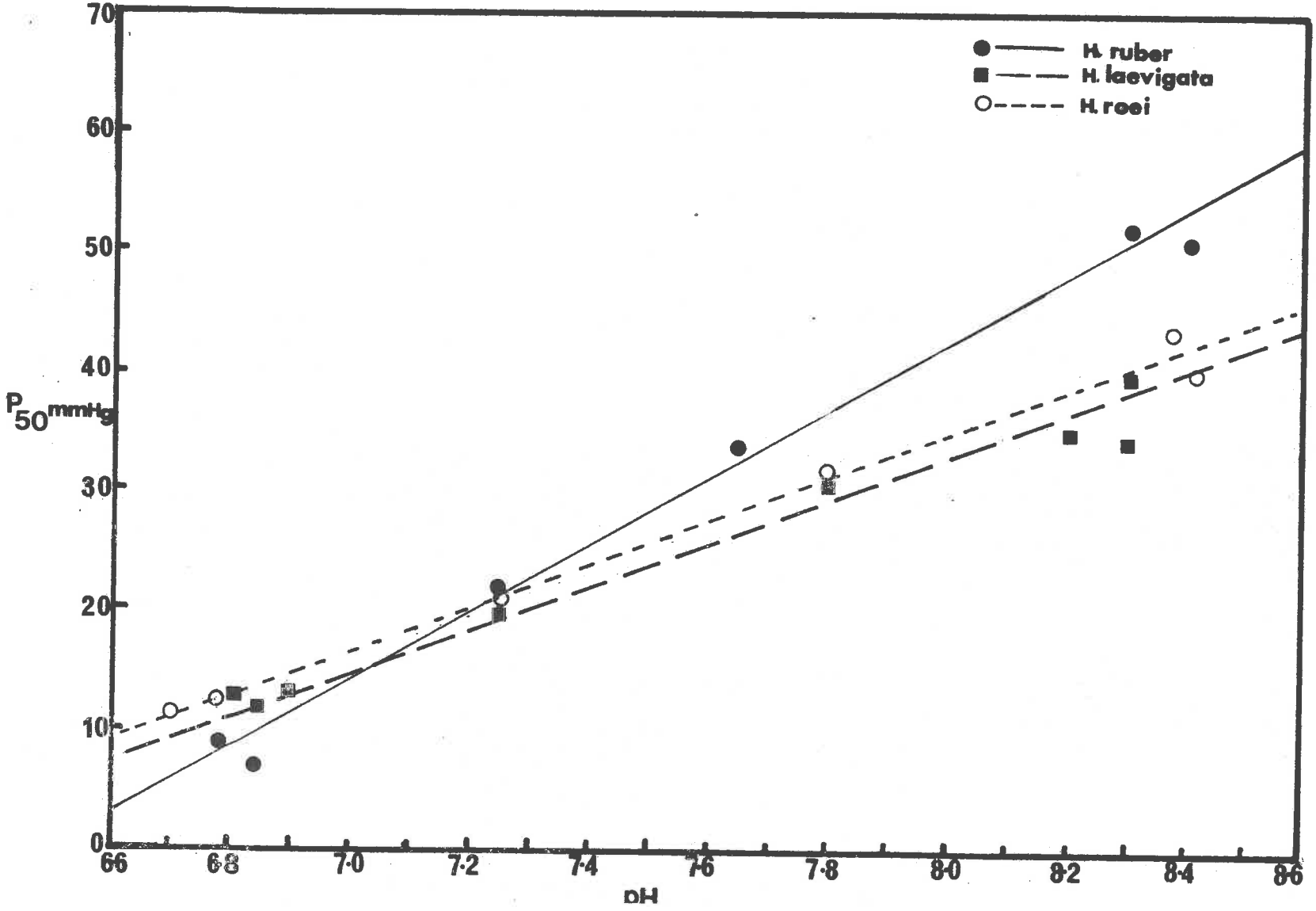

H. ruber
H. laevigata
H. roei
P_{50} mmHg
70
60
50
40
30
20
10
0
6·6
6·8
7·0
7·2
7·4
7·6
7·8
8·0
8·2
8·4
8·6
pH

Table 2.5 below gives a series of pH determinations for freshly drawn haemolymph of the three abalone species at 20°C.

Table 2.5

pH of fresh haemolymph at 20°C

	H. ruber	H. laevigata	H. roei
	7.35	7.30	7.30
	7.43	7.55	7.35
	7.35	7.25	7.55
	7.55	7.35	7.40
	7.37	7.28	7.42
	7.44	7.25	7.32
	7.35	7.38	7.55
	7.45	7.30	7.23
	7.30	7.25	7.40
	7.25		
	7.40		
	7.35		
	7.35		
Mean ± S.D.	7.38 ± .075	7.32 ± .096	7.37 ± .089

As can be seen, there is some variation in the pH of freshly extracted haemolymph. Some may be "real" variation, but some may be due to the previously mentioned difficulty in obtaining a stable pH reading on fresh abalone haemolymph. Nevertheless, the mean pH of freshly drawn haemolymph of all species is about 7.3 to 7.4.

Table 2.6 below compares the P_{50} values obtained using freshly drawn haemolymph in the absence of any further treatment, with P_{50} values of haemolymphs at the same pH but with the pH adjusted with small amounts of the two tris-HCl seawater buffers previously described.

Even allowing for a much larger variation in the pH of freshly drawn haemolymph than was observed, it can be seen that the small amount of tris-HCl seawater buffer used to adjust pH has caused an increase in the oxygen affinities of the haemocyanins, especially in the cases of H. ruber and H. laevigata. Further experience (not reported in detail here) with more concentrated tris buffers support the idea that tris itself caused the increase in oxygen affinity. I suspect that Ca^{++} in

Table 2.6

P_{50} values of freshly drawn haemolymph compared to P_{50} values of buffered haemolymph at same pH

Species	Mean P_{50} (mm Hg) of freshly drawn Haemolymph at 20°C	Mean pH of Freshly drawn Haemolymph (20°C)	P_{50} (mm Hg) at mean pH of fresh haemolymph - Buffer Adjusted (20°C)
H. roei	28.5	7.37	23.5
H. laevigata	38.8	7.32	21.5
H. ruber	44.7	7.38	25.0

the haemolymph is being chelated by the tris molecule. Reduction in the Ca^{++} concentration is known to cause subunit dissociation of haemocyanin with a concomitant increase in oxygen affinity (Larimer and Riggs, 1964).

These experiments using tris-HCl buffers to adjust the pH of the haemolymph provide support for the finding with CO_2 that the haemocyanins of all three species of abalone have a large reverse Bohr effect, however they emphasise that care must be taken in interpreting experiments with buffered haemocyanin.

2.3.2 Oxygen capacities of abalone haemocyanins

Table 2.7 gives the oxygen capacities (in volumes % carried by haemocyanin) and haemocyanin concentrations of the blood of individual H. roei, H. laevigata and H. ruber. The mean and standard deviation of both oxygen capacities and haemocyanin concentrations are given in the table.

It can be seen that for each species there is a wide range of haemocyanin concentration. Large variation in haemocyanin concentration has also been recorded for four species of California abalone, Haliotis fulgens, H. corrugata, H. rufescens, and H. cracherodii (Pilson, 1964),

and also in other gastropod molluscs, notably the conch Busycon canaliculatum (Betzer and Pilson, 1974). This large range in haemocyanin concentrations will be discussed further in reference to the field sampling of the haemocyanins of the three species in this study (Chapter 5).

Table 2.7

Oxygen capacities (in vol% carried by haemocyanin) and haemocyanin concentration of the blood of individual abalone

	H. roei		H. laevigata			H. ruber		
Animal No.	Haemocyanin concentration mgm/ml	Oxygen capacity Haemocyanin vol %	Animal No.	Haemocyanin concentration mgm/ml	Oxygen capacity Haemocyanin vol %	Animal No.	Haemocyanin concentration mgm/ml	Oxygen capacity Haemocyanin vol %
1	6.8	1.40	1	4.6	1.03	1	6.3	1.22
2	7.2	1.15	2	4.0	1.15	2	5.2	1.095
3	6.2	1.35	3	4.7	1.20	3	4.0	1.19
4	8.52	1.43	4	4.3	.97	4	1.1	.64
5	7.4	1.21	5	4.4	.97	5	3.8	.91
6	8.4	1.52	6	3.2	.75	6	4.0	.97
7	6.7	1.16	7	4.3	1.22	7	7.1	1.19
8	8.6	1.40	8	3.1	.79	8	3.9	.64
9	7.5	1.52	9	3.5	.85	9	2.6	.79
10	3.16	.79	10	3.0	.70	10	2.6	.78
11	7.9	1.40	11	2.9	.76	11	4.1	1.06
12	12.9	2.19	12	5.4	1.03	12	5.2	1.03
13	10.25	1.69	13	5.25	1.22	13	2.5	.75
14	9.6	1.99	14	3.16	.91	14	7.8	1.38
			15	6.4	1.55	15	3.4	1.56
			16	6.8	1.55			
Means ± S.D.	7.9±2.2	1.44±.35		4.3±1.2	1.04±.26		4.6±2.1	1.01±.27

H. ruber and H. laevigata haemocyanins have similar mean O_2 capacities (Table 2.7). H. roei haemocyanin has a significantly higher mean O_2 capacity than H. laevigata, $P < .005$, or H. ruber, $P < .001$, (Comparison of means of samples of unequal sizes - Students t test).

Table 2.8 compares the oxygen capacities determined for the haemocyanins of the three species of South Australian abalone with some of those known for other molluscs. From the table it can be seen that the O_2 capacities of the abalone compare reasonably closely with those of other gastropod mollusc, Busycon although it appears that they have a slightly lower average oxygen capacity than this species. The oxygen capacities of the abalone are considerably lower than those of the more active cephalopod mollusc, but greater than those of the amphineuran molluscs, the other "sluggish" molluscan group which has haemocyanin in the haemolymph.

Table 2.8

Typical oxygen capacities of some molluscan haemocyanins (excluding dissolved O_2)

Class	O_2 capacity of haemocyanin vol %	Reference
Cephalopods		
Loligo pealei	3.4-4.1	Redfield et al., 1926
Gastropods		
Busycon canaliculatum	1.65-2.90	Redfield et al., 1926
Haliotis roei	.79-2.19	Present study
Haliotis ruber	.64-1.56	"
Haliotis laevigata	.7 -1.55	"
Amphineurans		
Chiton tuberculatus	.50-1.13	Redmond, 1962b
Katherina tunicata	.55-.65	"
Nepalia muscosa	.61-.82	"
Amicula stelleri	.26-.36	"

In Figure 2.6 are plots of haemocyanin concentration against oxygen capacity of the haemocyanin for each of the three species. The dashed lines are fitted regression lines, the correlation coefficients are given on the figure.

The Maegraith et al. (1950) technique for measuring O_2 capacity measures the O_2 capacity of the respiratory pigment alone, (see Edmonds 1957, page 93) unlike the Van Slyke blood gas analysis, which by relying on an evacuation technique measures O_2 capacity of whole blood. For this reason it would be expected a priori that the regression lines fitted to the data obtained for the O_2 capacities and haemocyanin concentrations would pass through the origin. In other words, where there was no haemocyanin, no O_2 would be detected when the haemolymph was poisoned. From Figure 2.6 it can be seen that in fact the regression lines do not pass through the origin, but meet the Y axis slightly above the origin. This observation lead to the hypothesis that the relationship between haemocyanin concentration and oxygen capacity is not linear, the higher concentrations releasing proportionally less oxygen. The results of the serial dilution experiments are given on Table 2.9 and plotted on Figure 2.7. Again it is evident from these results that halving the concentration of the haemocyanin in the haemolymph does not result in halving the O_2 capacity of the haemocyanin in solution. In order to test if the poisoning process was incomplete the solutions were examined spectrophotometrically after the O_2 capacities had been determined. Figure 2.8 shows a typical absorbance spectrum of the oxygenated solution treated as described in section 2.1.21. In no case was there any evidence of oxygenated haemocyanin, which would have been expected to demonstrate a typical peak of absorbance at 345 *nm* (Chapter 6, Fig. 6.1 b). This suggests that at all concentrations the haemocyanin was being effectively poisoned to cause release of all oxygen, supporting the idea that at higher concentrations the haemocyanins of the abalone are combined

Table 2.9

Oxygen capacities, and haemocyanin concentrations of serial dilutions of abalone blood

		Pure haemolymph	Diluted x $\frac{1}{2}$ with .025M Tris HCl .5M NaCl pH 7.35	Diluted x $\frac{1}{4}$ with .025M Tris HCl .5M NaCl pH 7.35
H. ruber				
1	O_2 cap.(Vol %)	1.56	1.25	.78
	Hcy conc.(mgm/ml)	8.4	4.2	2.1
2	O_2 cap.	1.38	1.05	.65
	Hcy conc.	7.8	3.9	1.95
H. laevigata				
1	O_2 cap.	1.55	1.05	.75
	Hcy conc.	6.8	3.4	1.7
2	O_2 cap.	1.55	.95	.62
	Hcy conc.	6.4	3.2	1.6
H. roei				
1	O_2 cap.	1.69	1.18	.81
	Hcy conc.	10.25	5.125	2.56
2	O_2 cap.	1.99	1.40	1.0
	Hcy conc.	9.6	4.8	2.4

with proportionally less oxygen than at lower haemocyanin concentrations. It should be noted however that this effect is not very great at the lower haemocyanin concentrations usually found in the abalone.

It has long been accepted that haemocyanin combines with oxygen in the ratio of one molecule of oxygen to a quantity of haemocyanin containing exactly two atoms of copper (Redfield et al., 1928; Redfield, 1934). The logical extension of this, is that a doubling in the concentration of haemocyanin should exactly double the amount of oxygen which can be bound to the haemocyanin in the solution. However I suggest that this apparently is not the case with the haemocyanins of H. ruber, H. roei, and H. laevigata. It would seem that at higher concentrations

Figure 2.6

Variation of oxygen capacity of haemocyanin in the blood of *Haliotis ruber*, *H. roei*, and *H. laevigata*, with concentration of haemocyanin

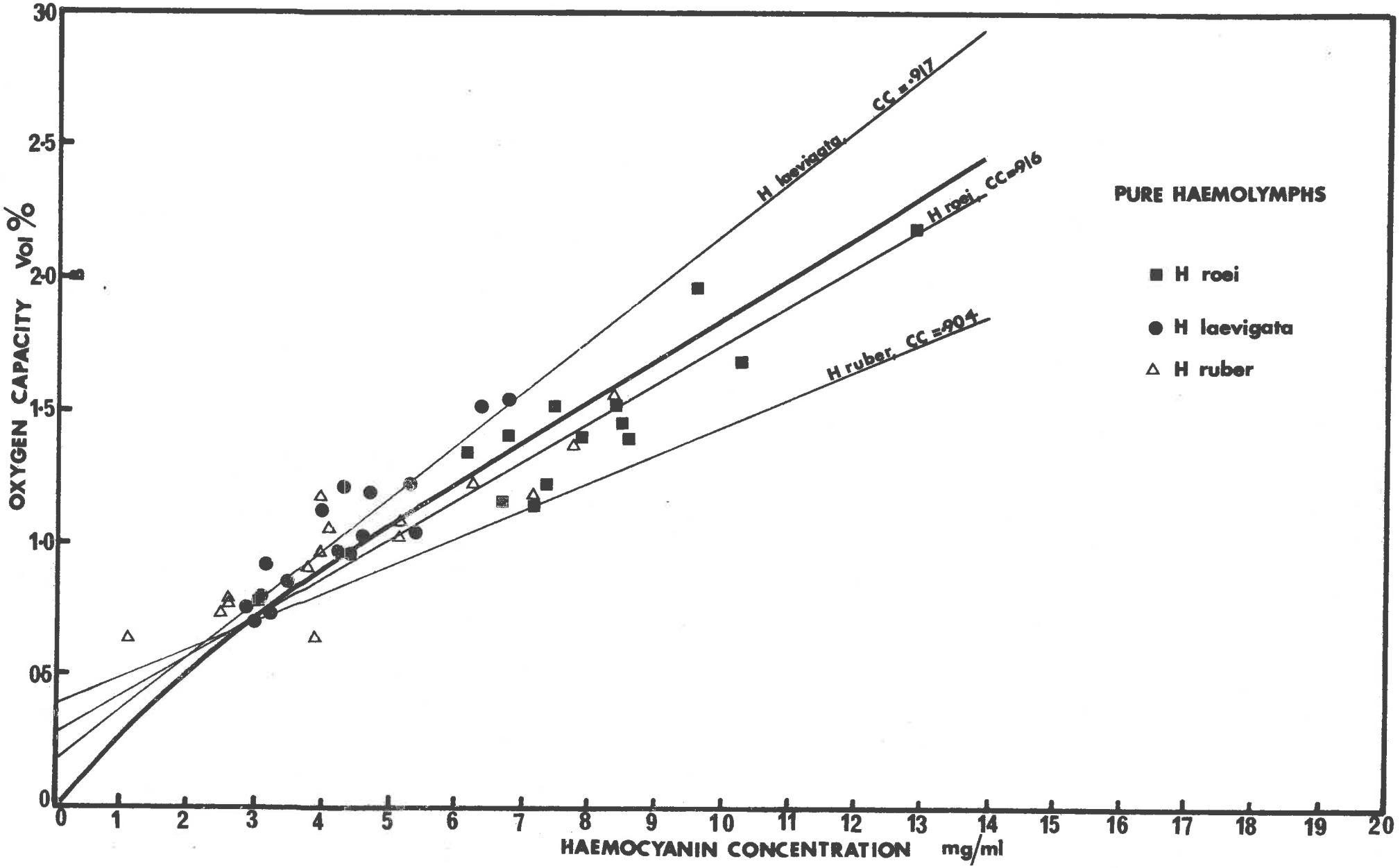

30
2·5
2·0
1·5
1·0
0·5
0
OXYGEN CAPACITY Vol %
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
HAEMOCYANIN CONCENTRATION mg/ml
H laevigata, CC =·917
H roei, CC=916
H ruber, CC =904
PURE HAEMOLYMPHS
H roei
H laevigata
H ruber

Figure 2.7

Effect of serial dilution of abalone blood on the relationship between oxygen capacity of haemocyanin and haemocyanin concentration

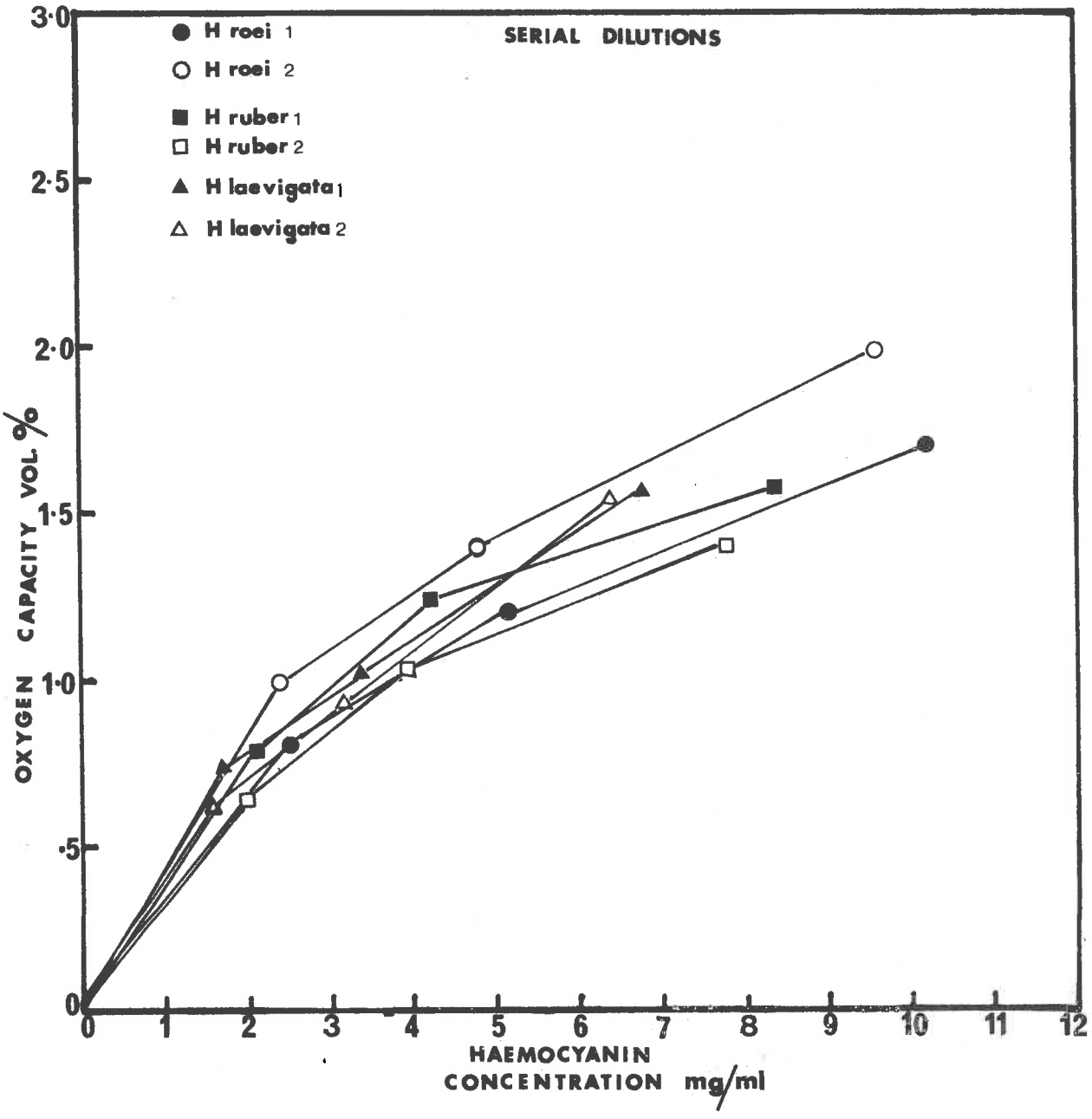
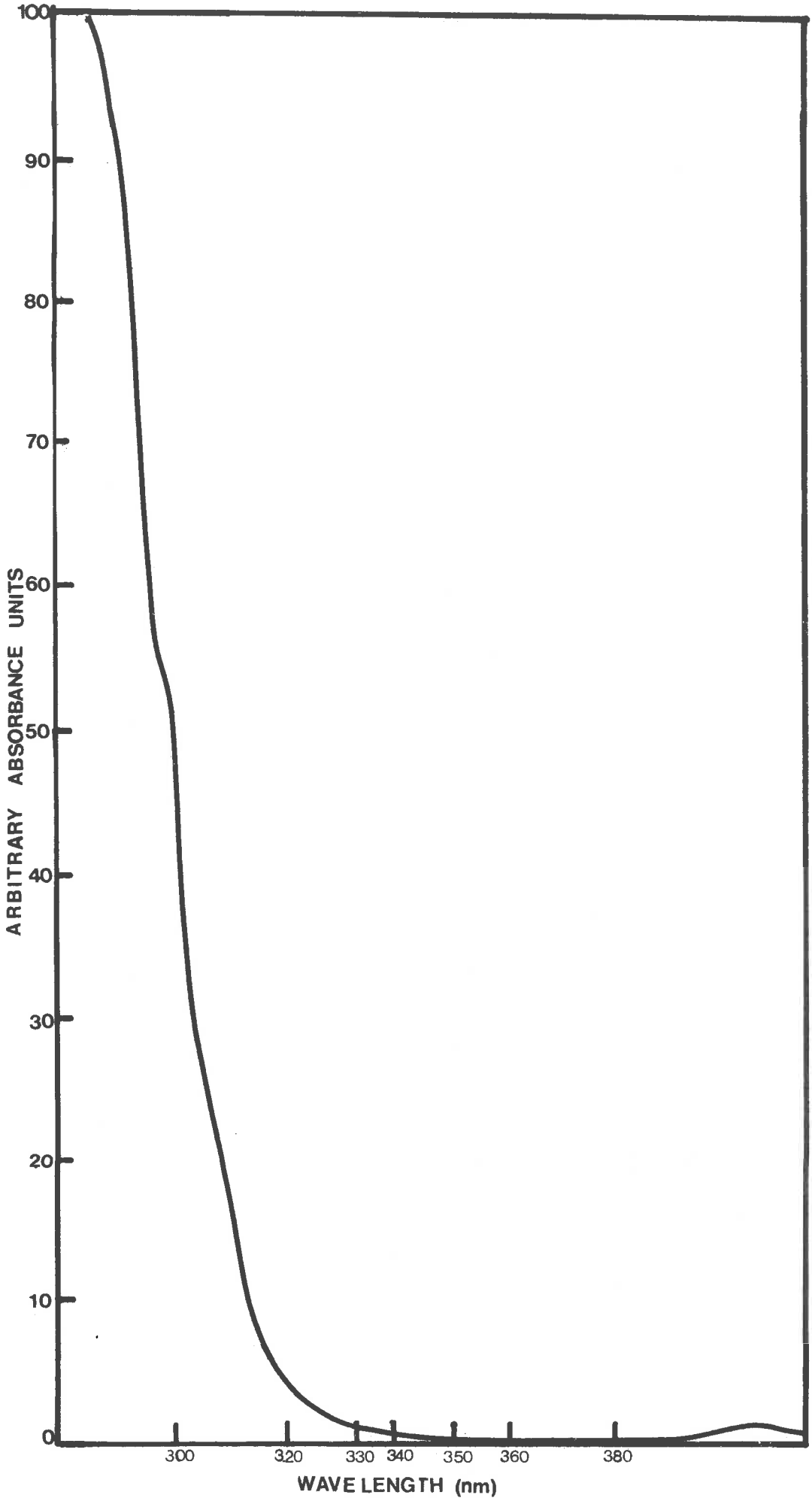

SERIAL DILUTIONS
H roei 1
H roei 2
H ruber 1
H ruber 2
H laevigata 1
H laevigata 2
OXYGEN CAPACITY VOL %
3·0
2·5
2·0
1·5
1·0
·5
0
0 1 2 3 4 5 6 7 8 9 10 11 12
HAEMOCYANIN CONCENTRATION mg/ml

Figure 2.8

Absorbtion spectrum of the supernatant obtained after centrifuging the products of the O_2 capacity determination procedure. Note the absence of a typical haemocyanin absorbing peak at 346 nm (cf. Figure 6.1 b)


100
90
80
70
60
50
40
30
20
10
0
ARBITRARY ABSORBANCE UNITS
300
320
330
340
350
360
380
WAVE LENGTH (nm)

of haemocyanin proportionally less copper atoms are available to combine with oxygen. This may be a result of aggregation of haemocyanin molecules at higher concentrations such that some copper atoms are "buried" and unable to combine with oxygen.

From Figure 2.6 it can be seen that where the haemocyanin concentrations of the three species overlap, there is much similarity in the O_2 capacities.

Although there are differences in the regression lines obtained for the three species, I believe that these differences can to some extent be accounted for by the different average haemocyanin concentrations of the three species - this is particularly true in the case of H. roei and H. laevigata. Combining the evidence of Figure 2.6 and Figure 2.7 suggests that the plot of O_2 capacity versus haemocyanin concentration might be more truly represented for all species by the unbroken curve on Figure 2.6 (fitted by eye).

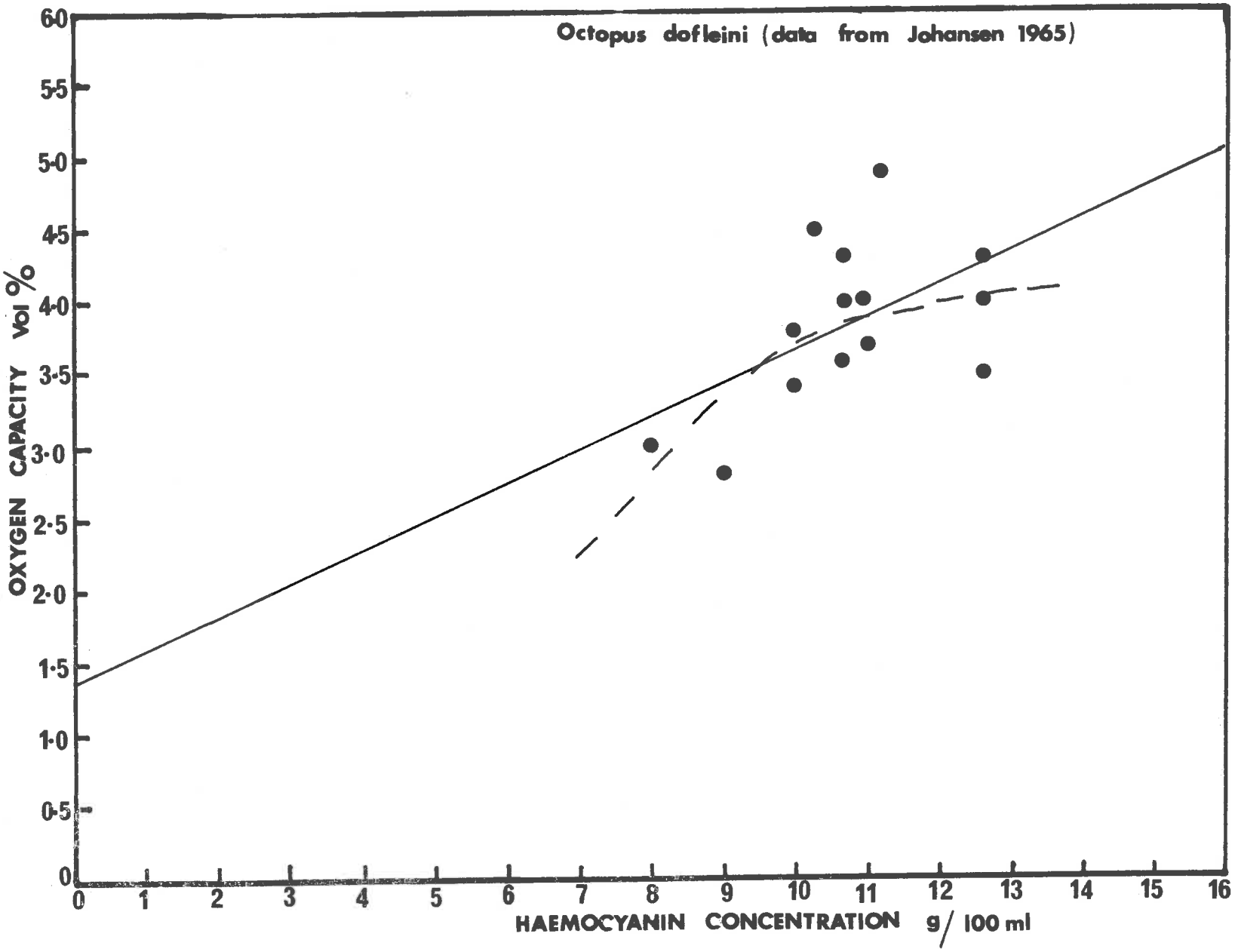
There is a lack of information in the literature of other attempts to directly relate the concentration of the haemocyanin in the haemolymph to the O_2 carrying capacity of the haemocyanin over a wide range of concentrations. For the most part it is generally accepted that O_2 capacity relates in a linear fashion to haemocyanin concentration.

However, in determining O_2 capacities of the blood of Octopus dofleini Johansen (1965) found that "It was surprising to find several inconsistencies in the expected correlation between the oxygen capacity and the haemocyanin content". (Underlining mine). A plot of Johansen's data (Figure 2.9) shows that animals having the highest haemocyanin concentration do not have O_2 capacities as high as expected from the O_2 capacities of animals with less concentrated haemocyanin.

The regression line fitted to Johansen's data intercepts the Y axis well above the origin. The lack of O_2 capacity determinations for very low concentrations of the Octopus dofleini haemocyanin make comparison to the data in this Chapter difficult, but the figures presented tend to suggest that a similar non-linear relationship between haemocyanin concentration and O_2 capacity may exist for the haemocyanin of this cephalopod.

Figure 2.9

Relationship between oxygen capacity, and haemocyanin concentration in *Octopus dofleini* (from the data of Johansen, 1965)


Octopus dofleini (data from Johansen 1965)
OXYGEN CAPACITY Vol %
HAEMOCYANIN CONCENTRATION g/ 100 ml
0
0·5
1·0
1·5
2·0
2·5
3·0
3·5
4·0
4·5
5·0
5·5
6·0
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16

2.3.3 Internal Gas Tensions

Tables 2.10-2.12 give the results of the measurements of the oxygen and carbondioxide partial pressures of abalone haemolymph taken from three sampling points. These points, as explained earlier in the Chapter, are the heart, containing blood directly from the ctenidia; the renal sinus containing immediately prebranchial blood, and the foot muscle, an intermediate sample.

Tables 2.10-2.12 give the results of gas tension determinations of animals "acclimated" to 20°C and examined at this temperature. Tables 2.13-2.15 give the results of 20°C acclimated animals subjected to an abrupt temperature increase to 25°C, two hours before sampling. The Tables also give the P_{O_2} and P_{CO_2} values of the seawater from which the animals were taken immediately before sampling. The means and standard deviations of all measured parameters are given in each Table.

The 20°C acclimated animals, sampled at 20°C will be considered first.

An examination of the mean values for each parameter gives a general picture of how P_{O_2} and P_{CO_2} vary within the three abalone species. More will be said about variation between individuals later.

Tables 2.10-2.12 show that for each of the three species at the temperature of acclimation (20°C) the mean immediate postbranchial P_{O_2} is almost 40 mm Hg (H. ruber, 38.8 ± 3.98; H. laevigata, 37.4 ± 3.7; H. roei, 36.7 ± 4.05).

This value compares closely with that of the only other species of gastropod mollusc for which internal P_{O_2} has been investigated - Busycon canaliculatum. Florkin (1934) calculated the arterial P_{O_2} of this gastropod from the data of other authors and obtained a figure of 36.0 mm Hg.

This similarity of internal arterial P_{O_2} of the abalone and the conch, is interesting in view of the differences in the

Table 2.10

Internal Gas Tensions of H. ruber at 20°C

Animal No.	Heart		Foot		Renal Sinus		Sea Water	
	O_2 mm Hg	CO_2 mm Hg	O_2 mm Hg	CO_2 mm Hg	O_2 mm Hg	CO_2 mm Hg	O_2 mmHg	CO_2 mm Hg
1	35	1.25	-	-	9	4	149	.25
2	34	1	16	2	9	5	149	.25
3	-	-	17	2.25	16	3	149	.25
4	45	1.1	25	2.75	17	3	158 +158	.5
5	41.5	1.5	17	2.75	14	6	153	.5
6	39.5	1.25	22	2.75	12	*	155	.5
7	39	1.5	13	3.25	14.5	4	151	.25
8	54	1	21	3	12	8	154	1.0
9	41.5	1.5	21	2.25	9.5	*	140	.75
10	46	1	26	2	-	-	154	.75
11	36	.75	28	2	10	5	-	-
12	40	1.25	28	3.25	14	*	154	.75
13	38	1.5	15	2.0	6	13	158	.25
14	35	1.0	32	2	7	9.0 then rising	155	.5
Means ± S.D.	38.8 ±3.98	1.2 ±.25	21.6 ±5.9	2.5 ±.49	11.5 ±3.4	6 ± 3.16	152.5 ±4.8	.5 ± .25

* in these renal sinus samples the CO_2 content was off scale. The readings were considered an abberation - see text

Table 2.11

Internal Gas Tensions of H. roei at 20°C

Animal No.	Heart		Foot		Renal Sinus		Sea Water	
	O_2 mm Hg	CO_2 mm Hg	O_2 mm Hg	CO_2 mm Hg	O_2 mm Hg	CO_2 mm Hg	O_2 mm Hg	CO_2 mm Hg
1	35 34	1 1	-	-	11	4.25	148.5	.5
2	28	1	18	2.5	7	4.25	148	.5
3	37.5	2.5	20	5	6.5	Rising off scale*	150	1.5
4	35	1	13	1	3.5	*	145.5	2
5	43.5	1.25	25	2.0	13	5.5	140	.75
6	58	1.5	30	2.75	8	*	149	.25
7	38	1.25	-	-	-	-	152	.25
8	38 42	1.3 1.1	21	2.0	10	5 and then creeping up	151	.25
9	36.5	1.25	16.5	3	14	4.5	157	.25
10	29	1.25	12	3.25	12	5	140	.5
11	40.5	1.0	19	2.0	13	4	-	-
12	38	.5	-	-	8	6	-	-
13	36 38	1.25 1.25	-	-	-	-	152	.25
Means ± S.D.	36.7 ±4.05	1.21 ±.40	19.4 ±5.6	2.1 ±1.0	9.6 ±3.3	4.3 ±.91	148.5 ±5.09	.64 ±.58

Table 2.12

Internal Gas Tensions of H. laevigata at 20°C

Animal No.	Heart O_2 mm Hg	Heart CO_2 mm Hg	Foot O_2 mm Hg	Foot CO_2 mm Hg	Renal Sinus O_2 mm Hg	Renal Sinus CO_2 mm Hg	Sea Water O_2 mm Hg	Sea Water CO_2 mm Hg
1	33.5 34	1.5 -	16	2.75	11	4.5	146	1
2	35	1.5	32	1.5	12	4.25	149.5	.5
3	32	1.25	18	2.25	14	-	148	.66
4	38	1.25	32	2.5	14	3.25	152	.75
5	38.5 34	1 1	-	-	17	2.5	147	.5
6	38	1	24	2	14	4.5	147	.75
7	36 33	1.25 1.25	20.5	2	15.5	2.25	141	.75
8	42 41	1.25 1.25	26	3.25	16	3	151	.3
9	42.5 42.5	1.0 1.15	22	2	11	3.75	151.5	.25
10	40.5	1.25	27	2.25	13	Moving up off scale *	153	.5
Means ± S.D.	37.4 ±3.7	1.21 ±.17	24.2 ±5.7	2.2 ±.36	13.0 ±2.0	3.5 ±.99	148.6 ±3.6	.6 ±.23

oxygen affinities of the haemocyanins at this temperature.

As has been explained, samples from the renal sinus would be expected to contain immediately prebranchial blood (Crofts, 1929), which has been deoxygenated by passing through the tissues. Tables 2.10-2.12 show that the mean P_{O_2} of the haemolymph from the renal sinus of all three species is considerably lower than the O_2 tension of the postbranchial (heart) bloods (H. ruber, 11.5 ± 3.4 mm Hg; H. laevigata, 13.8 ± 2.0 mm Hg; and H. roei, 9.6 ± 3.3 mm Hg). These values are somewhat higher than those predicted by Florkin (1934) for Busycon canaliculutum venous blood. Now, if the O_2 tensions of the samples taken from the foot muscle of the abalone are examined, it can be seen that in each case the blood has a mean P_{O_2} which is considerably less than that from the heart, but greater than that from the renal sinus. It should be noticed that the standard deviations about the means of the "foot blood" samples are greater than those of either the heart or renal sinus samples. This reflects the greater variability in the foot samples, resulting from the fact that in sampling the foot muscle blood was being collected from an area containing vessels and sinuses with both venous, and arterial blood. The mean P_{O_2} values of foot blood are: H. ruber, 21.6 ± 5.9 mm Hg; H. laevigata, 24.2 ± 5.7 mm Hg; and H. roei, 19.4 ± 5.6 mm Hg.

As has been continually emphasised in this Chapter, in order to determine the role of haemocyanin in delivering O_2 to the tissues, these internal oxygen tensions must be related to the characteristics of the oxygen equilibrium curves. In order to be able to do this it was necessary also to obtain a measurement of internal P_{CO_2} so that the in vivo shape and position of the oxygen equilibrium curves could be defined. Tables 2.10-2.12 give the P_{CO_2} values of the heart, foot and renal sinus haemolymphs, and the mean and standard deviations of all

measurements. From these tables it can be seen that for all three species, the partial pressure of CO_2 in the haemolymph from the heart is always low - H. roei, 1.21 ± .41 mm Hg; H. ruber, 1.20 ± .25 mm Hg; H. laevigata, 1.21 ± .17 mm Hg - but nevertheless higher than that in the seawater from which the animals were taken.

From the mean P_{CO_2} values in heart, foot, and renal sinus haemolymphs it can be seen that as the blood moves through the tissues to the renal sinus, before re-entering the ctenidia, the P_{CO_2} increases considerably. The mean P_{CO_2} values of the immediate prebranchial haemolymph of the three species are of the order of three to five times as high as the postbranchial P_{CO_2} values (H. ruber, 6 ± 3.16 mm Hg; H. roei, 4.3 ± .91 mm Hg; and H. laevigata, 3.5 ± .89 mm Hg). These values compare reasonably with the in vivo P_{CO_2} values calculated for Busycon canaliculatum (Henderson, 1928). In the conch arterial P_{CO_2} was calculated to be 2.0 mm Hg, whereas venous P_{CO_2} was 5.3 mm Hg. It can be seen that the arterial-venous difference in P_{CO_2} is larger for the abalone than calculated for the conch. However the arterial-venous difference in P_{CO_2} for abalone is of the same order as that recorded in the cephalopod mollusc Loligo pealei, where arterial and venous P_{CO_2} was determined at 2.2 mm Hg, and 6.0 mm Hg respectively (Redfield, 1929).

In some cases (see Tables 2.11 and 2.12) the P_{CO_2} of the renal sinus samples was not recorded. In these, instead of giving a normal response, the P_{CO_2} reading was off-scale, indicating a P_{CO_2} of over 80 mm Hg. Due to the sampling technique the renal sinus samples sometimes contained visible contaminants from the surrounding digestive gland; samples with such contaminants were observed to give the above described P_{CO_2} response. After such responses with contaminated sera, the CO_2 electrode had to be well cleaned before giving reasonable readings with calibrating gases. The P_{CO_2} values from contaminated renal sinus samples were therefore discarded.

In this study there is considerable individual variation in the P_{O_2} of pre and postbranchial samples. Despite this variation, in every case there is a distinct arterial-venous difference in P_{O_2}. There are no other series of *in vivo* P_{O_2} determinations of gastropod molluscs to compare with the results of the present study. However, where the *in vivo* P_{O_2} of other groups of haemocyanin bearing animals have been examined similar variability between individuals has been observed. This can be noted, for example in various crustaceans (Redmond, 1955, Table 2; Redmond, 1968b, Table 1; Johansen *et al.*, 1970, Table 2) and also in cephalopod molluscs (Johansen *et al.*, 1966, Table 1). Variation in the P_{O_2} of blood samples taken from the same region of individual chitons was also noticed by Redmond (1962b).

There may be several reasons for such variability in the P_{O_2} values of both arterial and venous blood samples. It is possible, with the sampling method used in this study that some contamination of the haemolymph occurred. Although particular care was taken to avoid this, the close connection between the pericardial cavity, kidney, and gonads, made it impossible to ensure that no contamination by fluids from these regions occurred. In this study it is impossible to assess the amount of variation in the results caused by such contamination.

Johansen *et al.* (1966) have shown that the partial pressure of oxygen in the "inhaled" water can directly influence the arterial oxygen partial pressure in the cephalopod *Octopus dofleini*; the greater the P_{O_2} of the inhaled water the greater the P_{O_2} in the arterial haemolymph. In the present experiments all animals were kept in highly aerated seawater prior to sampling. Hence there was little variation in the P_{O_2} of inhaled water - it was always saturated. Because of this fact it would be difficult to conclude whether the P_{O_2} of inhaled water has any significant affect on the P_{O_2} of abalone arterial haemolymph.

Appendix 2 shows the plots of P_{O_2} of inhaled water versus P_{O_2} of arterial haemolymph at 20°C. From the scatter of the plots it can be concluded that at least over this high, restricted range of P_{O_2} of inhaled water there is no direct relationship with the P_{O_2} of arterial haemolymph. However, it should be noted that the P_{O_2} of aquarium water may not be a true measure of the conditions prevailing at the ctenidia in the respiratory chamber of the abalone. The P_{O_2} inside the respiratory chamber will depend on the rate of ventilation. Unfortunately I was unable to measure either the rate at whixh water passed through the chamber, or the P_{O_2} of water inside the respiratory chamber.

Johansen et al. (1966) carried out their experiments on cannulated, free swimming octopi. They acknowledge that varying degrees of activity may have contributed to some of the variation in the P_{O_2} of the arterial haemolymph. In fact they regard the normally unsaturated conditions of the arterial blood as a potential oxygen reserve, hypothesising that with increased activity the arterial saturation may become complete. Presuming that the venous saturation does not change, this will mean a greater delivery of O_2 to the tissues. Johansen et al. (1970) demonstrated that in the crab Cancer magister activity indeed does influence the internal P_{O_2}. In this crab, rather than an increase in saturation as proposed for Octopus, increased activity caused a considerable lowering of arterial P_{O_2}, with a small (due to the shape of the oxygen equilibrium curve - see section 1.1.42 after Johansen et al., (1970), Figure 9) decrease in % saturation of the haemocyanin. The P_{O_2} of the venous blood was also lowered, with a large decrease in % saturation of the haemocyanin. The result of this was an overall increase in the oxygen delivered to the tissues during activity of the crab - largely as a result of lowered venous P_{O_2}.

In this study every attempt was made to exclude activity as a variable. During the day (when the experiments were conducted) abalone are normally quite inactive. Prior to examination for internal P_{O_2} and

P_{CO_2} all specimens were inactive. However during the brief process of sampling the animals were disturbed, and responded with various degrees of activity. It is possible that this "last minute" activity caused some of variation observed in either, or both the arterial and venous P_{O_2}.

In vivo gas tensions of animals acclimated to 20°C, and subjected to an abrupt temperature increase to 25°C, 2 hours after sampling

Although 25°C is well within the geographical temperature range of the abalone species it is about 2°C higher than any species experiences at West Island, even during mid summer. Hence the abrupt temperature increase might be expected to considerably stress the animals.

The results in section 2.3.11 indicated that the position of the oxygen equilibrium curve of abalone haemocyanin is shifted considerably to the right when the temperature is raised from 20 to 25°C. A priori it was thought that when the animal was transferred to the higher temperature, and allowed to briefly adjust to this temperature (two hours) the internal O_2 tensions might have significantly increased to compensate for the lowered oxygen affinity of the haemocyanin (cf. Falkowski, 1973). The internal gas tensions of animals subjected to an abrupt temperature increase from 20° - 25°C are presented on Tables 2.15-2.15.

In two species, H. roei and H. laevigata, there was no significant difference between the mean arterial P_{O_2} values of animals acclimated to 20°C and those subjected to an abrupt temperature increase to 25°C ($.2 > P > .1$ and $.4 > P > .2$, respectively; students t test comparing means of samples of unequal sizes). In H. ruber there was a significant increase in arterial P_{O_2} of the 25°C animals ($P < .001$). However, as will be seen, this increase is by no means large when related to the change in P_{50} of the oxygen equilibrium curve of H. ruber haemocyanin over the same temperature increase.

When the venous P_{O_2} values of 25°C stressed H. roei and H. laevigata were compared with those of 20°C animals there was no significant difference ($.4 > P > .2$). As in the arterial haemolymph,

the venous P_{O_2} of H. ruber at 25°C was higher than in those measured at 20°C. As in the case of the change in arterial P_{O_2}, the change in venous P_{O_2} in H. ruber, although significant, is not great in terms of the change in the P_{50} of the oxygen equilibrium curve of this species over the same temperature range. Tables 2.13-2.15 show that for each species at 25°C the P_{CO_2} increases from the heart through the tissues to the renal sinus, as in the 20°C animals.

2.3.4 In vivo function of Haliotis haemocyanin

This chapter has involved descriptions of (a) the oxygen equilibrium curves, (b) the oxygen capacities of the haemocyanins, and (c) the in vivo P_{O_2} and P_{CO_2} of the three species of abalone H. roei, H. ruber, and H. laevigata. Together these factors determine the role of haemocyanin in the supply of oxygen to the abalone. The results presented in this chapter indicate that the oxygen equilibrium curves determined on individual haemolymphs under the same conditions are characteristic of the given species and show little variation. However, in the other parameters considered, oxygen capacity and internal O_2 and CO_2 tensions, there is considerable variation between individuals of a given species.

Therefore, the role of haemocyanin in the oxygen transport of a hypothetical average individual of each abalone species will be described first, based on the mean for each parameter. When this hypothetical average oxygen exchange has been described, the source of variation in the system caused by individual differences will be examined. Two situations will be considered: (1) animals acclimated to 20°C, and examined at this temperature, and (2) animals acclimated to 20°C, and then subjected to an abrupt temperature change to 25°C a short period (two hours) before sampling.

Table 2.13

Internal Gas Tensions of H. ruber at 25°C

Animal No.	Heart		Foot		Renal Sinus		Sea Water	
	O_2 mm Hg	CO_2 mm Hg	O_2 mm Hg	CO_2 mm Hg	O_2 mm Hg	CO_2 mm Hg	O_2 mm Hg	CO_2 mm Hg
1	49	1.5	30	1.75	23	4	146	.25
2	46	1.5	-	-	19	3.5	146	.25
3	47	1.25	21	2	14	3	146	.5
4	45.5	1	-	-	16	3.5	146	.5
5	38	1	15	3.5	9	5.5	145	.5
6	47 49	1.25	-	-	15	4	142	1
7	43 42.5	1 1	14	4	13.5	3.25	147	.75
8	42	1.5	32	2.25	14	6	147	1
9	46	1.25	28	2.25	19	3	142	.3
Means ± S.D.	45.0 ±3.3	1.2 ±.22	23.3 ±7.8	2.6 ±.9	15.8 ±4.3	4.0 ±1.1	145.5 ±1.9	.56 ±.29

Table 2.14

Internal Gas Tensions of H. laevigata at 25°C

Animal No.	Heart		Foot		Renal Sinus		Sea Water	
	O_2 mm Hg	CO_2 mm Hg	O_2 mm Hg	CO_2 mm Hg	O_2 mm Hg	CO_2 mm Hg	O_2 mm Hg	CO_2 mm Hg
1	43	1.75	33	1.75	12	3	146	.25
2	38.5	1.5	22	3.25	10.5	5	144	.5
3	39	1.5	19	3	16	3	145.5	.5
4+	35	1.25	20	2	11	2.5	146	.5
5	41	1.5	18.5	1.5	14	2.5	145	1
6	34	1	-	-	10	3.25	157*	1.25
7	45	.75	26	1.5	13.5	3	146	.5
Means ± S.D.	39.4 ±4.0	1.3 ±.35	23.1 ±5.6	2.2 ±.77	12.4 ±2.17	3.2 ±.85	144.2 ±3.26	.64 ±.34

* Seawater frothy and polluted
\+ Animal unable to grip substrate lying upside down prior to sampling

Table 2.15

Internal Gas Tensions of H. roei at 25°C

Animal No.	Heart		Foot		Renal Sinus		Sea Water	
	O_2 mm Hg	CO_2 mm Hg	O_2 mm Hg	CO_2 mm Hg	O_2 mm Hg	CO_2 mm Hg	O_2 mm Hg	CO_2 mm Hg
1	34	1.25	16	2	12	3.75	144	.5
2	35.5	1.25	15	2.25	8.5	3	144	.5
3	33	1.25	12.5	2.75	6	7.5	142	.5
4	34	1.0	25	1.75	11	4	144	.25
5	37	1.25	-	-	12	3	144.5	.5
6	33	1	16.5	2.8	8	4.25	159	.5
Means ± S.D.	34.4 ±1.56	1.17 ±.12	17.0 ±4.7	2.3 ±.46	9.6 ±2.5	4.25 ±1.7	142.9 ±2.1	.46 ±.10

2.3.41 Oxygen exchange in abalone acclimated to 20°C

Figures 2.10-2.12 represent graphical summaries of oxygen exchange in the blood of Haliotis ruber, Haliotis roei, and Haliotis laevigata at 20°C. Each figures was compiled in the same way. To illustrate how the information in each figure was derived I will describe in detail Figure 2.10, which shows the oxygen exchange in the blood of the black abalone H. ruber at 20°C.

The first step was to determine the in vivo position and shape of the oxygen equilibrium curves. The temperature (20°C) and the mean in vivo P_{CO_2} of the abalone are known for sites where the blood can be considered to be immediately prebranchial and immediately postbranchial. The oxygen equilibrium curve A (Figure 2.10) is a hypothetical curve derived from the data on the influence of P_{CO_2} on the P_{50} of the haemocyanin at 20°C for H. ruber haemocyanin in the presence of 6 mm Hg CO_2; this is the mean P_{CO_2} found in the renal sinus samples of H. ruber specimens at 20°C (Table 2.10). In order to draw this curve a series of plots of

Figures 2.10, 2.11, and 2.12

Graphical summaries of oxygen exchange in the blood of *Haliotis ruber*, *H. laevigata*, and *H. roei*, at 20°C

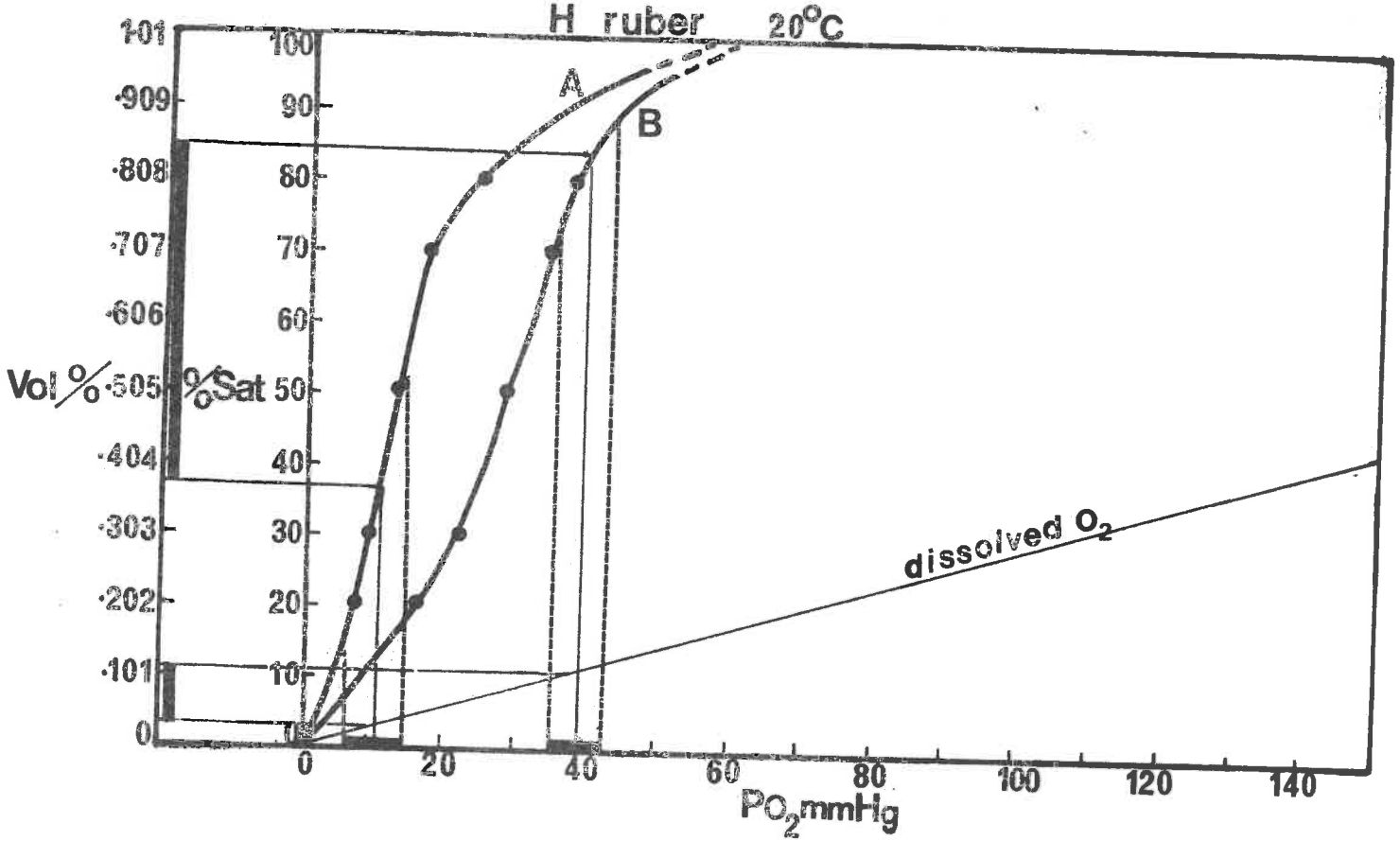

H ruber 20°C
A
B
dissolved O_2
Vol%
%Sat
PO_2mmHg
·101
·909
·808
·707
·606
·505
·404
·303
·202
·101
0
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
120
140

Fig. 2.10

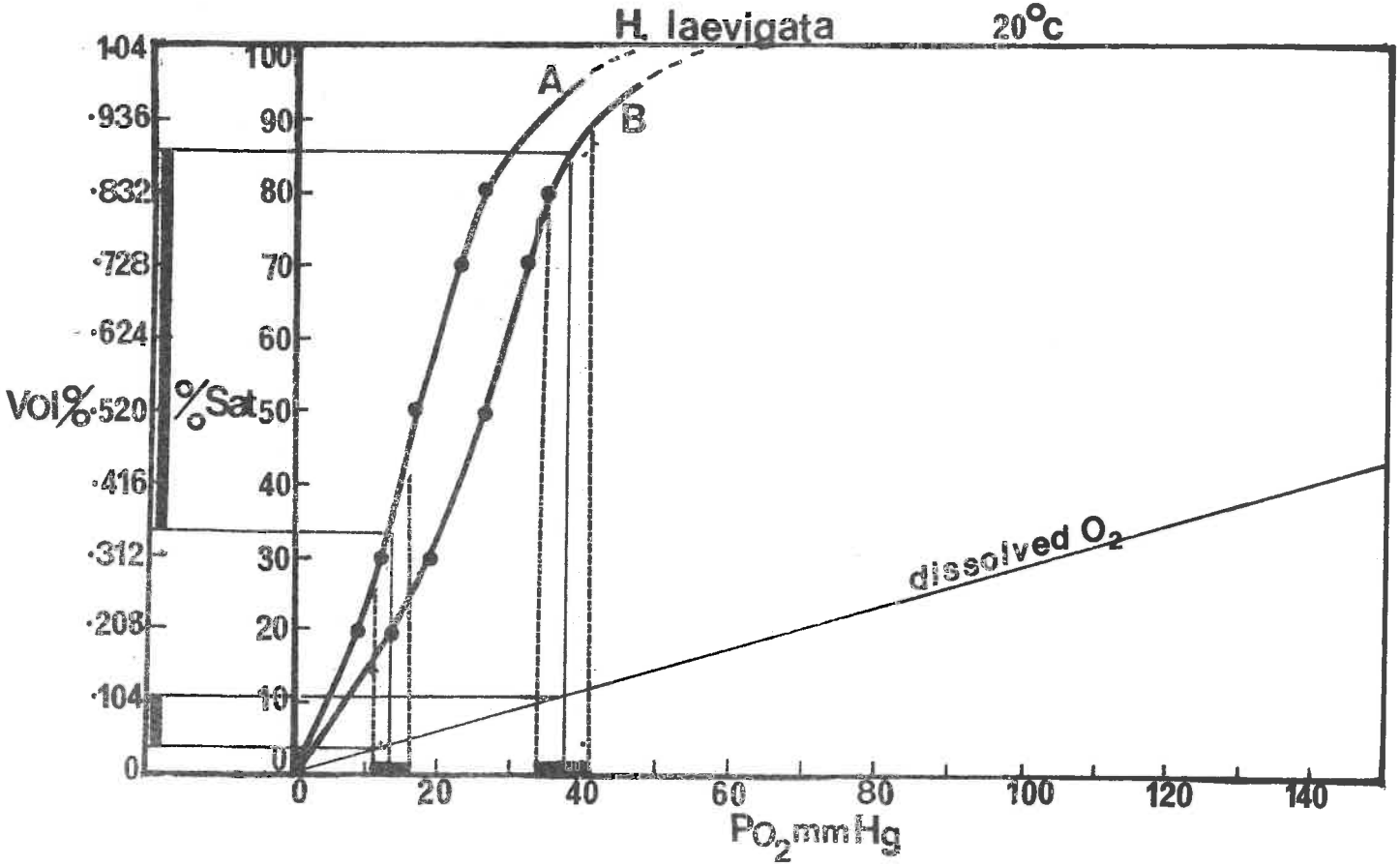

H. laevigata
20°c
A
B
dissolved O_2
Vol%
%Sat
1·04
·936
·832
·728
·624
·520
·416
·312
·208
·104
0
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
120
140
PO_2 mm Hg

Fig.211.

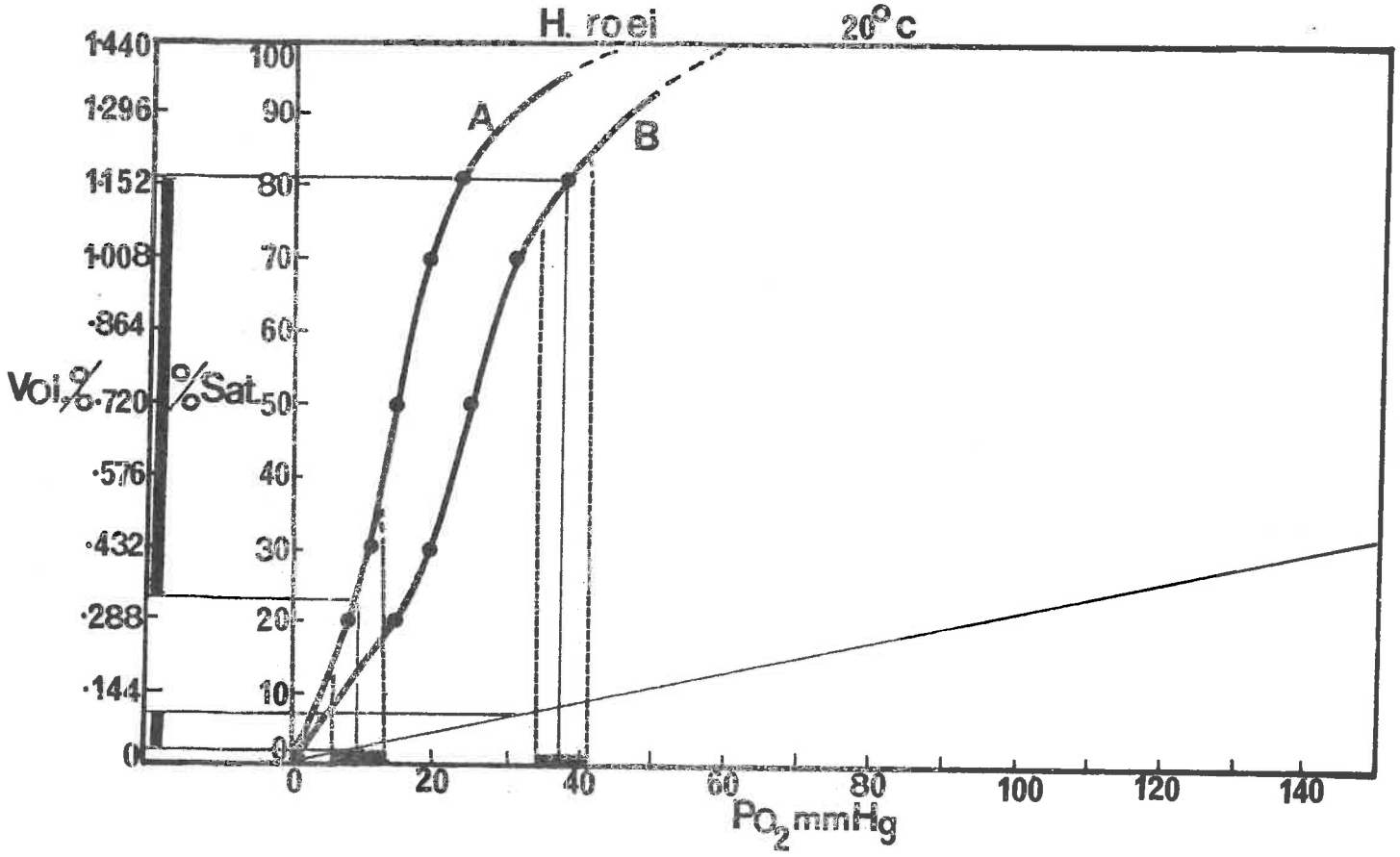

H. roei
20° c
A
B
Vol %
% Sat
1·440
1·296
1·152
1·008
·864
·720
·576
·432
·288
·144
0
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
120
140
PO2 mmHg

Fig.212.

P_{20}, P_{30}, P_{50}, P_{70}, P_{80} and P_{90} versus P_{CO_2} at 20°C were made using data extracted from the original oxygen equilibrium curves of H. ruber haemocyanin at 20°C in the presence of various partial pressures of CO_2 (Appendix 1 b). Typical examples of these graphs are the P_{50} versus P_{CO_2} summaries presented in this chapter (Figure 2.4). From these series of curves then, the position of the oxygen equilibrium curve at a known P_{CO_2} (6 mm Hg) can be derived. The oxygen equilibrium curve B, for blood in the ventricle of the heart (mean P_{CO_2} = 1.2 mm Hg) was derived in the same way.

Having by this means obtained the positions and shapes of the oxygen equilibrium curves of the pre and post branchial blood, the next step in estimating the role of haemocyanin in oxygen exchange is to relate these curves to the in vivo P_{O_2} of the animals. The unbroken vertical lines on figure 3.10 are extended from the mean P_{O_2} values of the ventricle and the renal sinus (Table 2.10). The dotted lines about these unbroken lines indicate the standard deviations about the means. Where the line indicating the mean ventricle P_{O_2} intercepts oxygen equilibrium curve B the mean % saturation of the immediate postbranchial ventricle haemocyanin can be read off the Y axis. Likewise, when the P_{O_2} of the renal sinus is related to the oxygen equilibrium curve A, the mean percent saturation of the haemocyanin immediately before entering the ctenidia can be obtained. (Horizontal lines drawn from the vertical dotted lines would indicate the variation in % saturation when the standard deviations about the mean P_{O_2} are considered. It will be noticed that because of the nature of the oxygen equilibrium curve of the haemocyanin relatively small variations in P_{O_2} lead to much larger variations in percent saturation of the haemocyanin).

Thus it is possible to obtain the difference in percent saturation of the "arterial" and "venous" haemocyanin of H. ruber. In order to find how much oxygen is being delivered to the tissues by the haemocyanin, this difference in percent saturation must be related to the oxygen capacity of the haemocyanin. The Y axis on the far left of the graph gives volumes % (ml of oxygen carried by haemocyanin or in solution in 100 ml of blood). For H. ruber the mean oxygen capacity of the haemocyanin was determined to be 1.01 vol %. At this point on the Y axis the haemocyanin of the hypothetical H. ruber is 100% saturated. From the figure it can be seen that the haemocyanin in the ventricle of the heart at the mean ventricle P_{O_2} is 84% saturation and thus carries about .85 vol % of oxygen (curve B). In the renal sinus the haemocyanin is about 36.5% saturated at the mean P_{O_2} and P_{CO_2} of this sinus and has .37 vol% oxygen bound to it (curve A). The difference between the ventricle and renal sinus is .48 vol %. This is the amount of oxygen delivered to the tissues by the haemocyanin in this hypothetical average H. ruber.

In order to estimate the effective contribution of the haemocyanin in the supply of oxygen to the tissues, the amount of oxygen delivered in physical solution must also be known. Redfield et al. (1926) found that the approximate solubility of oxygen in the blood of Limulus, Cancer, Busycon and Callinectes is about 90% of that of seawater at corresponding temperatures. More recent workers have also adopted this approximation for estimating oxygen carried in physical solution in haemocyanin bearing organisms (Redmond, 1955, 1968b; Talkowski, 1973). This approximation was also used in this study. The solubility of oxygen in the seawater in which the abalone were kept at 25°C and 20°C was derived from Harvey (1945, Table 1). Plotted on Figure 2.10 is a graph showing the oxygen carried in physical solution, derived from the above approximation.

From Figure 2.10, it can be seen that due to the relatively large difference in P_{O_2} between the heart and the renal sinus, an appreciable amount of oxygen (.08 vols %) is delivered to the tissues as oxygen in solution. The whole blood of this hypothetical H. ruber therefore delivers .56 vol % oxygen to the tissues, with haemocyanin making a much greater contribution (about 86% of the total) than the oxygen carried in physical solution.

The summaries of oxygen exchange in the other two species H. laevigata, and H. roei at 20°C were constructed in the same way.

In the hypothetical H. laevigata at 20°C (Figure 2.11) the oxygenated haemocyanin in the heart carries .89 vol % oxygen. The haemocyanin in the renal sinus is still carrying .35 vol % oxygen. Therefore, in this case the haemocyanin has delivered .54 vol % to the tissues. Over the same change in P_{O_2}, the amount of oxygen given up by the physical solution to the tissues is .070 vol %. Therefore the total amount of oxygen delivered to the tissues by the blood of this average H. laevigata at 20°C is .61 vol %, of which the haemocyanin is responsible for nearly 90%. The contributions of the haemocyanin to the oxygen supply to the tissues in these first two species then is very similar.

In the third species H. roei at 20°C (Figure 2.12) the haemocyanin in the heart carried 1.17 vol % oxygen. In the renal sinus this has been reduced to .33 vol %. The haemocyanin has therefore delivered .84 vol % to the tissues. Over the same change in P_{O_2} the oxygen delivered in physical solution is about .079 vol %. The total oxygen delivered by the blood then is about .92 vol %. This is considerably more than is delivered by the bloods of the other two species. By far the greatest part of this oxygen (91%) is carried by the haemocyanin.

2.3.42 Comparison of oxygen exchange of the three abalone species at 20°C

On Table 2.16, the factors describing haemocyanin's role in oxygen exchange in the three abalone species at 20°C are summarised. Perhaps the point which is most obvious from Table 2.16, is the overall similarity in the role which the haemocyanin plays in the delivery of oxygen to the tissues of each of the abalone species.

In each case, the arterial P_{O_2} and P_{CO_2} are such that, given the oxygen equilibrium characteristics of the haemocyanin of the species the arterial haemocyanin is highly saturated when it leaves the gills. There is only a small amount of variation (4.5%) between the mean arterial % saturations of the three species.

The venous % saturations are somewhat more variable. H. laevigata, and H. ruber show similar saturations of the venous haemocyanin, 33% and 36.5% saturated respectively, but the potentially most active of the three species H. roei, shows a considerably lower venous saturation, 23%. This results in the fact that the arterial-venous difference in % saturation of H. roei (58%) is larger than in the other two species (51.7% and 47.5% for H. laevigata, and H. ruber respectively). This greater arterial venous difference in % saturation of H. roei haemocyanin to a small extent accounts for the greater amount of oxygen delivered to the tissues by the haemocyanins of this species. For example if H. ruber, and H. laevigata both had the same arterial venous difference in % saturation of their haemocyanins as H. roei, their haemocyanins would deliver .55 vol %, and .56 vol % respectively, excluding dissolved oxygen. However, by far the greatest difference between H. roei and the other two species, in terms of the amounts of O_2 delivered to the tissues in a single respiratory cycle stems from the larger mean O_2 capacity of H. roei haemocyanin, which in turn results in the larger arterial-venous differences in vol % of oxygen of this species. It is important to

Table 2.16

Mean values of parameters describing haemocyanins role in oxygen exchange of abalone at 20°C

Species	Arterial P_{O_2} mm Hg	Venous P_{O_2} mm Hg	Arterial P_{CO_2} mm Hg	Venous P_{CO_2} mm Hg	Arterial P_{50} mm Hg	Venous P_{50} mm Hg	Arterial % sat	Venous % sat	A-V diff. %sat	O_2 capacity vol %	Arterial O_2 content haemocyanin	Venous O_2 content haemocyanin	Hcy A-V diff. Vol % O_2	Dissolved O_2 delivered	Total O_2 delivered	% of total O_2 delivered by haemocyanin
H. ruber	38.8	11.5	1.20	6.0	28	13	84	36.5	47.5	1.01	.85	.37	.48	.06	.56	86
H. laevigata	37.4	13.8	1.21	3.5	26	16.5	85.5	55	51.7	1.04	.89	.35	.54	.076	.61	90
H. roei	36.7	9.6	1.13	4.3	24	14	81	23	58.0	1.44	1.17	.33	.84	.079	.92	91

note that the greater O_2 capacity of H. roei haemocyanin is a direct result of the greater concentration of haemocyanin in the haemolymph of this species.

Despite the fact that there is a difference in the actual amount of oxygen delivered by the blood of the three abalone species in a single respiratory cycle, one thing bears emphasising: In each species by far the greatest proportion of oxygen delivered by the blood is carried by the haemocyanin (Table 3.16).

2.3.43 The reverse Bohr effect in abalone

In each of the three species of abalone a considerable arterial venous difference in P_{CO_2} was recorded. It should be noted that this difference was greatest in H. ruber, although it is difficult to offer any explanation as to why the venous P_{CO_2} of this species at 20°C should have been somewhat higher than those observed in the other species.

Nevertheless, the result of this arterial venous difference in P_{CO_2} is obvious in all species (Figures 2.10-2.12, Table 2.16), although the arterial venous change in P_{50} is most exaggerated in H. ruber. The reverse Bohr effect plays a marked role in reducing the amount of O_2 unloaded to the tissues in all three species. Simply to emphasise this point Table 2.17 below presents the venous % saturations derived taking account of the arterial venous P_{CO_2} increase, and compares them to hypothetical venous % saturations if no reverse Bohr effect is presumed.

Table 2.17

True venous % saturations compared to hypothetical venous % saturations in the absence of a reverse Bohr effect.

	H. ruber	H. laevigata	H. roei
Correct venous % saturations	36.5	33	23.0
Hypothetical venous % saturations in absence of reverse Bohr effect	12.0	20	12.5

It can be seen from this table that if the reverse Bohr effect had not been present, the saturation of the venous haemocyanins would have been greatly reduced in all cases.

It is difficult to imagine a way in which such a pronounced reverse Bohr effect can be of any physiological advantage to the abalone.

It has been hypothesised for some gastropods that the reverse Bohr effect may assist loading of O_2 at the respiratory surfaces in environmental conditions of low P_{O_2} and high P_{CO_2} (Redmond, 1955). An objection to this proposal, as indicated by Jones (1963), is that the displacement to the left of the oxygen equilibrium curve, although assisting the loading of O_2 would mean that unloading could only occur at extremely low P_{O_2}. This problem would be exaggerated if there was any increase in venous P_{CO_2}. The objection itself is only speculation, as it has been shown (e.g. Mangum and Weiland, 1975) that the P_{O_2} of venous bloods in some haemocyanin bearing organisms under stress (e.g. activity) can fall to very low levels.

It is unlikely that abalone in the field would ever experience environmental conditions of low P_{O_2} and high P_{CO_2}. However, it is possible that at times when abalone are clamped tightly to the substrate to avoid predator attack or some other disturbance, such conditions might prevail in the respiratory chamber surrounding the ctenidia. It is conceivable that under such circumstances the reverse Bohr effect might be of some advantage to the animals in assisting loading of O_2 at the respiratory surfaces, providing the venous P_{O_2} was sufficiently low to allow unloading of O_2 from the haemocyanin to the tissues.

In some gastropods, during periods of muscular inactivity the blood may tend to pool in sinuses of the open circulatory system. In such circumstances it has been proposed that a reverse Bohr effect could be advantageous in facilitating diffusion of oxygen to more active tissues with higher local P_{CO_2} (Redmond, 1968). All species of abalone are

inactive for long periods of time; even H. roei, which is at night a mobile, grazing species, has long periods of inactivity during daylight. Considering this mode of existence, it is tempting to hypothesise that the reverse Bohr effect may help distribute oxygen to the more active tissues of the abalone when the blood pools in sinuses in the absence of muscular assistance to a weak circulation. However, as is shown later in this study (Chapter 3, Part II) it is apparent that the abalone species have relatively effective circulatory systems, even when inactive. There must therefore be doubts as to the validity of the above hypothesis in the case of abalone. It is possible however, that even with an effective circulatory system in terms of volume of blood circulated in a given time, the inefficiency of "turnover" of blood in large sinuses might still lead to a reverse Bohr effect being advantageous in abalone.

It is shown later in this study that metabolism in abalone is primarily, if not wholly aerobic (Chapter 4). It may be then, that the reverse Bohr effect is simply a primitive feedback regulation of metabolism in these animals. When CO_2 accumulates, O_2 is withheld by the haemocyanin, thereby stopping metabolism in that tissue, until local CO_2 tension decreases.

2.3.44 Comparison of the oxygen delivery role of haemocyanin in abalone with other organisms

When the data obtained for abalone in this study are compared with that for Busycon canaliculatum, the only other gastropod for which an in vivo description of O_2 exchange is available, it can be seen that the abalone haemocyanins deliver considerably less oxygen to the tissues than does that of the conch.

The arterial venous difference in % saturation of the conch haemocyanin is only slightly larger than the average obtained for H. roei at 20°C, and is not a major contributor to the observed

difference between the abalone and conch with respect to the amount of O_2 delivered per respiratory cycle. Florkin (1934) gives the oxygen capacity of Busycon canaliculatum haemocyanin as approximately 3 vol %. This is considerably higher than the mean O_2 capacities of the abalone haemocyanins determined in this study, and accounts largely for the greater amount of O_2 delivered to the tissues of the conch compared to the abalone.

Comparison of the data obtained for the amount of oxygen delivered by the haemocyanin to the tissues of the abalone in one respiratory cycle (A-V difference in vol % O_2) with data from other groups of animals is made difficult because of the sometimes limited, and often contradictory data available from other studies (General Introduction).

For example Redmond (1968) reports that the arterial venous difference in % saturation of Cryptochiton (= Armicula) haemocyanin is about 45%. Petersen and Johansen's (1975) data on the other hand indicate that the difference in % saturation between arterial and venous haemocyanins is of the order of 18.6% (General Introduction Section 7.1.44).

As Redmond (1962) reports the O_2 capacity of the whole blood of this species to be less than 1 vol %, while the O_2 capacity of the haemocyanin is apparently less than .5 vol %, it would seem that the amount of O_2 delivered by the blood of this chiton species in a single respiratory cycle is likely to be considerably less than that delivered by the blood of any of the abalone in this study.

Again, there are differences of opinion concerning the details of the in vivo function of Limulus haemocyanin. (General Introduction Section 1.1.43). However, the A-V differences in % saturation recorded in two recent studies (Falkowski, 1973; Petersen and Johansen, 1975)

would indicate that in this sluggish arthropod which has a haemocyanin oxygen capacity of about 2 vol % O_2 (Prosser, 1956), the amount of oxygen delivered to this tissue in a single respiratory cycle will be similar to the values recorded for the abalone at 20°C. In all the early studies on decapod crustacea reported in the literature (General Introduction Section 1.1.42 Table 1.2) the A-V difference in vol % of oxygen of the same order as those reported for abalone in this study, with the possible exception of that for Gercarcinus lateralis (Redmond, 1968b) which has a somewhat larger A-V difference of .84 vol %.

The A-V difference in vol % of oxygen reported by Johansen et al. (1970) for Cancer magister is considerably larger than all of the values recorded in the earlier studies on other decapod crustacea (1.7 vol %). However as Mangum and Weiland (1975) point out this is almost entirely a direct result of the greater O_2 capacity of Cancer blood compared to other decapod crustaceans. Mangum and Weiland's recently obtained A-V difference value of .595 vol % O_2 for another species of crab, Callinectes sapidus, is again quite comparable with values obtained for abalone.

Not surprisingly the haemocyanins of the abalone do not deliver as much oxygen per respiratory cycle as the haemocyanins of the highly active cephalopod molluscs (Table 1.1 General Introduction).

2.3.45 Effect of individual variation of haemocyanin concentration on the oxygen transport system

In this study, as in other descriptions of the O_2 transport system involving haemocyanin (notably Redmond, 1955, 1962a, 1968b; Falkowski, 1973), a model describing the oxygen transport role of haemocyanin in the average animal has been developed for each of three abalone species. This was of course necessary so that general conclusions could be drawn. However, I now consider the effects of the observed

individual variation in haemocyanin concentration to show that, despite this variation, haemocyanin functions as a respiratory pigment in abalone, even when in low concentrations. The variation in the concentration of haemocyanin leads to variation of the O_2 capacity of the haemolymph. This factor is important as it is a stable difference between individuals (Chapter 6) unlike *in vivo* P_{O_2} and P_{CO_2} which might reasonably be expected to vary within an individual depending on such factors as environmental P_{O_2} and state of activity of the organism. It may be supposed that variations in haemocyanin concentration in other species are likely to cause similar variations in the O_2 capacities of their haemolymphs. For example, the O_2 capacity of the haemolymph of the conch *Busycon canaliculatum*, given by such authors as Hendersen, (1928), Redmond (1955, 1968), Florkin (1954), as 3 vol % on the basis of unpublished data of Redfield (1926), will vary considerably as it has been shown that large variations in the haemocyanin concentration of the blood occur in this species (Betzer and Pilson, 1974). This must in turn lead to variation in the A-V difference in vol % of oxygen. Similarly the A-V differences in vol % of oxygen in each of the chiton species so far examined would be expected to vary considerably with varying haemocyanin concentration in the haemolymph (Redmond, 1962).

In the abalone the variation in O_2 capacity is not as great as would be expected if there had been a direct linear relationship between O_2 capacity and haemocyanin concentration. Consider *H. roei*, the species with the highest mean haemocyanin concentration. One individual in the sample has a haemocyanin concentration of 3.16 mgm/ml, and an oxygen capacity of .79 vol %. The haemocyanin of this animal in the same P_{O_2} and P_{CO_2} conditions as those on Figure 2.12 at 20°C, delivers about .46 vol % O_2 to the tissues. This means that even at this low haemocyanin concentration the haemocyanin still carries about 85% of the total oxygen delivered to the tissues. As a result of the

O_2 capacity-haemocyanin concentration relationship apparent in the abalone, the haemocyanin of this animal although less than half as concentrated as that of the mean animal still delivers more than half (55%) as much oxygen to the tissues as the haemocyanin of the mean animal. Presuming the amount of dissolved oxygen will not change greatly with the concentration of haemocyanin, the A-V difference of the whole haemolymph of this animal will be .54 vol % which is nearly 60% of that of the mean animal.

Even if the haemocyanin concentration was related directly to the O_2 capacity, the haemocyanin of an animal with half the haemocyanin concentration of the mean animal would deliver .42 vol % O_2 to the tissues. Further, the haemocyanin of an animal with only quarter the haemocyanin concentration of the mean would still deliver .21 vol % O_2 to the tissues, which is 2.7x the contribution made by the O_2 in solution. There is no record of H. roei with a haemocyanin concentration nearly as low as this.

In H. ruber haemocyanin concentrations as low as 1.1 mgm/ml were recorded. At this extremely low haemocyanin concentration there is virtually no visible colour to the haemolymph, even when fully oxygenated. Spectrophotometric methods must be used to detect the haemocyanin present. Nevertheless, substituting the O_2 capacity obtained for this animal (.30 vol % - see solid fitted curve Figure 2.6) in Figure 2.10 at 20°C it can be seen that the haemocyanin delivers .14 vol % O_2, or over 60% of the total oxygen delivered to the tissues. Similar reasoning applies to the low O_2 capacity values observed in H. laevigata; and the same applies to all three species at 25°C.

It is apparent then, that although the contribution of the haemocyanin to the A-V difference in vol % O_2 varies according to the amount of haemocyanin in the haemolymph this variation is to some extent damped by the O_2 capacity - haemocyanin concentration relationship and,

even when the haemocyanin is extremely dilute the contribution made by the respiratory protein is significant when viewed in terms of the total O_2 supplied by the blood.

2.3.46 Oxygen exchange in animals acclimated to 20°C and subjected to a rapid temperature change to 25°C

Tables 2.13-2.15 give the internal gas tensions for H. roei, H. ruber, and H. laevigata subjected to an abrupt temperature change from 20°C to 25°C two hours before measurements were made. As with the 20°C animals the in vivo gas tensions must be related to the position and shape of the in vivo oxygen equilibrium curves, and the O_2 capacities of the haemocyanins in order to determine what contribution the haemocyanin makes to the O_2 supply of the animals under the new conditions.

Figures 2.13-2.15 diagrammatically show the O_2 exchange of the three species after being subjected to the temperature change. These figures were derived in the same way as those discussed for the 20°C animals, using oxygen equilibrium curves determined at 25°C and with various partial pressures of CO_2 and data for the in vivo gas tensions at 25°C.

I will consider first the role of haemocyanin in the transport of oxygen in H. ruber stressed by a temperature change from 20°C to 25°C. In this species there was a relatively small, but significant increase in the arterial P_{O_2} from a mean of 38.8 mm Hg at 20°C to 45 mm Hg at 25°C $P \ll .001$ (comparison of means - student's t test). Nevertheless when this P_{O_2} is related to the oxygen equilibrium curve at 25°C in the presence of 1.2 mm Hg CO_2 (the mean for the heart samples at this temperature), it can be seen that the haemocyanin in the immediate postbranchial sample is only 60% saturated (Figure 2.13 curve B), whereas at 20°C the postbranchial haemocyanin of H. ruber was 84% saturated (Figure 2.10, curve B). Again in H. ruber the venous P_{O_2} was significantly higher in the 25°C animals than in the 20°C animals -

Figures 2.13, 2.14 and 2.15

Graphical summaries of oxygen exchange in the blood of *Haliotis ruber*, *H. laevigata* and *H. roei*, after an abrupt temperature increase from 20°C to 25°C.

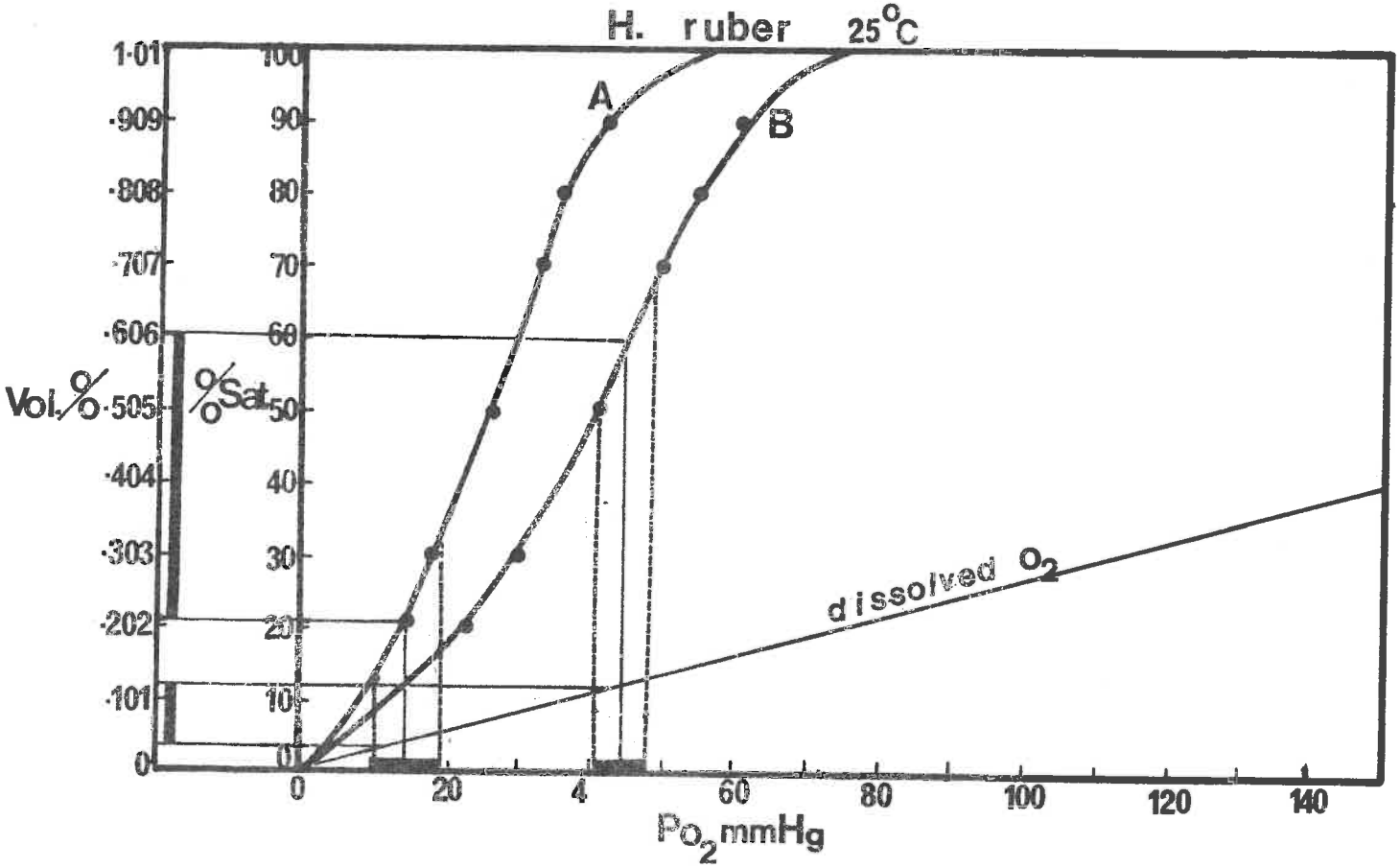

H. ruber 25°C
A
B
dissolved O_2
Vol.%
%Sat
1·01
·909
·808
·707
·606
·505
·404
·303
·202
·101
0
100
90
80
70
60
50
40
30
20
10
0
0
20
4
60
80
100
120
140
Po_2 mmHg

Fig.2.13

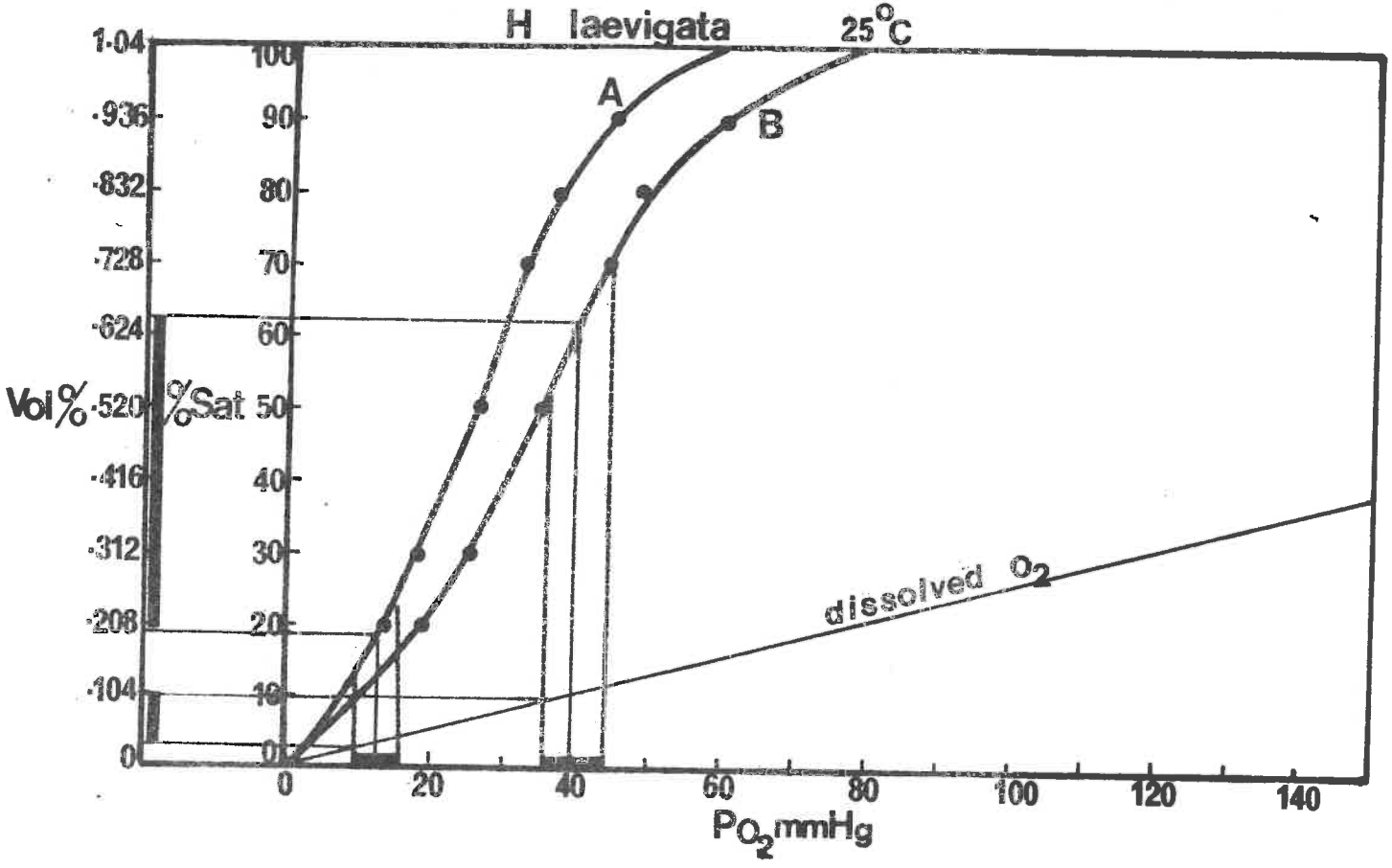

H laevigata
25°C
A
B
dissolved O2
Vol%
%Sat
1·04
·936
·832
·728
·624
·520
·416
·312
·208
·104
0
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
120
140
PO2mmHg

Fig 2.14

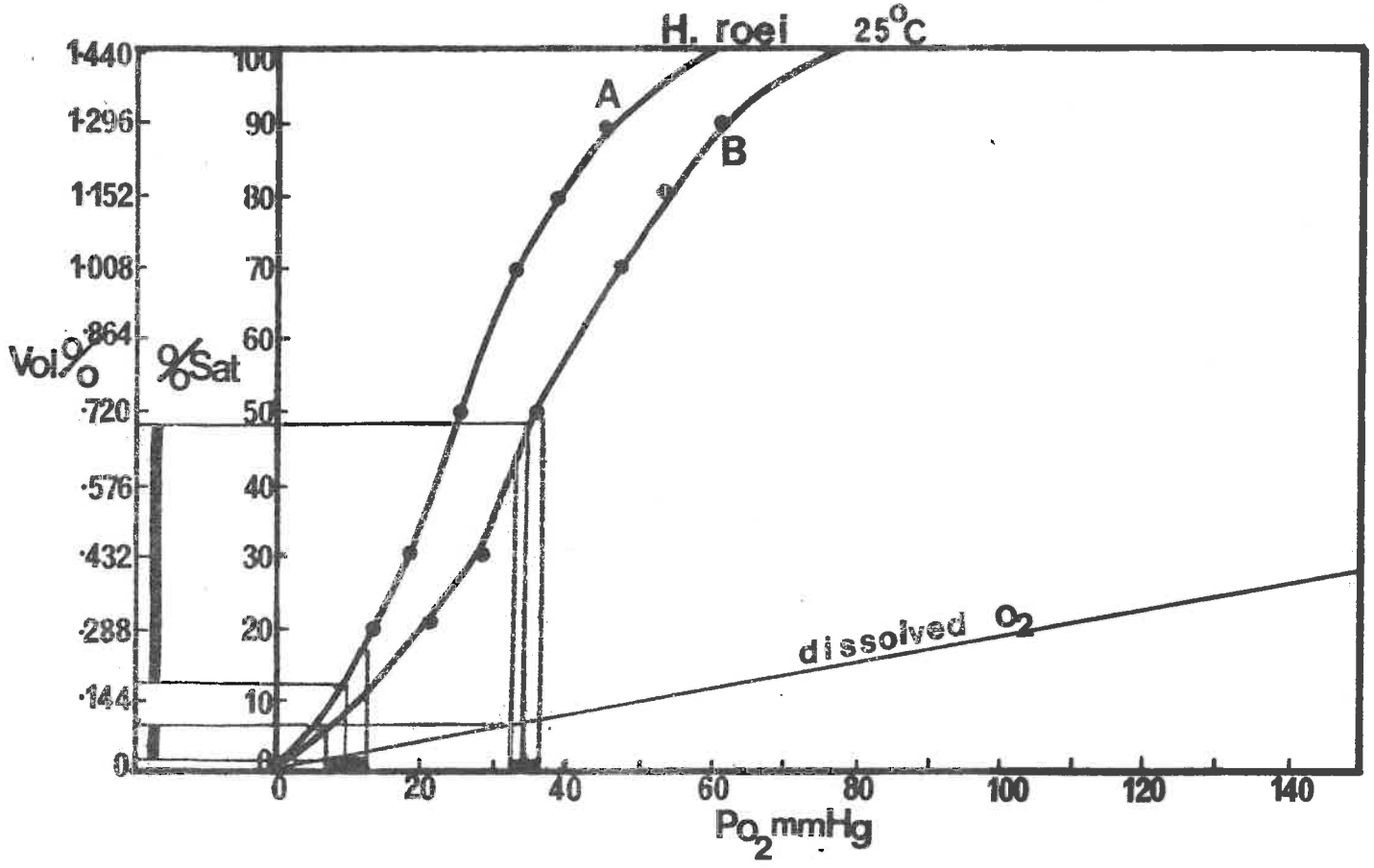

H. roei 25°C
A
B
dissolved O2
Vol%
%Sat
1·440
1·296
1·152
1·008
·864
·720
·576
·432
·288
·144
0
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
120
140
Po2 mmHg

Fig. 2 15

.025 > P > .01. However when this venous P_{O_2} is related to the mean oxygen equilibrium curve describing the in vivo renal sinus haemocyanin at 25°C and 4 mm Hg CO_2 (Figure 2.13 curve A) it can be seen that the haemocyanin is only 21% saturated. This is considerably less than the 36.8% saturation of the venous samples at 20°C (Figure 2.10 curve A). Now the oxygen equilibrium experiments in this study have shown that a given haemocyanin sample saturated at 25°C has the same absorbance as when saturated at 20°C. This indicates that the O_2 capacity of the haemocyanin at saturation is the same at both temperatures. The difference in % saturation of the arterial and venous haemocyanins of H. ruber at 25°C can therefore be related to the mean O_2 capacity determined for this species - far left y axis of Figure 2.13. Therefore, as there is a drop of 39% observed in the saturation of the haemocyanin between the heart and the renal sinus, 0.394 vol % of oxygen is delivered to the tissues by the haemocyanin. Over the same decrease in O_2 tension, approximately 0.075 vol % of oxygen is delivered in physical solution. (The approximate O_2 in solution at 25°C derived in the same manner as that at 20°C is shown on Figure 2.13). This means that the whole blood of the abalone delivers 0.47 vol % of oxygen about 84% of which is carried by the haemocyanin.

In H. laevigata there is no significant difference between the in vivo P_{O_2} found in the 20°C animals, and those transferred to 25°C (arterial .4 > P > .2; venous .4 > P > .2 - students t test) comparison of means). Relating the internal gas tension data (Table 2.14) to the oxygen equilibrium curves of the haemocyanin at in vivo P_{CO_2} (Figure 2.14) it can be seen that the arterial haemocyanin of this species is only about 62% saturated compared to 85.5% saturated at 20°C. The venous haemocyanin at 25°C is only about 18.5% saturated which is considerably less than the 55.8% saturation of the venous haemocyanin of H. laevigata at 20°C. The difference between arterial and venous haemocyanin

saturation in this species at 25°C is 45.5%. Relating this to the mean O_2 capacity of the haemocyanin of this species shows that the haemocyanin has given up 0.452 vol % of oxygen to the tissues. At the same time 0.068 vol % of oxygen has been delivered by the physical solution. Therefore a total of 0.520 vol % of oxygen is delivered by the haemolymph of H. laevigata in a single respiratory cycle. About 86% of this O_2 is delivered by the haemocyanin.

Like H. laevigata the in vivo gas tensions of H. roei measured after transfer to 25°C showed no significant difference to those at 20°C. When this data is related to the in vivo oxygen equilibrium curves of the haemocyanin (Figure 2.15) it can be seen that the arterial haemocyanin of this species is about 48.5% saturated under these conditions, which is considerably less than the 81% saturation of the arterial haemocyanin at 20°C. However, as in the other live species the venous % saturation at 25°C (12%) is also considerably lower than at 20°C (23%). Relating the arterio-venous difference in % saturation (36.5 at 25°C to the mean O_2 capacity of this species it is found that the haemocyanin of H. roei at 25°C delivers about 0.53 vol % of oxygen to the tissues. Over the same drop in P_{O_2} the physical solution has supplied 0.069 vol % of oxygen to the tissues. The total haemolymph therefore has delivered 0.599 vol % of oxygen to the tissues. Again although this total is smaller than that supplied by the whole haemolymph at 20°C, the proportion of oxygen delivered by the haemocyanin is little different.

Table 2.18 summarises the role of haemocyanin in the delivery of oxygen to the tissues in abalone acclimated to 20°C, and subjected to an abrupt temperature increase to 25°C.

The first thing which is obvious from the table is that haemocyanin still contributes most of the oxygen supplied to the tissues by the whole haemolymph (84%, 86% and 88% of total O_2 delivered, in H. ruber, H. laevigata and H. roei respectively).

Table 2.18

Mean values of parameters describing haemocyanin's role in oxygen exchange in abalone subjected to an abrupt temperature increase from 20°C to 25°C

Species	Arterial P_{O_2} mm Hg	Venous P_{O_2} mm Hg	Arterial P_{CO_2} mm Hg	Venous P_{CO_2} mm Hg	Arterial P_{50} mm Hg	Venous P_{50} mm Hg	Arterial % sat.	Venous % sat.	A-V diff. % sat.	O_2 capacity vol %	Arterial O_2 content haemocyanin vol %	Venous O_2 content haemocyanin vol %	A-V diff. vol % O_2 Hcy	Dissolved O_2 delivered vol %	Total O_2 delivered vol %	% of total O_2 delivered by haemocyanin
H. ruber	45.0	15.8	1.2	4.0	41.0	26.5	60	21.0	39	1.01	.606	.212	.394	.075	.47	84
H. laevigata	39.4	12.4	1.3	3.2	35.0	26.0	62	18.5	43.5	1.04	.645	.192	.453	.068	.570	86
H. roei	34.4	9.6	1.2	4.3	35.5	25.0	48.5	12.0	36.5	1.44	.698	.175	.535	.069	.599	88

However, it is also evident that the total amount of oxygen delivered by the haemolymphs of each of the three species in one respiratory cycle after an abrupt temperature increase to 25°C is considerably less than the amount delivered at 20°C. In H. ruber the haemolymph delivers 85% of that delivered at 20°C. In H. laevigata and H. roei the figures are 85.5% and 65% of the 20°C values respectively. As the dissolved oxygen contributes so little to the total oxygen delivered, the most important fact is that there is a decrease in the amount of oxygen delivered by the haemocyanin after an abrupt temperature increase.

When poikilothermic organisms are transferred to a higher temperature, they often exhibit an increase in the rate of respiration (Prosser, 1966, 1973). (As is shown later in this thesis, abalone do exhibit an increased oxygen consumption after an abrupt temperature increase). A priori it might therefore be expected that ideally the contribution of the respiratory pigment to the oxygen supply to the tissues might also increase under such circumstances. This obviously does not occur in the abalone. From Table 2.18, it can be seen that although after an abrupt temperature increase the oxygen equilibrium curves of abalone haemocyanin show a typical shift to the right (reduction in O_2 affinity of the haemocyanin), the in vivo P_{O_2} do not adjust to maintain the level of arterial haemocyanin saturation observed at 20°C. Moreover, the lower saturation of the venous haemocyanin (also a result of the relatively stable venous P_{O_2} but increased P_{50} of venous haemocyanin) does not completely compensate for this lowered saturation of the arterial haemocyanin. In each species the net result is a lower arterial venous difference (vol % of oxygen). This net effect is most exaggerated in H. roei, where the mean arterial P_{O_2} after the abrupt temperature transfer was in fact lower than the mean arterial P_{O_2} of 20°C animals.

It is only possible to hypothesise why the internal P_{O_2} does not change when abalone are subjected to the temperature change.

Redmond (1955, 1962, 1968b), Larimer (1964) and Jones (1963) have all proposed that the gills of some haemocyanin bearing organisms have high diffusion barriers which help in maintaining low arterial P_{O_2} found in several species. They further hypothesised that the maintenance of these low arterial P_{O_2} values in turn leads to the maintenance of a high diffusion gradient. Johansen et al. (1970) found high arterial P_{O_2} values in Cancer magister, and have criticized the above theory, claiming that "A steep diffusion gradient across the gills, resulting in only partial arterialisation of the blood imposes excessive demands on blood flow." In more recent work (Mangum and Weiland, 1975) it has however been shown that low in vivo arterial P_{O_2} values can be associated with high % saturations of the haemocyanins, in animals which nevertheless live in high external P_{O_2} conditions.

Theoretically at least, some sort of diffusion barrier across the gills would seem possible as it has been shown that whereas the rate of diffusion of oxygen in water is about 300,000 times slower than in air, the rate of diffusion of oxygen in animal membranes can range up to several million times slower than in air (Krogh, 1941).

Unlike Redmond's (1955) decapod crustaceans, the abalone in this study showed high saturations of arterial haemocyanin at their acclimation temperatures. It might therefore be hypothesised in accordance with Johansen's criticism of Redmond's "diffusion barrier" theory that the diffusion characteristics of the abalone gills would be such that nearly maximal saturation of the blood could occur under the conditions to which the animals were adapted (e.g. 20°C). This is what is observed. When the animals are transferred suddenly to a higher temperature, it is possible that the inherent "diffusion barrier characteristics" of the gills adapted to lower temperatures prevent a rise

in internal P_{O_2}, at least until after a reasonable acclimation period.

In proposing this hypothesis it must be remembered that with an increase in temperature the rate of diffusion of oxygen increases. However the partial pressure of oxygen in the environment also slightly decreases. In general these two factors, increase in rate of diffusion, and decrease in solubility of oxygen are thought to virtually compensate for each other at biologically viable temperatures (Wood, 1974).

The observation that the *in vivo* P_{O_2} of an organism does not change immediately after an abrupt temperature change is not without precedent. Falkowski (1973) also observed that in the horseshoe crab *Limulus polyphemus*, no change in internal P_{O_2} occurred immediately after acute temperature change. However, after a period of acclimation the *in vivo* P_{O_2} changed considerably. It is interesting to note however that in *Limulus*, even at the P_{O_2} associated with acclimation to a high temperature ($24^{\circ}C$), the haemocyanin contributes less oxygen to the tissues (per respiratory cycle) than it does in low temperature ($8^{\circ}C$) acclimated animals. Although Falkowski has also demonstrated that the oxygen consumption of *Limulus* has a "low sensitivity to thermal fluctuation, the metabolic rate of this organism is at least as great at $24^{\circ}C$ as it is at $8^{\circ}C$". This means that with a smaller amount of oxygen being delivered by the blood per respiratory cycle the blood flow must be greater at this higher temperature.

I propose that the same situation must hold in abalone subjected to acute temperature change.

Such interaction between oxygen delivered by the haemolymph, the oxygen consumptions of the animals, and the role played by the circulatory system in maintaining this oxygen supply are examined in detail in the following chapter.

2.3.47 Summary of haemocyanin function in relation to temperature change in the three abalone species

H. roei is the most active abalone species. Moreover, because of its geographical distribution, and habitat, this species is likely to experience more fluctuating, and higher, temperatures than either of the other species. A priori then the function of its haemocyanin might have been expected to adjust more readily to an increase in temperature.

In this chapter it has been demonstrated that H. roei haemocyanin is more resistant to heat denaturation than the haemocyanins of the other two species. However, it is no less sensitive to temperature change when viewed in terms of change in P_{50} for a given temperature change. Further, although both at 20°C, and 25°C the haemocyanin of this species delivers more oxygen to the tissues per respiratory cycle (Table 2.19) the relative loss in efficiency of this species' haemocyanin after an abrupt temperature increase to 25°C is greater than in the other two species. At this stage then it would appear that the O_2 delivery system of H. roei is no better adapted, or perhaps less well adapted, to conditions of abrupt temperature change, than the systems of the other two species. However, the overall significance of the haemocyanin's contribution to the O_2 supply to the tissues can only be assessed meaningfully in the light of data for oxygen consumption, and circulation in these organisms.

Table 2.19

Vol % oxygen delivered by abalone haemocyanin at 20°C, and after an abrupt temperature change to 25°C

Species	Vol % O_2 delivered by haemocyanin at 20°C - (A)	Vol % O_2 delivered by haemocyanin after abrupt temp. increase to 25°C - (B)	B expressed as a percentage of A ("efficiency")
H. ruber	.48	.394	82
H. laevigata	.54	.453	84
H. roei	.84	.535	64

Chapter 3

1. Oxygen consumption of H. ruber, H. laevigata and H. roei.
2. Heart function of H. ruber, H. laevigata and H. roei.
3. Relationship between in vivo haemocyanin function and heart function in the maintenance of the oxygen supply in the abalone.

3.1 General Introduction to Chapter 3 part I and II

"Much more should be done with the interaction of respiratory and circulatory physiology, ideally with unrestrained, intact organisms. The effect of temperature change on these interactions should be determined". (Redmond, 1968a).

It has been demonstrated (Chapter 2) that haemocyanin contributes significantly to the supply of oxygen to the tissues of the abalone. Values for arterial venous differences in vol % of oxygen have been determined for each of the three abalone species at the acclimation temperature 20°C, and after an abrupt temperature increase from 20°C to 25°C.

As the above quotation from Redmond (1968) suggests, I feel that for a more complete understanding of the overall physiological role of haemocyanin it is necessary to relate this oxygen supplied to the tissues in a single circulation of the blood, to the oxygen consumption of the animal under various conditions.

The oxygen consumption of an organism is limited by (1) the oxygen delivered to the tissues in a single circulation of the blood (A-V difference in vol % of oxygen) and (2) the rate of circulation of the blood as determined by the cardiac output (ml/minute).

Thus in general the O_2 consumption of an organism can be described by the equation

$$O_2 \text{ consumption} = \frac{A-V}{100} \times C.O.$$

where A-V = arterial venous difference in O_2 content (ml O_2/100 ml of blood)

and C.O. = cardiac output in ml/min

and O_2 consumption is expressed as ml/min

This relationship is sometimes known as Fick's principle.

As A-V difference is in fact the difference in O_2 content between pre and postbranchial bloods the equation obviously considers only the oxygen gained through the gills or ctenidia of an organism. There may therefore be some error in this relationship in organisms in which O_2 is also gained through respiratory surfaces other than gills or ctenidia.

The Cardiac output of an organism's heart is determined by

two parameters,

(1) the rate at which the heart is beating, and

(2) the volume of blood pumped at each stroke.

C.O. ml/min = H.R. (beats/min) x S.V. ml/beat

Thus, knowing cardiac output, if a value for either heartrate or stroke-volume can be obtained the remaining parameter can also be calculated.

3.1.1 Oxygen consumption, A-V difference, Heart Function Relationships in Haemocyanin Bearing organisms (Fick Principle)

Few studies have examined the broad relationship between haemocyanin function, heart function and oxygen consumption of haemocyanin bearing organisms.

Redmond (1955) used his A-V difference data for the decapod crustacean Panulirus, and oxygen consumption figures taken from Weymouth et alis (1944) compiled data on the influence of body weight on oxygen consumption of a series of decapod crustaceans, to estimate the cardiac output of a theoretical 750 gm crayfish. He obtained a value of 60 ml/min (80 ml/kgm/min).

He then estimated the stroke volume of his theoretical Panulirus, using heartrate values derived from Burger and Smythe's (1955) data on Homarus americanus. The stroke volume thus indicated was about .6 - 1.0 ml/beat which was somewhat larger than that measured by Burger and Smythe at .2 - .3 ml/beat. In view of the relatively primitive manner in which the latter authors measured their stroke volumes, Redmond was justified in considering his calculated value a reasonable figure.

Chapman and Martin (1957) briefly reported the measurement of oxygen consumption of the cephalopod Octopus dofleini. They also measured the A-V difference in O_2 content of the blood in several specimens, using indwelling catheters in the afferent and efferent vessels of the ctenidia, and found this parameter to be quite variable. For one 18 kgm

specimen the A-V difference was related to the oxygen consumption using the Fick principle, and a cardiac output of 570 ml/min (17.8 ml/kgm/min) was obtained.

This compares closely with values later calculated by Johansen (1965), who also measured oxygen consumption, and blood oxygen contents of Octopus dofleini. Johansen obtained an average cardiac output (via Fick principle) of 14.3 ml/kgm/min. It should be noted that there was considerable variation about this mean figure, although the cardiac outputs calculated for Octopus were considerably lower than Redmond's (1955) values for Panulirus, leading Johansen to say that, "A detailed discussion of the results obtained is limited by the lack of comparable information for other invertebrates". Johansen also measured heartrate of Octopus, and interestingly found that the average heartrate for these animals was 16 beats/minute, "regardless of size and state of activity". This constant heartrate necessarily results in a variable stroke volume (2.2 - 18.1 ml/beat) to maintain the calculated cardiac outputs.

Lenfant (1966), also working with Octopus dofleini, described in detail the role of haemocyanin in the delivery of O_2 to the tissues of this animal. He also calculated oxygen consumptions, based on differences between O_2 content of inhaled water and that of exhaled water, and the volume of efferent water from the siphon of this large cephalopod. Lenfant did not measure any functional parameters of the animal's heart. However when discussing the role of CO_2 in the unloading of oxygen to the tissues in this animal, which like all cephalopods exhibits a positive Bohr effect, Lenfant acknowledges that if O_2 utilization was to increase greatly (e.g. at times of vigorous activity), it would mean a necessary increase in cardiac output. From Johansen's (1965) observations of a relatively constant heartrate in Octopus dofleini, regardless of state of activity, this again suggests that the octopus heart must have a highly

variable stroke-volume.

Johansen et al. (1970) made a comprehensive study of the oxygen transport system of the large crab Cancer magister. They described the in vivo haemocyanin function in this crab, obtaining measurements for A-V difference in O_2 content which were considerably larger than those obtained in earlier studies (Redmond, 1955, 1962, 1968). Measurements of O_2 consumption of Cancer were also made, and the Fick principle was used to calculate a cardiac-output of 29.5 ml/kgm/min for this crab. Having obtained this value Johansen et al. strongly criticised Redmond's (1955) data claiming that the earlier author's experimental technique was faulty, leading to erroneously low arterial P_{O_2} values, and hence low A-V difference values (Chapter 2). The low A-V difference values necessarily resulted in high C.O. values to maintain the oxygen consumption of Panulirus. As a further criticism of Redmond's data, Johansen et al. propose that his O_2 consumption data for Panulirus, derived from Weymouth et al. (1944), is suspiciously low. Using more recently obtained values for Panulirus' oxygen consumption (Winget, 1969), and Redmond's A-V difference data, Johansen et al. calculate a high C.O. value of 400 ml/kgm/min for this decapod crustacean. Even ignoring this latter criticism Johansen et al. (1970) claim that a C.O. of 80 ml/kgm/min is likely to be too large, placing an unreasonable strain on an invertebrate heart. This claim by Johansen et al. (1970) is to some extent refuted by recent work on another crab species Callinectes sapidus (Mangum and Weiland, 1975). In this species of crab, calculation of C.O. using oxygen consumption, and A-V difference data yields a value of 207-238 ml/kgm/min. Mangum and Weiland propose that this value is in fact well within the mechanical capacity of the crab heart. Moreover they point out that Johansen et al. (1970) obtained a large A-V difference value for Cancer not through any exceptionally large A-V difference in % saturation of the haemocyanin of Cancer but primarily through the large O_2 capacity of the

haemocyanin of this species. Most decapod crustaceans, simply by virtue of the smaller O_2 capacities of their haemocyanins could not have such high A-V difference in vol % of oxygen as Cancer. Hence, most decapod crustaceans are likely to have higher C.O. values than Cancer.

Although the role which the heart must play in circulating the blood to maintain the oxygen supply is obviously not well understood in haemocyanin bearing organisms it should be seen from the above discussion how important it is to consider A-V difference in vol % of oxygen in the light of the overall oxygen consumption of the organism. The calculation of an obviously unreasonable C.O. value from measured A-V difference and O_2 consumption data could mean, for example that some error has been made in the measurement of these parameters, or alternatively that some other considerations must be taken into account; for example, the organism may be gaining a substantial amount of oxygen through respiratory surfaces other than the gills.

3.1.2 Application of Fick principle in gastropod molluscs

Because of the lack of reasonable data for A-V difference in vol % of oxygen for gastropod molluscs, there has been little opportunity for previous workers to examine the relationship between this factor, oxygen consumption, and heart function in this group of haemocyanin bearing organisms. In this study however, the in vivo function of haemocyanin in the abalone has been described.

In part I of this chapter the oxygen consumption of the three species of abalone will be examined under controlled conditions. The A-V differences previously obtained will then be related to the oxygen consumption data via the Fick principle to obtain values for the cardiac outputs of the abalone species. These values will be discussed in the light of the limited data in the literature describing cardiac outputs of other invertebrates.

In part II of this chapter, measurements of heartrates of the abalone species are described. This data is then related to the calculated cardiac output data to obtain values for stroke volumes of the abalone heart.

Chapter 3

Part I

3.2 Oxygen Consumption

3.2.1 Introduction

There is a vast amount of literature describing the way in which numerous factors may modify the oxygen consumption of a given invertebrate organism. The references given below are only a few examples of work which has been done concerning the influence of secondary, or modifying factors on the oxygen consumption of arthropods and molluscs. In many cases the influence of more than one modifying factor on oxygen consumption has been described.

Factors which may modify oxygen consumption include

1. temperature (Read, 1962; Davies, 1966; Newell and Northcroft, 1967; Newell, 1969; Newell et al., 1970; Widdows, 1973; Pye and Newell, 1973)
2. oxygen tension of the environment (Von Brand, 1953; Weins and Armitage, 1961; Larimer and Riggs, 1961; Teal, 1967; Kushins and Mangum, 1971).
3. salinity (Lance, 1965; McFarland and Pickens, 1965; McLusky, 1969; Hagerman, 1969; Bayne, 1973 (a); Engel et al., 1974).
4. size of the organism (Body and Proctor, 1932; Weymouth et al., Body, 1945; Zeuthe, 1947, 1953; Hemmingsen, 1960; Roa et al., 1954; Bertalanffy, 1957; Prosser, 1966, 1973; Davies, 1966; Winget, 1964; Newell, 1970; Newell and Ray, 1973).
5. nutrition (Mcpherson, 1968: Bayne, 1973 (b); Marsden et al., 1973)

6. activity (Van Dam, 1954; Mcfarland and Pickens, 1965; Hagerman, 1969)
7. numerous other factors such as time of day or photoperiod (Webb et al., 1958; Dehnel, 1958; Sandeen et al., 1954; Hamman, 1974), stages in life cycles, moulting cycles etc. (Prosser, 1973), season (Mcfarland and Pickens, 1965; Berg, 1958; McMahon, 1973), and sex and breeding condition of the individual organism (Prosser and Brown, 1966; Prosser, 1973).

It should be emphasised that not all of these factors necessarily modify the oxygen consumption of any given species.

In the section of this project dealing with the contribution of haemocyanin to the oxygen supply to the tissues (Chapter 3), animals of varying sizes were used. In order to arrive at a general assessment of haemocyanin's contribution to the oxygen supply of the animals, the relationship between

(1) size and oxygen consumption is examined for each abalone species. Further, the influence of temperature change on the contribution of the haemocyanin to the oxygen supply of the abalone has been examined (Chapter 3 section 3.3.47). Therefore

(2) the influence of temperature on the rate of oxygen consumption by the abalone of a range of sizes is also determined.

3.2.11 Influence of body size on oxygen consumption

There have been numerous summaries of the vast literature which has accumulated describing the relationship between metabolic rate and body size for various organisms (see references above).

From all of this work it can be seen that the general form by which the oxygen consumption of a given species can be related to the size of the organism is expressed by the equation $M = KW^b$. This equation can also be expressed in the form $\log M = \log K + b \log W$.

In other words, the metabolism is proportional to a fractional power of the body weight where

$M = O_2$ consumed/unit time

W = the weight of the tissues

K = a constant giving the intercept on the y axis of the logarithmic regression line relating log M to log W, and

b = a constant describing the slope of this logarithmic regression line.

If oxygen consumed per unit weight is required the function becomes $\log \frac{M}{W} = (b - 1) \log W + \log K$ (cf. Bertalanffy, 1957; Newell, 1971 (b); Prosser and Brown, 1966; Prosser, 1973).

It can be seen that the value of the constant b defines the way in which the oxygen consumption of the particular organism being investigated changes with size.

Prosser (1973 pp. 192-193 Table 5-2) lists b values obtained from all the vertebrate classes and includes examples from most invertebrate phyla. In the examples listed for molluscs alone, b ranges from 1 for the snail Lymnaea (which means that metabolism is directly proportional to weight) to .696 for another gastropod Patella.

In some groups of organisms b values have been shown to vary even more widely than this. For example, in a species of barnacle Balanus balanoides the oxygen consumption at low temperatures has been shown to be almost independent of body weight at some times of the year (January in Scotland), b = .0138.

However at higher temperatures the value of b increases quite markedly in this species (Barnes and Barnes, 1969). Under some temperature conditions Newell and Northcroft (1967) obtained b values as high as 1.14 for the same species of barnacles. This is merely one example of the way in which the slope of the regression line relating oxygen consumption to body weight may change, not only between apparently closely related species (cf. Barnes and Barnes, 1969), but also within a given species depending on, for example, the temperature at which the

oxygen consumption measurements are being made. Numerous other factors, e.g. salinity (Rao, 1958; Edwards and Irving, 1943; Edwards, 1940) time of year and acclimation temperature have also been shown to influence the value of b (Dehnel, 1960; Newell and Pye, 1970 (a); Newell and Pye, 1970 (b)).

5.2.12 Influence of temperature on oxygen consumption

A change in temperature, as well as possibly causing a change in the slope of the regression line relating log oxygen consumption to log weight, may also have an affect on the "height" of this regression line. Perhaps the most usually accepted method of quantifying the effect of temperature on oxygen consumption rates is by the Q_{10} approxiration (e.g. Prosser, 1966, 1973). The Q_{10} approximation is defined as the factor by which a reaction velocity is increased for a 10°C rise in temperature. The Q_{10} for an oxygen consumption change is calculated by

$$Q_{10} = \left(\frac{K_1}{K_2}\right)^{10/(t_1-t_2)}$$

where K_1 = rate of O_2 consumption at t_1 and

k_2 = rate of O_2 consumption at t_2

Recently, as in the study of O_2 consumption-weight relationships, a large body of work describing O_2 consumption-temperature relationships has accumulated, particularly concerning intertidal, freshwater, and marine invertebrates (Grainger, 1956; Dehnel, 1960; Berg et al., 1962; Courtney and Newell, 1965; Davis, 1966, 1967; Newell, 1966, 1969, 1970; Newell and Northcroft, 1967; Newell and Pye, 1970 a,b.). Whereas in the past it has generally been accepted that in most poikilotherms the metabolism rises and falls with body temperature with a Q_{10} of about 2.5 in the physiological range (cf. Prosser, 1973), as a result of this work it has

been shown that there is a great deal of variation in the magnitude of the temperature effect on the oxygen consumption on various organisms. Moreover the conditions under which oxygen consumption is being measured may greatly influence the magnitude of the Q_{10} in any given species.

It has long been known that the change in oxygen consumption observed for a given organism in response to a temperature change, may be influenced by the temperature to which the animal is acclimated (Bullock, 1955; Precht, 1958; Prosser, 1958; Berg, 1958). Many other factors, such as the season during which the oxygen consumption measurements are being made may influence the observed response of oxygen consumption to temperature change in some organisms (Edwards and Irving, 1943 a,b; Newell and Pye, 1970 a; Barnes and Barnes, 1969). As starvation may affect the way in which O_2 consumption changes with temperature change (Barnes et al., 1963 ; Marsden et al., 1973) the seasonal influence on Q_{10} may not be simply due to acclimation to different field temperatures, but may be complicated by other factors such as the nutritional state of the organisms due to different food availabilities at different times of the year (Barnes et al., 1963). Another factor which influences the way in which temperature change affects the oxygen consumption of organisms is the degree of activity of the organism. In many intertidal invertebrates in particular, it has been shown that although the oxygen consumption of active organisms is remarkably dependent on temperature, the standard oxygen consumption is relatively independent of temperature change over a considerable temperature range (Newell and Northcroft, 1965, 1967; Newell, 1966, 1969, 1970; Newell and Pye, 1970 a; Newell and Pye, 1971). However, subtidal organisms have not been found to demonstrate this temperature independence of standard metabolic rate (cf. Courtney and Newell, 1965).

It is also possible for a given species that animals of different sizes may have different Q_{10}'s. (Read, 1962; Newell and Pye, 1971).

Obviously this ties in with the previous discussion on the influence of bodyweight on oxygen consumption; different Q_{10}'s for different sized animals will lead to a change in b, the slope of the regression line, with temperature.

It is intended in this chapter to describe the influence of size, and temperature change on the oxygen consumption of each of the three abalone species, where possible accounting for the numerous other factors which may modify the oxygen consumptions of these animals. A mathematical model will be developed by which, knowing the species, size, and temperature of a given abalone specimen, its oxygen consumption can be predicted.

5.2.2 Apparatus for oxygen consumption measurements

The oxygen consumption measurements were carried out using a closed respirometer system, based on that described by Ulbricht et al. (1972) for measuring the metabolic rate of sea urchins (Figure 5.1).

Several respiratory chambers of different sizes were used, to enable measurement of O_2 uptake of various sized abalone. The chambers were constructed from glass dessicators. The lid, a flat glass plate, was sealed to the ground glass top of the dessicator using vacuum grease. The water in the chamber was kept circulating using a large stirring bar and powerful magnetic stirrer. The stirring bar was protected from interference by the abalone by a plastic mesh shield. Filtered, aged sea water was used in the respirometer to ensure that bacterial or algal contamination did not affect oxygen levels in the apparatus.

The oxygen uptake from the water in the respirometer was measured using a Titron oxygen probe Type 500 MB, sealed through an opening in the glass lid of the respiratory chamber. The oxygen

Figure 3.1

Photograph of the closed respirometer system used to measure oxygen consumption of abalone.

Note: (1) sealed glass respiratory chamber, with stirring bar protected by open mesh.

(2) Titron oxygen probe (marked 111)

The apparatus was used in constant temperature rooms.

concentration of the water in parts per million was read on a Titron portable oxygen meter (Titron Instruments, Victoria). The oxygen electrode was calibrated following the recommended procedure in the Titron Technical Bulletin 500 MB. Calibration was checked before and after every experiment. It was found that with careful assembly and maintenance the electrode would maintain exact calibration over a period of two weeks or more. The salinity of the sea water (necessary for O_2 electrode calibration) was measured using an Autolab Model 602 salinometer.

3.2.3 Measurement of oxygen consumption

All oxygen consumption experiments were carried out in constant temperature rooms - air temperature fluctuation $\pm$ 1°C. The water temperature showed no significant fluctuation during the course of an experiment.

Prior to the commencement of O_2 uptake determinations, the abalone were placed in the aerated respirometer for a one hour "settling period".

This settling period was considered necessary for two reasons.

(1) Immediately after transfer to the respirometer from the aquaria, particularly if this involved a temperature change (see later experiments), some abalone became agitated, moving rapidly around the respirometer.

In this project, as no method was available to quantify degree of activity, standard metabolism only was considered. After the one hour settling period it was usually found that animals had ceased all noticeable movement. Moreover it should be noted that settled abalone cannot be readily induced to activity. It is most likely then that the oxygen consumption measured after this time can be considered standard oxygen consumption.

(2) When an animal is transferred abruptly from one temperature to another, even if not agitated, the oxygen consumption sometimes temporarily reaches an abnormally high level (overshoot response). This usually

lasts a few minutes, but not longer than one hour before the oxygen consumption returns to the stable level which will last at the new temperature for several hours or days (cf. Grainger, 1956; Prosser, 1960, 1973).

After the one hour settling period, the respirometer was topped up, and carefully sealed with the glass lid. Any remaining air bubbles were bled from the apparatus through a small hole in the lid, using a large syringe. The hole was then sealed with a greased rubber bung, and O_2 uptake measurements were commenced. The assembled apparatus is shown in Figure 3.1.

For measurement of standard oxygen consumption, O_2 uptake was measured over a period during which the rate of reduction of the oxygen content of water remained constant, usually for approximately one hour (see section 3.2.41).

At the completion of the O_2 uptake measurements, the animal was taken from the respirometer, and after ensuring that all excess water was removed, weighed. The animal was then killed and the shell removed and weighed. From the total weight, and the shell weight, the wet weight of the soft parts of the animal was calculated.

Knowing (1) the volume of water in the respirometer, (2) the difference in concentration of respirometer oxygen between commencement and completion of the experiment, and (3) the time over which this difference was measured the oxygen uptake of an animal of known weight could be calculated.

O_2 uptake ul/hr =

$$\frac{\text{time in minutes}}{60} \times \frac{\text{change in } O_2 \text{ conc'n(ppm)} \times 22.4 \times \text{Vol respirometer (ml)}}{32}$$ *

By dividing the above answer by the weight of the animal (g) the oxygen consumption in terms of ul/g/hr can be obtained.

* 32 = M.W. O_2
22.4 = VOLUME (L) OCCUPIED BY 1 MOLE O_2 AT STP.

3.2.4 Preliminary Experiments - Introduction

Because of the numerous factors which may modify the oxygen consumption of a given organism, before commencing the investigation of the effects of size and temperature on the oxygen consumption of each of the three abalone species, a brief preliminary investigation of the effect of (1) oxygen tension (2) time of day and (3) captivity on the O_2 uptake of the abalone was carried out.

This was thought to be necessary because the effects of such modifying factors may mask the effects of those factors with which this study is primarily concerned.

3.2.41 Effect of oxygen tension

The oxygen consumptions of various organisms are affected in various ways by the oxygen tensions of the environment. Many organisms regulate their oxygen consumption in decreasing O_2 tensions, down to a critical oxygen tension (Pc) below which the oxygen consumption falls off rapidly. This Pc may vary within a given species depending on such factors as temperature of the environment, and state of activity of the animal.

Those animals which show independence of environmental oxygen tension over a wide range of O_2 tensions are known as oxygen regulators. Many molluscs have been shown to be oxygen regulators (Van Dam, 1954; Bayne, 1967; Moon and Pritchard, 1970 etc.).

In other organisms however the oxygen consumption is dependent on the oxygen tension of the environment - as the environmental oxygen tension rises so does the oxygen consumption of the organism. These animals, known as oxygen conformers also include some molluscs (Berg, 1952; Prosser, 1973). It should be noted that the distinction between O_2 regulators and O_2 conformers is not always clear cut, and a given organism's ability to regulate its O_2 consumption may be affected

by factors such as starvation, or some other stresses related to collection and maintenance in the laboratory (Bayne, 1971).

Now, in using the closed respirometer system to investigate the O_2 consumption in this study, the animals are subjected to a continual decrease in the environmental oxygen concentration as they progressively use up the oxygen in the chamber. If the animals are oxygen regulators, the oxygen consumption measured would, at least over part of the O_2 concentration range, be expected to be independent of the O_2 concentration, and thus be the same as that measured in a continuous flow or open system. However if the animals are oxygen conformers, with the progressive decrease in oxygen concentration in the chamber, the oxygen uptake will also decrease. This will lead to a final oxygen uptake which is not equivalent to that which would have been measured in a system where the O_2 tension is maintained at a level similar to that of air equilibrated seawater.

A further consideration must be made. It has been documented (e.g. Redfield et al., 1929) that increased CO_2 concentrations in the environment can affect the O_2 uptake of some organisms. In the closed respirometer system there will be an increase in CO_2 concentration as the organism respires. Any affect on the O_2 uptake of an organism likely to be caused by such an increase should be considered in evaluating the effectiveness of the system in measuring oxygen consumption (Larimer and Gold, 1961; Courtney and Newell, 1965).

In order to determine whether the closed respirometer system was likely to have any effect on the final oxygen consumption of the abalone, the rate of uptake of oxygen from the water of the sealed respirometer was closely monitored over a wide range of oxygen concentrations. Although the CO_2 concentration may be presumed to be increasing in these sealed systems, no measurements were made of this parameter. Several experiments were carried out with different animals of each species at each of three experimental temperatures.

Method

The method used to investigate the response of the oxygen consumption of the abalone to declining oxygen tensions and increasing CO_2 tension was the same as that used by Newell and Courtney (1965) in their study of oxygen consumption of lancelets (Branchiostoma lanceolatum).

Animals of various sizes were placed in respirometers of various sizes, and allowed to settle for one hour before the commencement of O_2 uptake readings.

The respirometers were then sealed, and the oxygen tension in the respirometers were read at short intervals for the duration of the experiment.

Results and Discussion

The results are presented graphically on figures 3.2, 3.3 and 3.4. These figures give plots of O_2 concentration in the respirometer, against time (cf. Newell and Courtney, 1965, Figure 1). The weights of the animals are given on the figures, as are the temperatures of the experiments. It can be seen that for individuals of each species at each of the three experimental temperatures (15°C, 20°C, 25°C) the rate of uptake of oxygen from the respirometers is constant over a wide range of oxygen concentrations. The slopes of the lines differ largely because of different sized animals and volumes of water. It is evident that the three abalone species can be considered oxygen regulators. Although some individuals of each species were subjected to low O_2 concentrations (down to, and less than 1 ppm) in no case could an abrupt critical O_2 tension be defined: however, at the lower O_2 tensions there was a gradual reduction in the steepness of the O_2 uptake curve (Figures 3.2, 3.3, 3.4). This is similar to Valvata pincinalis (Berg, 1961).

From an examination of the figures it was decided that in each species the O_2 uptake was unaffected by O_2 concentration at least down to an oxygen concentration in the region of 2.8 ppm O_2 at all temperatures.

Figures 3.2, 3.3, and 3.4

Graphs of the variation of oxygen concentration with time for Haliotis ruber, H. roei and H. laevigata in closed respirometers containing abalone of various sizes.

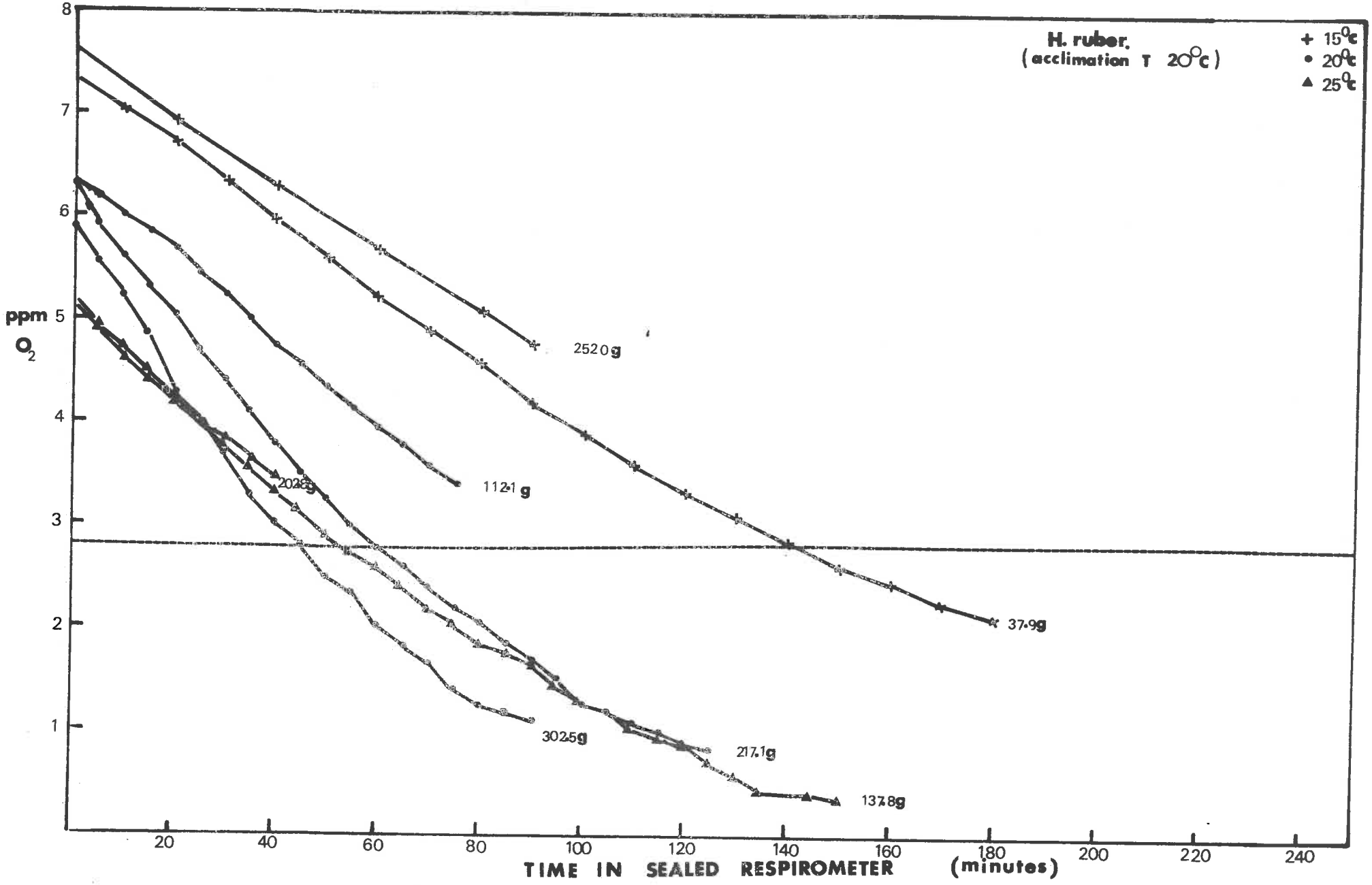

H. ruber.
(acclimation T 20°C)
+ 15°C
• 20°C
▲ 25°C
ppm O_2
8
7
6
5
4
3
2
1
2520g
1124g
202.8g
37.9g
302.5g
217.1g
137.8g
20
40
60
80
100
120
140
160
180
200
220
240
TIME IN SEALED RESPIROMETER (minutes)

Fig 3.2.

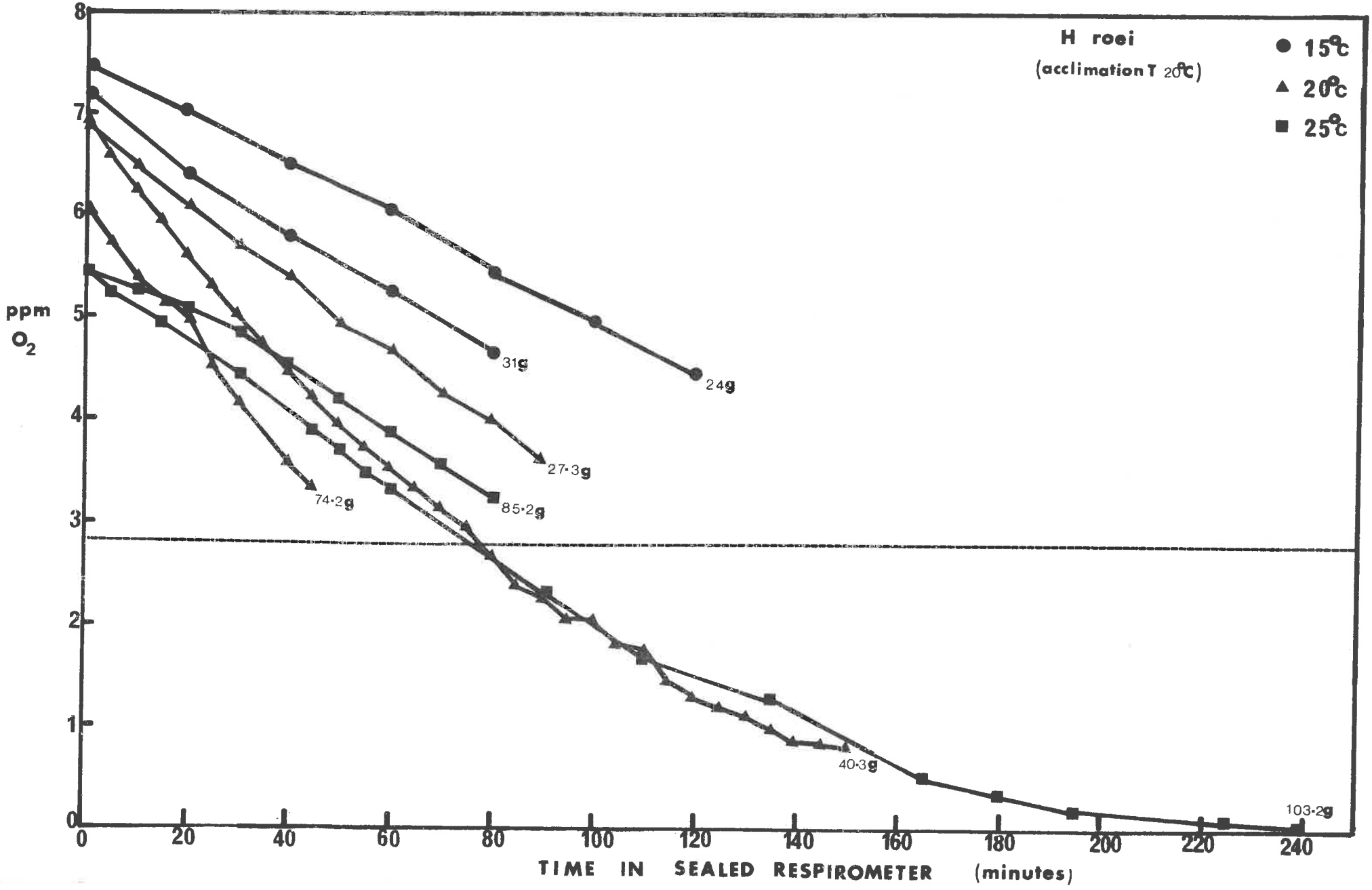

H roei
(acclimation T 20°C)
15°c
20°c
25°c
ppm
O_2
31g
24g
27·3g
74·2g
85·2g
40·3g
103·2g
TIME IN SEALED RESPIROMETER (minutes)

Fig-3.3.

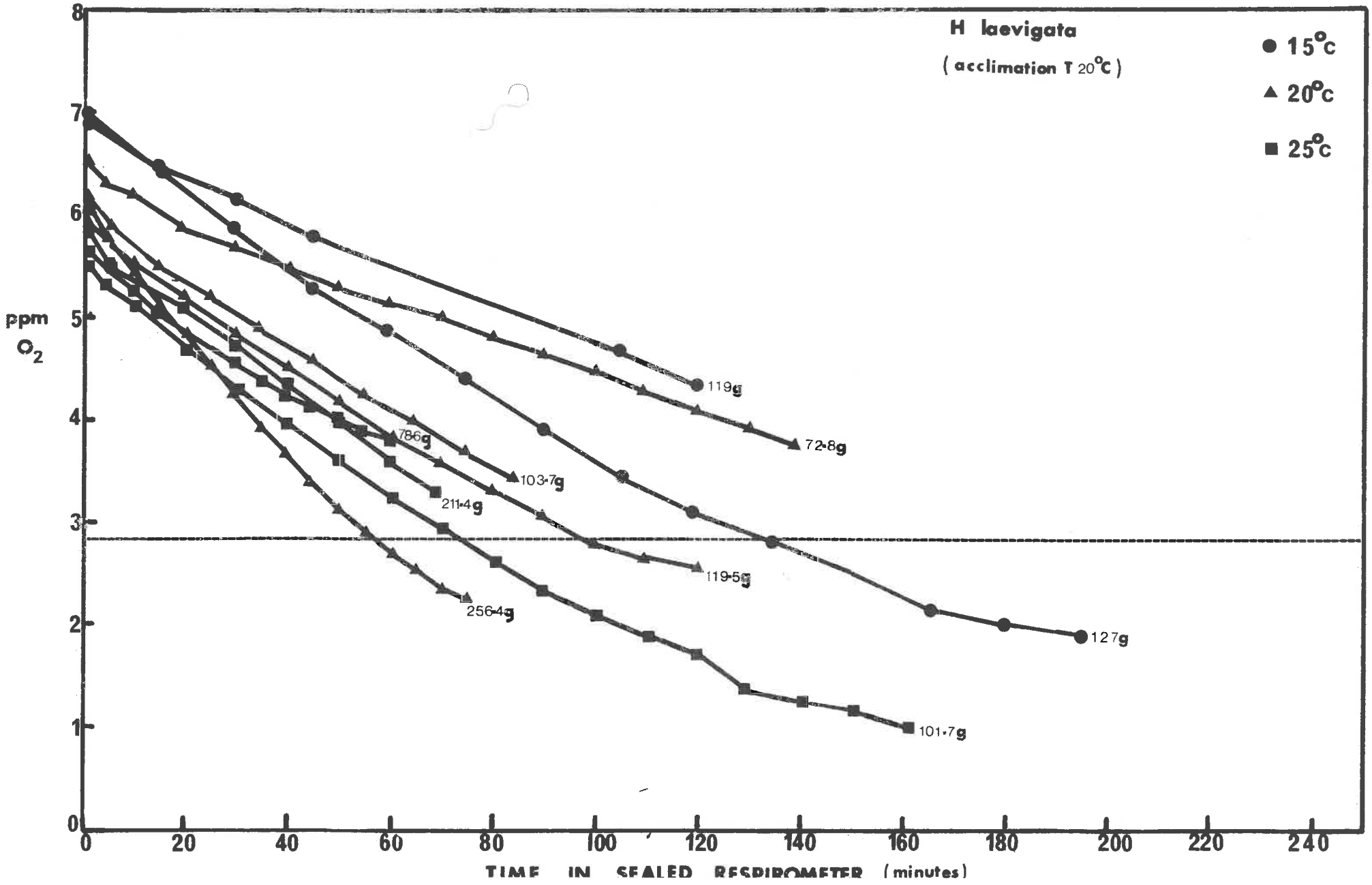

H laevigata
(acclimation T 20°C)
● 15°C
▲ 20°C
■ 25°C
ppm O2
119g
72·8g
78·6g
103·7g
211·4g
119·5g
256·4g
127g
101·7g
0
1
2
3
4
5
6
7
8
0
20
40
60
80
100
120
140
160
180
200
220
240
TIME IN SEALED RESPIROMETER (minutes)

Fig. 34.

This point, 2.8 ppm was arbitrarily chosen as the "cut-off" point for all oxygen consumption experiments.

This oxygen concentration corresponds to a P_{O_2} of from 65 mm Hg to 55 mm Hg depending on the temperature, 25°C to 15°C respectively, and depending on the salinity of the water - 37°/oo in this case.

Abalone haemocyanin has a strong reverse Bohr effect. Thus slightly increased CO_2 tensions in the respirometer will promote loading of O_2 at the gills rather than hinder it. Taking this into account, even presuming that abalone gills present a considerable diffusion barrier P_{O_2} values from 65-55 mm Hg should be easily high enough to maintain high saturation of abalone haemocyanin at the gills. It is not therefore surprising that the O_2 uptake of these animals remains constant at least down to these P_{O_2} values.

At the lower oxygen concentrations the abalone showed obvious signs of distress. At about 2-3 ppm the animals often demonstrated a typical escape response identical to that described by Shepherd (1973) as an escape response to predator attack. At lower O_2 tensions (less than 2 ppm) the shells were raised from the epipodia, and spasmodically clamped down again. The epipodia assumed a typical "buckled" appearance, Figure 3.5, and the animals eventually lost their grip on the substrate. The response of abalone to prolonged anoxia is discussed in more detail in chapter 4.

As a result of this preliminary investigation, the O_2 uptake of abalone of all three species was considered independent of external O_2 concentration down to the arbitrarily defined limit of 2.8 ppm.

3.2.42 Circadian rhythms in oxygen consumption of abalone

Circadian rhythms have been shown to affect various aspects of plant and animal life (Webb and Brown, 1959; Prosser and Brown, 1966; Prosser, 1973).

Figure 3.5

Haliotis ruber specimen stressed by low oxygen tensions. The epipodium shows the typical 'buckled' appearance described in the text. The epipodium also looks like this in animals stressed by abrupt temperature changes.

More specifically, with regard to this project, circadian rhythms have been shown to affect various aspects of the lives of molluscs. For example, the daily rhythm of water propulsion in Mytilus was found to be related to tidal water movement (Rao, 1954), and diurnal rhythms in the opening and closing of the valves of some bivalve molluscs have been described (Brown, 1954; Bennett 1954). Recently circadian locomotor activity has been described in the sea hare Aplysia californica (Jacklett, 1972). More specifically still, circadian rhythms in the uptake of oxygen have been found in several species of molluscs. Sandeen et al. (1954) demonstrated persistant tidal rhythms in the oxygen consumption of two species of intertidal snails Littorina littorea and Urospinx cinereus. In an early paper Gompel (1937) reported the existence of a tidal rhythm in oxygen consumption for three other littoral molluscs. These molluscs include a species of abalone, which Gompel claims demonstrates a circadian oxygen consumption rhythm with a periodicity of about 12.4 hours.

Now, the abalone used in the oxygen consumption experiments in this study were maintained in aquaria in an aquarium room continuously illuminated with artificial light. However it is known that some diurnal rhythms persist even in unvarying environmental conditions (Prosser, 1973). In view of this fact it was deemed necessary in this study to determine whether any such persistant diurnal rhythm in oxygen consumption might occur, which might complicate the results of investigations of the effect of weight and temperature change on oxygen consumption. Accordingly, the oxygen consumptions of six specimens (two of each species) were monitored over several hours, with particular emphasis on the daylight hours, in which future O_2 consumption experiments would be conducted.

Method

The animals used in this experiment were collected near the end of the winter month of June, from water at a temperature of about 16°C. They were transferred to the aquaria at 20°C. The 24 hour experiments were not commenced until the animals had been in the laboratory for one week. At the time the experiments were conducted (July 2,3,4) it was dark by 5.30 p.m. and light again at about 6.30 a.m.

All oxygen consumption measurements were conducted as described in section 3.2.3. After each oxygen consumption determination was made, the respirometer was opened and about 50% of the seawater was replaced with fresh oxygenated filtered seawater at the same temperature, and then the respirometer was reoxygenated for approximately 15 minutes.

In this way the animal was disturbed as little as possible between experiments. All experiments were conducted in a constant temperature room, air temperature 22°C ± 1.5°C, water temperature 19-20°C. At the end of the experiments, the animals were weighed, and the oxygen consumptions calculated as shown in section 3.2.3.

Results and Discussion

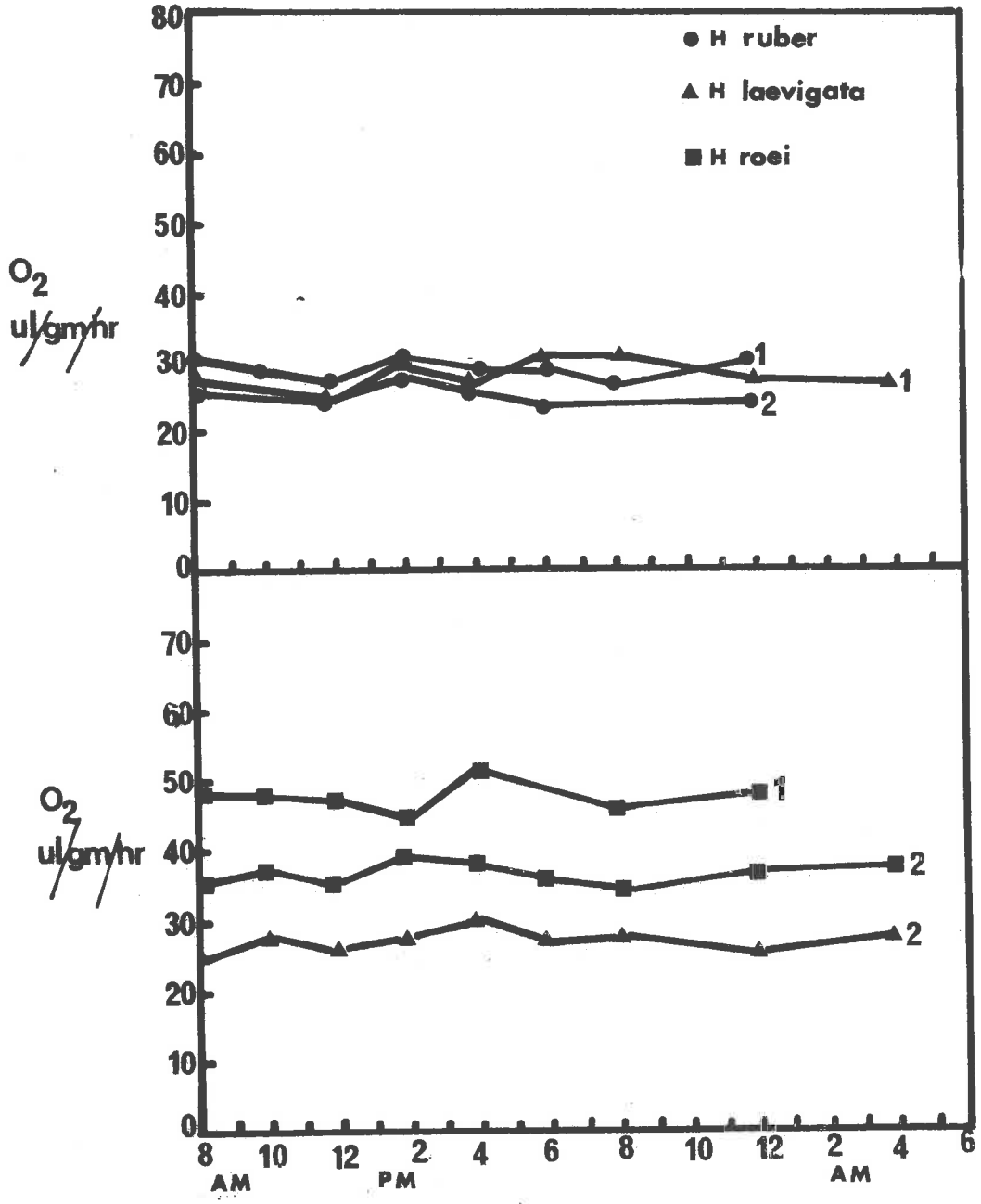
The results of the experiments are plotted on Figure 3.6. As can be seen from the graphs, although there were noticeable fluctuations in the oxygen consumption from time to time, there was no clearly discernable rhythm in these fluctuations, as was reported by Gompel (1937) for an intertidal species of abalone. There is certainly no evidence of the large clearly defined diurnal rhythms in O_2 consumption such as found in the intertidal gastropods Littorina littorea and Urospinx cinereus (Sandeen et al., 1954).

The variation in O_2 consumption between measurements, although showing no pattern, was large when compared to those found recently for the crayfish Orconectes limorus, which apart from the single high peak at

Figure 5.6

Diurnal variation of oxygen consumption

of abalone



H	ruber	1	81·4 g
H	ruber	2	64·6 g
H	laevigata	1	160·2 g
H	laevigata	2	172·0 g
H	roei	1	119·0 g
H	roei	2	126·0 g

the onset of the dark phase showed an extremely steady rate of oxygen consumption (Hamannd, 1973).

However the variation is relatively small compared to that observed in the sea urchin Strongylocentrotus over a period of 56 hours (Ulbricht et al., 1972).

It is possible that keeping the abalone in the constant light conditions in the laboratory has removed a stimulus which might have maintained a rhythm in oxygen consumption such as observed in another species of abalone (Gompel, 1957). However this brief experiment indicates that at least in the captive specimens, experiments on oxygen consumption can be carried out during the whole day without risk of modification by diurnal rhythms in O_2 uptake.

Long term rhythmical patterns of some aspects of the behaviour and physiology of various animals, associated with such factors as phases of the moon or tides, have been observed on numerous occasions (Nayler, 1958; Brown, 1959; Fingerman, 1960; Hausenchild, 1960; Enright, 1965 a,b; Chandrashcharan, 1965). It is possible that such a long term of fluctuation in O_2 consumption may occur in the abalone species considered in this thesis. This possibility is not investigated in this project.

5.2.43 Effect of captivity on the oxygen consumption of the abalone

In the introduction to this chapter the conditions under which the animals used in the oxygen consumption experiments were maintained in the laboratory were described. The animals were maintained in the aquaria without feeding. Some animals were used in oxygen consumption experiments as many as ten days after the commencement of the experiments.

The effect of starvation on the oxygen consumption of several invertebrates has been investigated (e.g. Barnes et al., 1963 ; Marsden et al., 1973). The degree to which starvation affects the metabolism differs considerably between organisms. For example starvation

of the crab Cancer maenas in the laboratory "results in a progressive suppression of the metabolism compared with fully fed crabs. This effect becomes apparent after two weeks at 15°C...." (Marsden et al., 1973). In a species of cirripede (barnacle) however, periods of up to 124 days starvation do not affect the oxygen consumption of animals collected during winter, and only slightly lower the oxygen consumption of animals collected during summer (Barnes et al., 1963,).

Abalone are known to survive long periods without food (Pilson, 1965; Boolootian, 1965). Crop analyses of H. ruber and H. laevigata at West Island showed that the animal ate little or nothing during calm weather (Shepherd, 1973, Figure 28). It would seem then that under natural conditions, in calm summer weather, abalone probably go several days without feeding. During this study animals were found to survive up to three months in the laboratory without feeding. The circumstantial evidence therefore all points to the fact that abalone might be able to tolerate several days of starvation without any adverse affects.

During this study, for six specimens, the oxygen consumption was measured after starvation for various lengths of time. The results of the O_2 consumption determinations are presented on Table 3.1 and are illustrated graphically on Figure 3.7.

It can be seen that in specimens for which the oxygen consumption was measured after seven days and then after 14 days in the laboratory there was no significant difference in the O_2 consumption.

However, after 30 days in the laboratory the O_2 consumption had dropped considerably, being only about 2/3rds as large as after seven days starvation. From this data it might be suggested that the standard metabolism of the abalone is relatively unaffected by starvation for one or two weeks but after longer periods gradually decreases.

The solid line fitted to Figure 3.7 graphically illustrates this statement: the dotted vertical lines define the time limits during which

Table 3.1

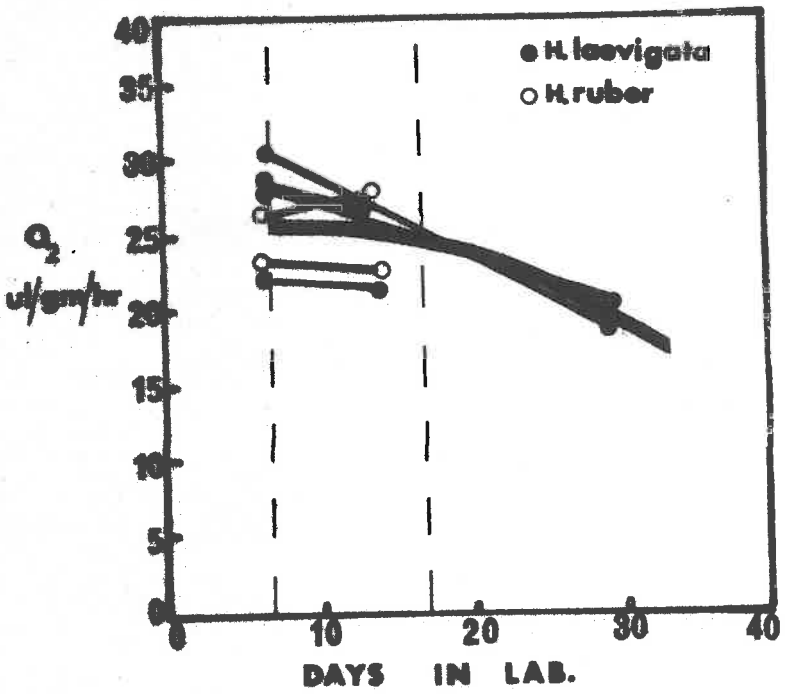
The effect of captivity on the oxygen consumption of abalone

Animal no.	Species	Weight (gm)	Time in laboratory (days) 7	14
			oxygen consumption μl/gm/hr	
1	H. ruber	142.5	23.5	22.7
2	H. ruber	103.2	26.7	26.6
3.	H. laevigata	135.7	22.1	21.5
4	H. laevigata	130.5	28.8	25.7
	mean O_2 consumptions		25.28*	24.65*
5	H. laevigata	126.0	27.9	20.9
6	H. laevigata	119.0	30.9	13.4

* N.S.D. t = .29 6df

Figure 3.7

Graphical representation of data on Table 3.1



animals were used in oxygen consumption experiments.

These few results therefore suggest that the laboratory starvation of the abalone did not greatly modify the results of the oxygen consumption experiments reported in this study.

5.2.5 The effect of body weight, and temperature change on the oxygen consumption of the three abalone species H. roei, H. ruber and H. laevigata

5.2.51 Methods and Materials

(a) Animals

The animals used in the weight-temperature experiments reported here were collected at West Island during the summer months 1972-1973. During these months, mid November through to mid April, the water temperature at West Island did not differ from 20°C by more than 5°C (Figure 5.8); all animals were transferred to the laboratory and kept in aquaria at 20°C ± 1.5°C, for at least one week before the commencement of the oxygen consumption measurements. Animals were not used in experiments more than ten days after the commencement of any particular set of experiments (i.e. 17 days after captivity).

It has been pointed out that in interspecific comparisons care must be taken that the species are at comparable stages of their breeding season (Berg et al., 1956; Barnes et al., 1963).

Shepherd and Laws (1974) studied the spawning of H. roei, H. ruber, and H. laevigata at West Island, examining the gonad indices to determine the reproductive cycle. Figure 5.9 is reproduced from their summary of spawning seasons of abalone throughout the world (Shepherd and Laws, 1974, Figure 10). It can be seen that through much of the year the three abalone species do not coincide with regard to their reproductive state. The summer months, when the animals in this study are compared is the only time when all three species from West Island show a coinciding period of little or no spawning activity. By comparing the species at

Figure 3.8

Variation of sea temperature throughout a typical year at West Island

Figure 3.9

Comparison of periods of maximum spawning activity of Haliotis ruber, H. roei, and H. laevigata

Fig.3.8

MEAN MONTHLY SEA TEMPERATURES AT WEST ISLAND

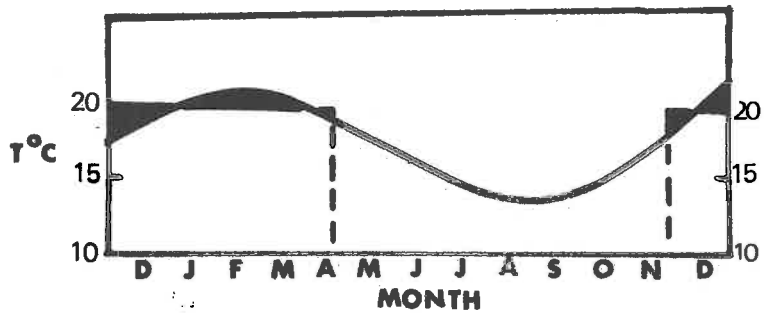


Fig.3.9

	SPRING S O N	SUMMER D J F	AUTUMN M A M	WINTER J J A
H ruber	———	- - -	- - -	
H reei	———		———	———
H laevigata	———	- - -	———	- - -

——— SPAWNING

(Figures after Shepherd and Laws 1974)

this time it was anticipated that any modification of oxygen consumption due to the stage of the reproductive cycle would be at least reasonably similar in all three species.

(b) Measurement of oxygen consumption

The oxygen consumptions of the abalone were determined as described in Section 3.2.3. After transfer from the aquaria to the respirometer animals were given 60-80 minutes to settle down before commencement of O_2 consumption measurements (see Section 3.2.3). If any animal showed signs of movement during the course of an experiment the results were discarded. For each species, at each experimental temperature the oxygen consumption of nine animals of a range of sizes was determined. (Each animal was only used in one oxygen consumption determination).

At the completion of the oxygen consumption run, the oxygen consumption for each animal was calculated as shown in Section 3.2.3.

3.2.52 Results and Discussion

Table 3.2, gives the results of the oxygen consumption measurements of various sized animals of H. roei, H. ruber and H. laevigata at the experimental temperatures 15°C, 20°C and 25°C. As is common in oxygen consumption weight analyses, all calculations were made using logarithmic data (in this case ln, ($\log_e$) - some other references mentioned in this chapter use $\log_{10}$; the conclusions drawn in either case are quite comparable). The data on table 3.2 are given later as logarithms on tables 3.6.1-3.6.3.

An analysis of covariance was carried out on the data to see if oxygen consumption varied with species, temperature or weight. The first step in the analysis involved determining the effect of weight on the oxygen consumption of each of the three species under the various experimental conditions. The slopes of the regression lines b, relating ln oxygen consumption to ln weight were calculated for each species at

Table 3.2

Oxygen consumptions of *H. roei*, *H. laevigata* and *H. ruber* at 15°C, 20°C and 25°C

	H. roei			*H. laevigata*			*H. ruber*		
	Animal No.	Wgt (gms)	O_2 consumption l/hr	Animal No.	Wgt (gms)	O_2 consumption l/hr	Animal No.	Wgt (gms)	O_2 consumption l/hr
15°C	1	55.400	1495.800	1	17.200	509.120	1	164.500	3059.700
	2	49.500	1395.900	2	58.600	1716.980	2	252.000	4561.200
	3	78.300	1996.650	3	119.200	2980.000	3	79.900	1717.850
	4	55.200	1396.560	4	295.000	4536.500	4	121.400	2245.900
	5	25.500	764.060	5	69.400	1846.040	5	104.300	2524.060
	6	29.000	875.800	6	127.000	3263.900	6	63.300	1810.380
	7	24.100	867.600	7	119.000	2058.700	7	57.900	1099.100
	8	31.500	1061.070	8	71.900	1545.850	8	35.500	1196.350
	9	20.500	754.400	9	202.000	3575.400	9	31.500	954.450
20°C	1	57.800	2155.940	1	114.400	3683.680	1	217.100	5557.760
	2	27.500	1250.350	2	46.000	1554.800	2	112.100	3878.660
	3	60.500	1948.100	3	193.700	4164.550	3	302.500	6080.250
	4	20.100	1015.040	4	72.800	1951.040	4	95.300	2509.770
	5	55.900	2124.200	5	117.000	3439.800	5	48.500	1668.400
	6	57.200	2448.160	6	79.300	2244.190	6	182.300	5250.240
	7	15.900	1149.570	7	103.700	2519.910	7	53.100	1855.190
	8	38.000	1862.000	8	256.400	5102.360	8	54.400	2001.920
	9	46.800	1778.400	9	119.500	3023.350	9	275.000	6600.000
25°C	1	64.900	3050.300	1	24.400	1144.360	1	137.800	4354.480
	2	54.500	3491.490	2	113.800	3994.380	2	46.700	1905.360
	3	55.700	2652.780	3	48.200	2175.820	3	118.400	4264.400
	4	60.500	3935.830	4	35.700	1786.100	4	114.500	3858.650
	5	41.700	2118.360	5	16.300	1059.500	5	121.400	4006.200
	6	45.000	2631.600	6	24.800	1361.520	6	58.700	2406.700
	7	85.200	3101.280	7	82.500	3052.500	7	202.200	6632.160
	8	54.700	2691.240	8	211.400	5390.700	8	237.500	6105.750
	9	105.200	5395.280	9	178.700	4574.720	9	79.000	3088.900

each temperature. Table 3.3 below gives the individual b values calculated for each species temperature combination, i.e. for each cell.

These b values were then compared to see if there were any significant differences either between treatments for a given species, or between species. Table 3.4 gives the results of this analysis of variance. As a result of this analysis it is clear that the relationship between ln oxygen consumption and ln weight can be accepted as being the same for each species at all temperatures.

Accepting a common b, the analysis of covariance was carried out to determine whether (1) the species to which an individual abalone belongs will effect its O_2 consumption, relative to similar sized individuals from the other species, and (2) whether temperature has a significant affect on the oxygen consumption of abalones of each species. The model investigated by the analysis of covariance is expressed by the general equation

$$E\ (\ln O_{ijk}) = u + s_i + t_j + (st)_{ij} + b\ (\ln W_{ijk} - \overline{\ln W_{ij}})$$

where

b = the common slope of the oxygen consumption-weight regression lines

u = that part of the oxygen consumption which is common to each cell, usually referred to as the general mean.

s_i = the factor representing that part of the oxygen consumption determined by the fact that an animal belongs to the "i^{th}" series.

t_j = the factor representing the influence of the "j^{th}" temperature on the oxygen consumption of the animal.

$(st)_{ij}$ = the "interaction factor" which accounts for any specific influence that the particular species-temperature combination may have on oxygen consumption.

W_{ijk} = the weight of the "k^{th}" animal in the ij cell and

O_{ijk} = the observed oxygen consumption of the animal W_{ijk}.

Table 3.3

Individual b values calculated for each species-temperature combination

Cell	Species	Temperature	Individual b's	Variance of b
1	H. roei	15	.7266	.0075
2	H. roei	20	.5827	.0064
3	H. roei	25	.3729	.0186
4	H. laevigata	15	.7584	.0024
5	H. laevigata	20	.7206	.0062
6	H. laevigata	25	.6546	.0019
7	H. ruber	15	.6946	.0051
8	H. ruber	20	.7505	.0031
9	H. ruber	25	.7475	.0057

Table 3.4

Coefficients of weight covariate (data converted to logarithms)

	Sums of Squares	Degrees of Freedom	Mean Squares	Variance Ratio
Common b	.1530	8	.0191	1.47
residual	.8178	63	.0130	
total	.9708	71		

F 1.47 8, 63 df N.S. at 5% level

It should be noted that if only a single cell is considered at any given time other factors (si, tj etc) and the term b ln Wij can be included with u, thus reducing the equation to

$$E \ln O = u + b \ln W$$

which is the general expression of oxygen consumption-weight relationships. The results of the analysis of covariance are given on Table 4.5 below.

Table 3.5

Analysis of covariance; model described by

$$E(\ln O_{ijk}) = u + s_i + t_j + (st)_{ij} + b(\ln W_{ijk} - \ln \overline{W}_{ij})$$

Source	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio
Grand mean	4863.28	1		
Species	3.42	2	1.71	125.16 **
Temperature	5.12	2	2.56	187.58 **
Interaction	2.27	4	.57	41.72 **
Slope	14.38	1	14.38	1052.56 **
Residual	.97	71	.0137	
Total	4889.44	81		

** All highly significant

It is evident from this analysis that all the investigated factors have a significant influence on the oxygen consumption of the abalone.

This means

1. Temperature has a significant affect on the oxygen consumption of the abalone, regardless of species.
2. Over the whole range of temperatures there is a significant difference in the oxygen consumptions of the three species.
3. There is a significant species-temperature interaction factor which has an influence on the oxygen consumption of the abalone and

4. Weight has a significant affect on the oxygen consumption of all species at all temperatures (b significantly different from 0).

From the analysis of covariance carried out given the observed ln oxygen consumption and ln weight data for each cell, values for u, s_i, t_j, (st) and b were calculated. These values are given for each cell on Tables 3.6.1 - 3.6.3.

These values in turn were used to estimate the theoretical oxygen consumption of the animals used in the experiments ($\hat{C}_{ijk}$) and the 95% confidence limits about these theoretical O_2 consumptions. These latter values are also given in Tables 3.6.1 - 3.6.3.

Figures 3.10 to 3.12 are the plots of calculated ln O_2 against ln W for each species at each experimental temperature (closed symbols). Clearly all these regression lines must demonstrate the common slope, b = .697. The 95% confidence limits about these regression lines are plotted on the figures. The observed ln O_2 consumption, ln weight data are also plotted on the figures (open symbols).

As a general statement it should be noted that substitution of any range of weights of theoretical animals into the equation

$$E\ (\ln O_{ijk}) = u + s_i + t_j + (st)_{ij} + b\ (\ln W_{ijk} - \ln \overline{W}_{ij})$$

for a given cell, where the values of b, u, s_i, t_j and $(st)_{ij}$ are defined, will enable calculation of points determining the regression line relating ln O_2 consumption and ln weight. The intercept u on the Y axis of the general equation relating these factors $E\ (\ln O_2) = u + b \ln W$, will be defined for a given cell where ln W = 0, i.e. W = 1. The ln O_2 consumption, ln weight relationship in each individual cell can be expressed by the equations given below in Table 3.7.

Table 3.6.1 H. roei

Logarithmic expressions of measured and calculated values for the parameters in the model describing oxygen consumption in the abalone

T°C	Animal No.	u general mean	si species factor	tj temperature factor	$(st)_{ij}$ species-temperature interaction factor	b common slope oxygen consumption-weight regressions	$\bar{w}$ mean weights of animals in cell	W_{ijk} weights of animals	O_{ijk} absorbed oxygen consumption	$\hat{O}_{ijk}$ theoretical oxygen consumption	95% confidence limits about $\hat{O}_{ijk}$ lower	upper
	1	7.749	-.269	-.345	-.116	.697	3.615	4.015	7.310	7.297	7.217	7.377
	2	7.749	-.269	-.345	-.116	.697	3.615	3.902	7.241	7.219	7.140	7.298
	3	7.749	-.269	-.345	-.116	.697	3.615	4.361	7.599	7.538	7.454	7.623
	4	7.749	-.269	-.345	-.116	.697	3.615	4.011	7.242	7.295	7.215	7.374
15	5	7.749	-.269	-.345	-.116	.697	3.615	3.231	6.639	6.751	6.671	6.830
	6	7.749	-.269	-.345	-.116	.697	3.615	3.367	6.775	6.846	6.767	6.924
	7	7.749	-.269	-.345	-.116	.697	3.615	3.182	6.766	6.717	6.636	6.797
	8	7.749	-.269	-.345	-.116	.697	3.615	3.444	6.967	6.899	6.821	6.977
	9	7.749	-.269	-.345	-.116	.697	3.615	3.020	6.626	6.604	6.522	6.686
	1	7.749	-.269	.099	-.153	.697	3.643	4.057	7.676	7.714	7.634	7.794
	2	7.749	-.269	.099	-.153	.697	3.643	3.307	7.151	7.191	7.112	7.270
	3	7.749	-.269	.099	-.153	.697	3.643	4.103	7.575	7.746	7.665	7.826
	4	7.749	-.269	.099	-.153	.697	3.643	3.001	6.921	6.977	6.895	7.060
20	5	7.749	-.269	.099	-.153	.697	3.643	4.024	7.661	7.691	7.611	7.770
	6	7.749	-.269	.099	-.153	.697	3.643	4.047	7.803	7.707	7.627	7.786
	7	7.749	-.269	.099	-.153	.697	3.643	2.766	7.047	6.814	6.727	6.900
	8	7.749	-.269	.099	-.153	.697	3.643	3.638	7.529	7.421	7.343	7.499
	9	7.749	-.269	.099	-.153	.697	3.643	3.646	7.463	7.567	7.488	7.645
	1	7.749	-.269	.247	.269	.697	4.092	4.175	8.023	8.052	7.974	8.130
	2	7.749	-.269	.247	.269	.697	4.092	3.995	8.158	7.927	7.849	8.005
	3	7.749	-.269	.247	.269	.697	4.092	3.983	7.883	7.920	7.842	7.998
	4	7.749	-.269	.247	.269	.697	4.092	4.099	8.291	8.000	7.922	8.078
25	5	7.749	-.269	.247	.269	.697	4.092	3.751	7.658	7.745	7.664	7.823
	6	7.749	-.269	.247	.269	.697	4.092	3.761	7.875	7.765	7.685	7.844
	7	7.749	-.269	.247	.269	.697	4.092	4.445	8.040	8.242	8.162	8.321
	8	7.749	-.269	.247	.269	.697	4.092	4.002	7.998	7.932	7.854	8.011
	9	7.749	-.269	.247	.269	.697	4.092	4.637	8.130	8.375	8.294	8.457

Table 5.6.2 H. laevigata

Logarithmic expressions of measured and calculated values for the parameters in the model describing oxygen consumption in the abalone

T°C	Animal No.	u general mean	s_i species factor	t_j temperature factor	$(st)_{ij}$ species temperature interaction factor	b common slope of O_2 consumption weight regressions	$\bar{w}$ mean weight of animals in cell	W_{ijk} weights	O_{ijk} observed O_2 consumption	$\hat{O}_{ijk}$ theoretical oxygen consumption	95% confidence limits about $\hat{O}_{ijk}$ lower	upper
	1	7.749	.039	-.345	.204	.697	4.537	2.845	6.235	6.467	6.360	6.574
	2	7.749	.039	-.345	.204	.697	4.537	4.071	7.448	7.322	7.241	7.402
	3	7.749	.039	-.345	.204	.697	4.537	4.781	8.000	7.817	7.738	7.896
	4	7.749	.039	-.345	.204	.697	4.537	5.687	8.375	8.449	8.357	8.541
15	5	7.749	.039	-.345	.204	.697	4.537	4.240	7.521	7.440	7.361	7.519
	6	7.749	.039	-.345	.204	.697	4.537	4.844	8.091	7.861	7.782	7.940
	7	7.749	.039	-.345	.204	.697	4.537	4.779	7.650	7.816	7.737	7.895
	8	7.749	.039	-.345	.204	.697	4.537	4.275	7.343	7.465	7.386	7.543
	9	7.749	.039	-.345	.204	.697	4.537	5.308	8.182	8.185	8.100	8.270
	1	7.749	.039	.099	.082	.697	4.692	4.740	8.212	8.001	7.923	8.079
	2	7.749	.039	.099	.082	.697	4.692	3.829	7.349	7.366	7.280	7.452
	3	7.749	.039	.099	.082	.697	4.692	5.266	8.354	8.369	8.287	8.450
	4	7.749	.039	.099	.082	.697	4.692	4.288	7.576	7.686	7.606	7.766
20	5	7.749	.039	.099	.082	.697	4.692	4.762	8.143	8.017	7.939	8.095
	6	7.749	.039	.099	.082	.697	4.692	4.375	7.716	7.746	7.667	7.825
	7	7.749	.039	.099	.082	.697	4.692	4.642	7.852	7.933	7.855	8.011
	8	7.749	.039	.099	.082	.697	4.692	5.547	8.537	8.564	8.476	8.650
	9	7.749	.039	.099	.082	.697	4.692	4.785	8.014	8.052	7.954	8.110
	1	7.749	.039	.247	-.286	.697	4.051	3.195	7.045	7.165	7.079	7.251
	2	7.749	.039	.247	-.286	.697	4.051	4.734	8.295	8.259	8.155	8.323
	3	7.749	.039	.247	-.286	.697	4.051	3.875	7.684	7.340	7.562	7.718
	4	7.749	.039	.247	-.286	.697	4.051	3.517	7.488	7.390	7.309	7.471
25	5	7.749	.039	.247	-.286	.697	4.051	2.791	6.966	6.884	6.799	6.978
	6	7.749	.039	.247	-.286	.697	4.051	3.211	7.216	7.176	7.091	7.262
	7	7.749	.039	.247	-.286	.697	4.051	4.415	8.034	8.015	7.935	8.094
	8	7.749	.039	.247	-.286	.697	4.051	5.554	8.592	8.671	8.574	8.767
	9	7.749	.039	.247	-.286	.697	4.051	5.186	8.438	8.554	8.461	8.646

Table 3.6.3 H. ruber

Logarithmic expressions of measured and calculated values for the parameters in the model describing oxygen consumption in the abalone

T°C	Animal No.	u general mean	s_i species factor	t_j temperature factor	$(st)_{ij}$ species temperature interaction factor	b common slope of O_2 consumption weight regressions	$\bar{w}$ mean weight of animals in cell	W_{ijk} weights	O_{ijk} observed O_2 consumption	$\hat{O}_{ijk}$ theoretical oxygen consumption	95% confidence limits about $\hat{O}_{ijk}$ lower	upper
	1	7.749	.230	-.345	-.088	.697	4.362	5.103	8.026	8.061	7.977	8.145
	2	7.749	.230	-.345	-.088	.697	4.362	5.529	8.425	8.359	8.266	8.451
	3	7.749	.230	-.345	-.088	.697	4.362	4.381	7.449	7.558	7.480	7.635
	4	7.749	.230	-.345	-.088	.697	4.362	4.799	7.717	7.849	7.769	7.929
15	5	7.749	.230	-.345	-.088	.697	4.362	4.647	7.854	7.743	7.664	7.822
	6	7.749	.230	-.345	-.088	.697	4.362	4.148	7.501	7.395	7.317	7.474
	7	7.749	.230	-.345	-.088	.697	4.362	3.635	7.002	7.037	6.953	7.121
	8	7.749	.230	-.345	-.088	.697	4.362	3.570	7.087	6.992	6.907	7.077
	9	7.749	.230	-.345	-.088	.697	4.362	3.450	6.861	6.908	6.821	6.996
	1	7.749	.230	.099	.071	.697	4.780	5.380	8.623	8.567	8.485	8.649
	2	7.749	.230	.099	.071	.697	4.780	4.719	8.263	8.106	8.028	8.184
	3	7.749	.230	.099	.071	.697	4.780	5.712	8.715	8.798	8.711	8.886
	4	7.749	.230	.099	.071	.697	4.780	4.536	7.828	7.978	7.899	8.057
20	5	7.749	.230	.099	.071	.697	4.780	3.882	7.420	7.522	7.435	7.609
	6	7.749	.230	.099	.071	.697	4.780	5.206	8.566	8.445	8.365	8.525
	7	7.749	.230	.099	.071	.697	4.780	5.972	7.525	7.585	7.500	7.670
	8	7.749	.230	.099	.071	.697	4.780	3.996	7.602	7.602	7.517	7.687
	9	7.749	.230	.099	.071	.697	4.780	5.617	8.795	8.732	8.646	8.818
	1	7.749	.230	.247	.017	.697	4.701	4.926	8.579	8.399	8.321	8.478
	2	7.749	.230	.247	.017	.697	4.701	3.944	7.552	7.645	7.559	7.731
	3	7.749	.230	.247	.017	.697	4.701	4.774	8.558	8.294	8.216	8.372
	4	7.749	.230	.247	.017	.697	4.701	4.741	8.258	8.270	8.192	8.348
25	5	7.749	.230	.247	.017	.697	4.701	4.799	8.296	8.311	8.233	8.389
	6	7.749	.230	.247	.017	.697	4.701	4.072	7.786	7.804	7.722	7.887
	7	7.749	.230	.247	.017	.697	4.701	5.309	8.800	8.667	8.585	8.749
	8	7.749	.230	.247	.017	.697	4.701	5.470	8.717	8.779	8.694	8.864
	9	7.749	.230	.247	.017	.697	4.701	4.369	8.036	8.011	7.932	8.091

Table 3.7

ln oxygen consumption, ln weight relationship in each individual cell

Cell (see table 3.3)	$\ln O_2 = u + b \ln W$
1	$\ln O_2 = 4.498 + .697 \ln W$
2	$\ln O_2 = 4.385 + .697 \ln W$
3	$\ln O_2 = 5.142 + .697 \ln W$
4	$\ln O_2 = 4.485 + .697 \ln W$
5	$\ln O_2 = 4.696 + .697 \ln W$
6	$\ln O_2 = 4.957 + .697 \ln W$
7	$\ln O_2 = 4.503 + .697 \ln W$
8	$\ln O_2 = 4.815 + .697 \ln W$
9	$\ln O_2 = 4.964 + .697 \ln W$

The mean value of b obtained in this experiment for the three species of abalone (.697) is quite comparable to values of b obtained for other gastropod molluscs (cf. Prosser, 1973; Table 5.2). Prosser lists b values of .67 for prosobranch snails, and .73 for a species of freshwater limpet (from Berg et al., 1962). The mean b values determined for Patella undulata, which like abalone is a marine archaeogastropod is .696 (Davis, 1966).

As in this study Davis (1966) found that although the value of b varied to some extent, the variation was not significant, and he was able to use the mean value of b to describe the log oxygen consumption, log weight relationship over a wide range of temperatures.

Barnes and Barnes (1969) found, for the barnacle Balanus balanoides, that at all temperatures, and throughout the year (except in January) the oxygen consumption weight relationship can be described by a common regression coefficient b = .6119. It is interesting to note that having demonstrated this fact statistically Barnes and Barnes

Figures 3.10, 3.11 and 3.12

Regression lines relating calculated ln O_2 ($\hat{O}_{ijk}$) and ln W for each species at each experimental temperature (15°C, 20°C and 25°C) - closed symbols. The 95% confidence limits about these regression lines are also plotted on the figures.

The open symbols are plots of the observed ln oxygen consumption, ln weight data.

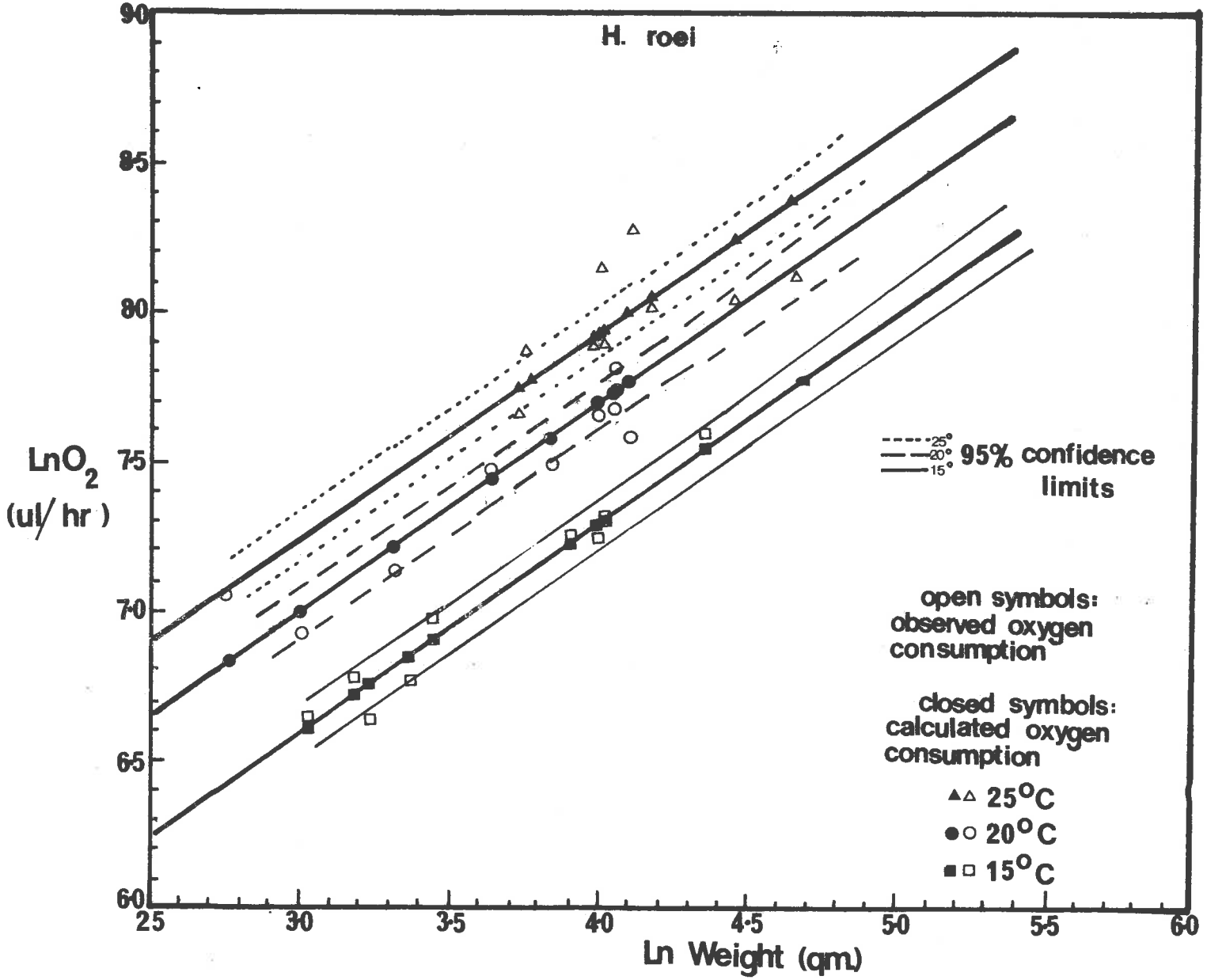

H. roei
LnO2 (ul/hr)
Ln Weight (gm)
25°
20°
15°
95% confidence limits
open symbols: observed oxygen consumption
closed symbols: calculated oxygen consumption
▲△ 25°C
●○ 20°C
■□ 15°C
90
85
80
75
70
65
60
25
30
35
40
45
50
55
60

Fig-3.10

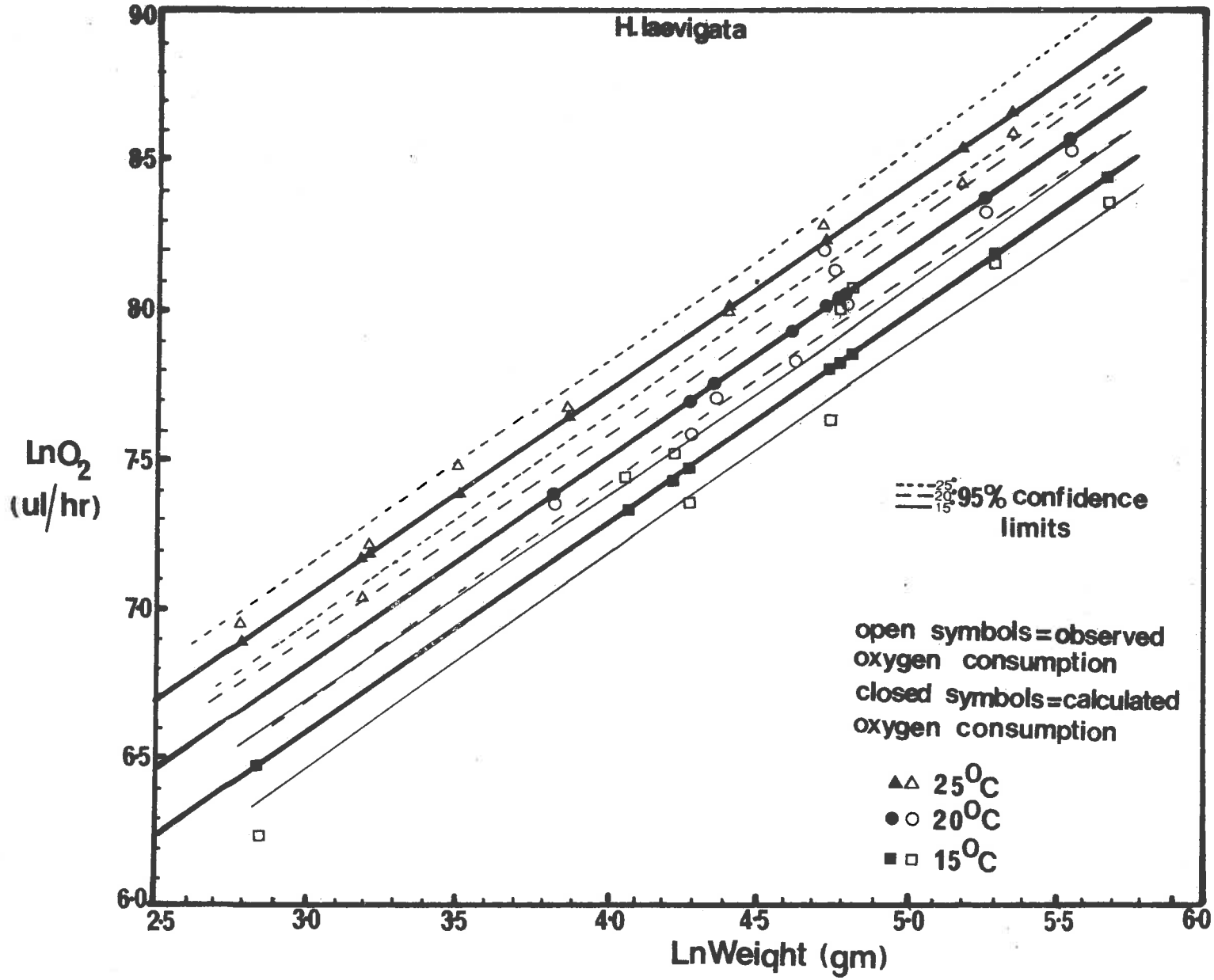

H. laevigata
LnO2 (ul/hr)
LnWeight (gm)
9·0
8·5
8·0
7·5
7·0
6·5
6·0
2·5
3·0
3·5
4·0
4·5
5·0
5·5
6·0
25°
20°
15°
95% confidence limits
open symbols = observed oxygen consumption
closed symbols = calculated oxygen consumption
▲△ 25°C
● ○ 20°C
■ □ 15°C

Fig-3.11

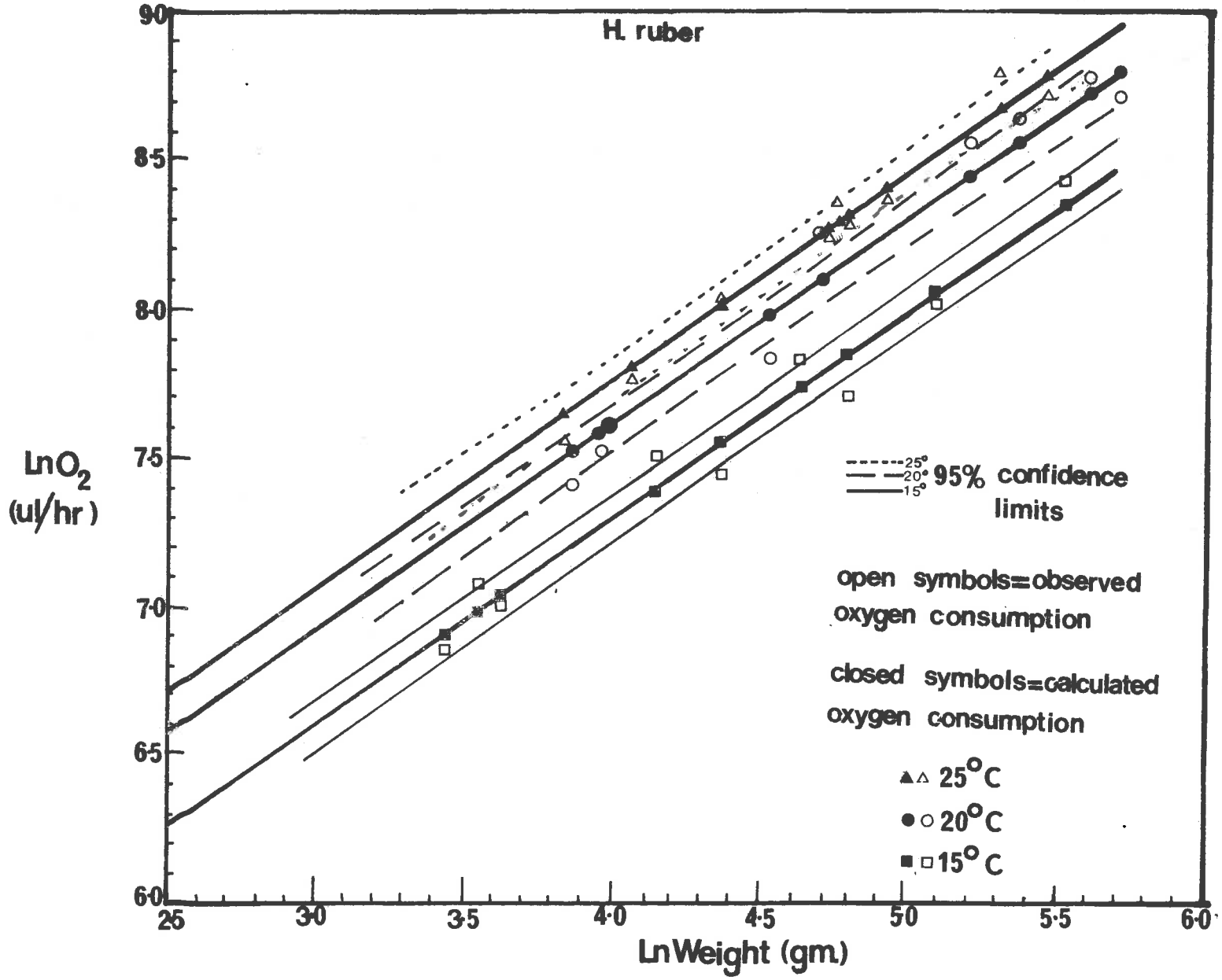

H. ruber
9·0
8·5
8·0
7·5
7·0
6·5
6·0
Ln O2 (ul/hr)
2·5
3·0
3·5
4·0
4·5
5·0
5·5
6·0
Ln Weight (gm.)
25°
20°
15°
95% confidence limits
open symbols=observed oxygen consumption
closed symbols=calculated oxygen consumption
▲△ 25°C
● ○ 20°C
■ □ 15°C

Fig. 12.

(1969) do not use the common regression lines when plotting their figures. In the discussion of this paper the authors consider the question as to whether or not the slope of the log oxygen consumption versus log weight regression line varies with temperature in different organisms. They conclude that no general rule holds true. In some organisms the relationship apparently remains the same over a wide range of temperatures (Ellenby, 1951; Edwards, 1946; Scholander et al., 1953; Davis, 1966; Barnes and Barnes, 1969; this study), while in other cases Q_{10} obviously varies with size, leading to different b values at different temperatures (Rao and Bullock, 1954; Read, 1962; Newell, 1969).

In this study however, because the regression line relating ln O_2 consumption to ln weight for all species at all temperatures can be considered to have a common slope, the Q_{10}'s will be the same for animals of any size. Further, the same sized animals of each of the three species are directly comparable. To simplify the comparisons of oxygen consumption of the three species and the responses of oxygen consumption to temperature change an arbitrary "standard" size of 90.02g ln 90.02 = 4.5) was selected (cf. Barnes and Barnes, 1969; Newell and Pye, 1971).

For each species the theoretical oxygen consumption of a specimen of this size at 15°C, 20°C and 25°C was read from figures 3.10-3.12. These oxygen consumptions, expressed as ul/gm/hr, are given in Table 3.8 and plotted on Figure 3.13. The Q_{10} values, derived from these O_2 consumption figures are also given in Table 3.8.

Consider first the oxygen consumptions of the abalone species at the acclimation temperature 20°C. From Figure 3.13 it can be seen that the oxygen consumptions of the three species appear quite different at this temperature, H. roei's oxygen consumption being markedly higher than that of H. ruber, which is in turn higher than that of H. laevigata. As the common regression line slope was calculated on the basis of the ln O_2 consumption-ln-weight data at all temperatures a statistical test

Table 3.8

Oxygen consumption of "standard" animals, wet weight 90.02 gm (ln 90.02 = 4.5)

Temperature	Oxygen consumption ul/gm/hr		
	H. roei	H. ruber	H. laevigata
15°C	23.1	23.1	22.7
20°C	34.5	31.8	28.2
25°C	43.4	36.6	35.2

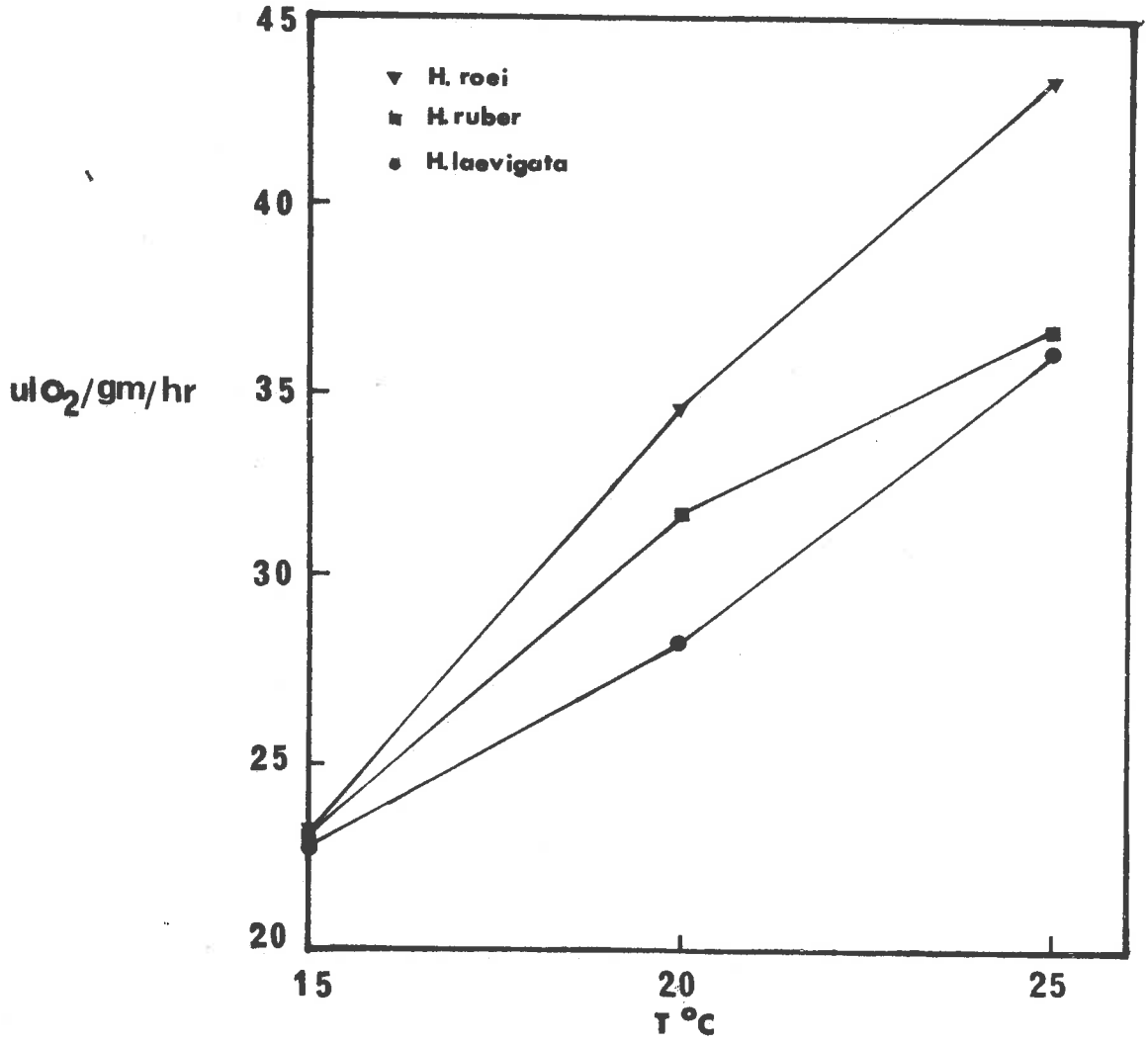
	Q_{10} values for oxygen consumption of abalone		
	H. roei	H. ruber	H. laevigata
15-20°C	2.23	1.89	1.54
20-25°C	1.58	1.55	1.56

comparing the O_2 consumptions represented on Figure 3.13 at 20°C alone is not valid. However, the analysis of covariance has shown that over the range of temperatures the oxygen consumptions of the species are significantly different, and it is probably also safe to say that at 20°C there is a significant difference between the oxygen consumptions of the same sized animals of each species.

It should be remembered in the comparisons of the species that the size range of H. roei does not extend as far as that of the other two species. Consequently the 90.02 gm "standard" animal of this species is likely to be more mature than similar sized animals of the other two species. This may to some extent influence its oxygen consumption. However, any error made by comparing standard sized animals will be a conservative one. Comparison of animals of mean weights of each species would only serve to emphasise the higher oxygen consumption.

Figure 3.13

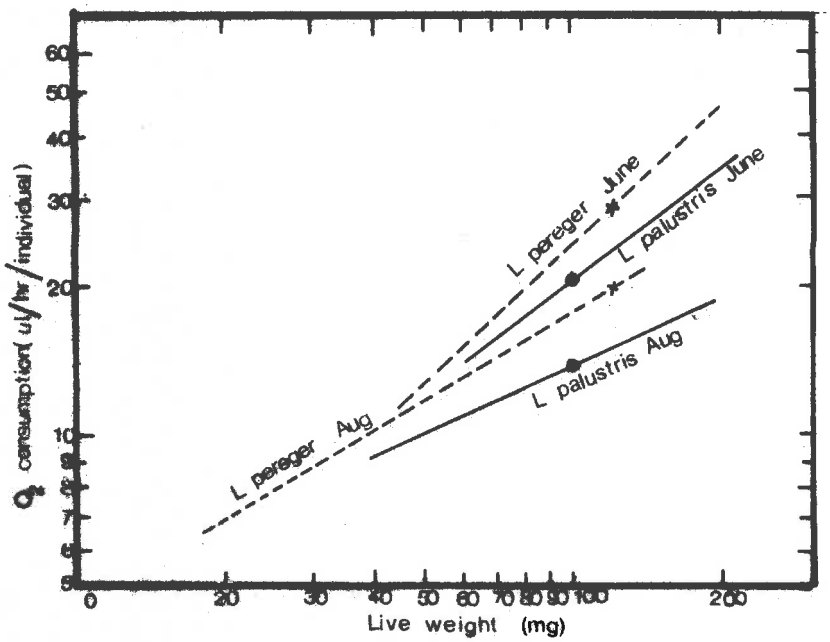
Oxygen consumptions of "standard" weight (90.02g) specimens of *Haliotis roei*, *H. ruber*, and *H. laevigata* at 15°C, 20°C (the acclimation temperature), and at 25°C


45
40
35
30
25
20
15
20
25
T °C
ul O2/gm/hr
H. roei
H. ruber
H. laevigata

Despite the fact that a general examination of the metabolic rates of a range of metazoan poikilotherms indicates that similar sized organisms examined under the same conditions show similar metabolic rates (Hemmingsen, 1960; Newell, 1970), the differences in oxygen consumption between the three abalone species is not unusual. For example, examination of the metabolic rates of several species of barnacle under the same conditions has shown that the metabolic rates of similar sized specimens differ considerably between the species (Barnes and Barnes, 1969). Moreover, such different oxygen consumptions of similar sized animals of closely related gastropod species have been described below. Figure 3.14 below demonstrates how similar sized specimens of two species of the freshwater genus Lymnaea show interspecific difference at the same temperatures, as well as intraspecific differences at different times of the year (Berg and Ockelman, 1959).

Figure 3.14

Oxygen consumption plotted against live weight for two species of snail Lymnaea pereger, and L. palustris, at different times of the year (after Berg and Ockelman, 1959; Figure 3)



As all the abalone species in this study are acclimated to the same temperature, the differences in their oxygen consumptions at this temperature are not related to their previous thermal experience. Some unaccounted modifying factor, such as stage of the breeding cycle, may be causing the differences between the species, although as far as possible this sort of variation was eliminated in the experimental design. Therefore, I hypothesise that the differences in the metabolic rates of the three species are associated with the differences in their life styles, H. roei, as has been reported earlier being an active grazing animal, while H. ruber and particularly H. laevigata rely almost entirely on drift algae for food, and rarely move.

Prosser (1973) (Table 5-1) lists the standard metabolisms of a number of organisms at specified temperatures. The data on Table 3.9 below is that for the molluscs in Prosser's Table. The comments about the activity associated with the life styles are mine.

Table 3.9

Standard metabolism of molluscs (after Prosser, 1973, Table 5.1)

Animal	Standard Metabolism $ml O_2/gm/hr$	Temperature	General Comments on activity associated with life style
Ancylus	.117	16	freshwater gastropod, moderately active
Lymnaea	.24		"
Mytilus	.076	15	bivalve - sessile
Octopus	.28	25	cephalopod, highly active
Pecten	.67		bivalve - highly active
Anodonta	.002	10	bivalve - sessile

Overall, from this table it can be seen that the oxygen consumptions of the more active species are higher than that of the more sluggish or sessile animals. This interpretation of the data on this table must be made with some caution in view of the differences in sizes of the

organisms, and the different temperatures at which the standard metabolisms were recorded. However, for example, it is evident that the bivalve Pecten which is regarded as being an active, mobile animal (Ricketts and Calvin, 1960, page 219) has a much higher oxygen consumption than the other bivalves Mytilus, a sessile mussel, or Anodonta which is often anchored to the substrate. The explanation for this may be that the same enzymes are involved in both standard (resting), and active metabolism, and thus there will be limited flexibility in the metabolism of a given organism. The lower end of this flexibility range (standard metabolism) will be logically higher in highly active animals such as Octopus and Pecten, than in sessile animals such as Mytilus, and Anodonta. In a similar way I propose that the higher standard metabolism of H. roei is an indication of the potentially higher active metabolism in this most active species. A similar argument can be used to explain why H. ruber in turn has a higher standard metabolism than the almost completely sessile H. laevigata.

In one sense, H. roei is, by virtue of its higher resting oxygen consumption, a less efficient animal than the other two species, using up more energy just to maintain its life processes. However, lack of efficiency in this respect is offset by the fact that the animal actively moves around to graze, thus ensuring a steady food supply. The other two species, while using less energy in their mode of existence rely almost entirely on drift algae for food - as Barnes and Barnes (1969) point out, a reduction in metabolic rate could be one way in which species might overcome a restriction in feeding time.

Considering the way in which the metabolic rates of the abalone species change with temperature, it can be seen that the Q_{10} values for the abalone in general (Table 3.8) are quite comparable to those of numerous other marine invertebrates over the same temperature ranges (Read, 1962; Davis, 1966; Newell and Roy, 1973).

It is evident that the standard oxygen consumptions of all three species respond to temperature change in a way typical of subtidal molluscs (Courtney and Newell, 1965), showing no evidence of the temperature independence of standard metabolic rate associated with intertidal molluscs (Newell and Northcroft [illegible] 1967; Newell, 1966, 1969, 1970; Newell and Pye, 1970 a, 1971).

However, as the analysis of covariance indicates, and as is evident from Table 3.8 and Figure 3.13, the metabolic rates of the three species do not respond to temperature changes in the same way. When the three species are subjected to a 5°C temperature drop from 20° to 15°C their metabolic rates drop considerably. However, this drop is most marked in H. roei, and is greater in H. ruber than in H. laevigata. The result of these differential rates of decrease in metabolic rate is that at 15°C the oxygen consumptions of the three species are very similar. In fact, it is probably safe to say that at 15°C there is no significant differences between the oxygen consumptions of the three species. In H. ruber, with an abrupt temperature increase from 20°-25°C there is quite a large drop in the Q_{10} compared to the 15°-20°C value. H. roei exhibits a small decrease in the Q_{10} value from 20°-25°C compared to the 15°-20°C value, but the rate of increase of its oxygen consumption is still greater than that of either of the other two species over the higher temperature range. H. laevigata exhibits an increased Q_{10} from 20°-25°C compared to that for 15°-20°C. This difference in response to temperature change of the metabolisms of the three abalone species would seem not to be too surprising. Unrelated species of the same size may differ quite markedly in the responses of their resting metabolisms to temperature (Newell and Northcroft, 1967), and even specimens for single species acclimated to slightly different conditions can exhibit considerable variability in the responses of their metabolisms to temperature change (Rao and Bullock, 1954).

3.2.6 Relationship between oxygen consumption, and haemocyanin function at acclimation temperature 20°C, and also after an abrupt temperature transfer from 20°-25°C, in the three abalone species H. roei, H. ruber and H. laevigata

From the model developed in this chapter, relating oxygen consumption to size and temperature, it is possible to predict the oxygen consumption of an abalone of known species and size, at a given temperature. In this section, for the purposes of argument, a standard animal (90.02 gm $\ln W = 4.5$) will be considered for each species.

H. roei

In section 3.3.41 it was shown that the oxygen delivered by the blood of H. roei in a single circulation was .92 vol % of oxygen at 20°C. Most of this oxygen was carried by the haemocyanin.

The oxygen consumption of the standard H. roei at 20°C can be derived from Figure 3.13, and is found to be 3.1 ml/hr. The oxygen consumption is related to the A-V difference by the Fick principle equation

$$\frac{O_2 \text{ consumption ml/hr}}{60} = \frac{\text{A-V difference vol \%}}{100} \times \text{C.O. ml/min}$$

Therefore the cardiac output of the abalone heart necessary to maintain the observed oxygen consumption given the measured A-V difference can be calculated for the standard H. roei. The equation becomes

$$\text{C.O. ml/min} = \frac{3.1 \times 100}{60 \times .92}$$

$$= 5.62 \text{ ml/min}$$

This, expressed in terms of a theoretical 1 kgm animal (cf. Johansen et al., 1970) becomes 62.2 ml/min/kgm.

Similar calculations can be made for H. laevigata and H. ruber

H. laevigata 20°C

$$\text{C.O. ml/min} = \frac{\text{oxygen consumption ml/hr x 100}}{\text{60 x A-V difference vol \%}}$$

$$= \frac{2.54 \times 100}{60 \times .61}$$

= 6.94 ml/min for a standard animal

This can also be expressed as 76 ml/kgm/minute

H. ruber 20°C

$$\text{C.O. ml/min} = \frac{\text{oxygen consumption ml/hr x 100}}{\text{60 x A-V difference vol \%}}$$

$$= \frac{2.86 \times 100}{60 \times .56}$$

= 8.52 ml/min for a standard animal

This, similarly can be expressed as 94 ml/kgm/minute.

It is immediately evident that the calculated cardiac outputs are not in proportion to the measured oxygen consumptions of the animals. Although the oxygen consumption of the active species H. roei, is markedly larger than that of the other two species at 20°C, the A-V difference is also considerably larger, and this results in the observed smaller cardiac output in this species than in H. ruber or H. laevigata. In chapter 5, it was demonstrated that the larger A-V difference of H. roei was due almost entirely to the greater concentration of haemocyanin in the haemolymph of this species.

It would seem a logical argument then, that the most active of the three abalone species H. roei, maintains a high standard metabolism than the other two species, with less energy expendature by the heart (judged by magnitude of cardiac output) largely through the fact that it has a consistently higher concentration of haemocyanin in the haemolymph than the other two species. This argument will be discussed further in

Part II of this chapter when the parameters which determine cardiac output have been described in more detail for the three abalone species.

3.2.61 Comparison of cardiac output values obtained for abalone with those for other invertebrates

The cardiac output values obtained for abalone in this study are all similar to that calculated by Redmond (1955) for Panulirus (80 ml/kgm/min). Although Johansen et al. (1970) criticised this value as being unrealistically high, after obtaining a value of 30 ml/kgm/min for the crab Cancer magister (see section 3.1.1), more recently a cardiac output of 207-238 ml/kgm/min was calculated for another crab species Callinectes sapidus (Mangum and Weiland, 1975). Mangum and Weiland in turn suggest that Johansen et al.'s (1970) cardiac output value for Cancer magister may be unrepresentatively low.

Quote:

"The mean oxygen carrying capacity reported by Johansen et al. (1970) predicts 11.4 gm haemocyanin/100 ml blood, which is 2-5 times the amount in a variety of other decapods (Florkin '60). The increase in this parameter means that the denominator in the Fick equation is so large that the solution for cardiac output gives only 29.5 ml/kgm/minute."

Johansen (1966) also found a low average cardiac output for the cephalopod, Octopus dofleini (14.5 ml/kgm/minute). However, two facts emerged from Johansen's study: (1) the heartrate of octopus seemed to be constant regardless of the size of the animal, or its state of activity, and (2) the calculated stroke volume varied enormously 2.2-13.7 ml/beat). It is easy to see, considering that the cardiac output is determined by these two factors, heartrate and stroke volume, that in this species alone cardiac output must have up to 9 fold variation. In some specimens cardiac output must be of the same order as those found in this study, and by Redmond, (1955).

The cardiac outputs determined in this study for the abalone are much smaller than those which can be calculated for the sluggish arthropod Limulus polyphemus on the basis of Falkowski's (1973) data.

For example, consider the 24°C acclimated animals described in Falkowski's recent paper. Falkowski Table 3 records the A-V difference in % saturation of the haemocyanin of these animals to be 20%. He also states (page 4) that the "vol % capacity of Limulus haemocyanin for oxygen is about 2.0 ml O_2 per 100 ml haemolymph". On these figures it would seem that .56 vol % O_2 should be unloaded to the tissues by the haemocyanin. (Falkowski in fact records a value of .28 vol % O_2 unloaded to the tissues at 24°C). Falkowski records the average oxygen consumption of Limulus at 24°C to be .45 ± .06 mls O_2/gm/hr. Unfortunately he does not record the weights of the animals used in his oxygen consumption experiments. However in the introduction to the paper he indicates that animals of 45-90 gm wet weight were maintained in the aquaria.

Assuming therefore, an average weight of 67.5 gms, an average animal would consume 31.05 mls of oxygen per hour. Therefore using Falkowski's A-V data for Limulus at 24°C the minute volume calculated would be

$$\frac{31.05 \times 100}{.56 \times 60}$$

$$= 92.4 \text{ ml/min}$$

or 1367.7 ml/kgm/min, which definitely would seem too large for such a sluggish animal. It would appear that in Limulus, oxygen must be gained through respiratory surfaces other than the gills.

In the abalone in this study too, it is most likely that there will be some oxygen gained through surfaces other than the gills. The mantle area of the abalone is highly vascularised, and it has been suggested as a possible region of gas exchange (Crofts, 1929). This may cause the calculated cardiac output of the abalone to be slightly

exaggerated, as the Fick principle assumes that all oxygen is gained through the gills.

It is interesting to note that in the sessile urochordate Ciona, the cardiac output has recently been estimated at 66 ml/kgm/minute (Kriebel, 1968). Although Ciona is not a haemocyanin bearing organism this cardiac output compares closely with that found for the abalone in this study, adding support to the fact that this is a realistic value, by no means beyond the mechanical capacity of an invertebrate heart.

3.2.62 Validity of comparisons of values for cardiac output from various studies

As with oxygen consumption, the magnitude of the cardiac output of a given organism will depend on the conditions under which it is measured. Caution must therefore be exercised when comparing cardiac outputs measured under different conditions.

For example, ideally cardiac outputs should be compared at the same temperature. Johansen et al.'s (1970) estimation of the cardiac output of Cancer magister was made on animals respiring at 10°C. The determinations made on the animals discussed in this chapter were made at 20°C. Unfortunately O_2 consumption data and A-V difference data are not available for abalone at 10°C. However extrapolating from the O_2 consumption data for H. roei on the basis of the 15°-20°C Q_{10} value, the oxygen consumption of a standard H. roei at 10°C would be expected to be approximately 1.5 ml/hr.

Assuming a similar A-V difference for the 10°C animal as the 20°C animal this would lead to a cardiac output of only about 30 ml/kgm/min which is the same as that found by Johansen et al. (1970) for Cancer magister. This example has an admitted fault in that it considers the O_2 consumption of the abalone at 10°C on the basis of the Q_{10}'s obtained in this study, that is, on the basis of the predicted oxygen consumption after an abrupt temperature change. It is possible that abalone

acclimated to the low temperature of 10°C might not demonstrate such a low oxygen consumption. Nevertheless, the example illustrates the difficulty of drawing direct comparisons of cardiac output values obtained under different conditions.

In any event, it would appear reasonable to conclude from the above examples, that the calculated cardiac outputs for the abalone hearts at 20°C, are quite feasable values for invertebrate hearts.

3.2.63 Cardiac outputs after an abrupt temperature increase from 20°C to 25°C

In chapter 2, values were given for A-V difference in vol % of oxygen for each of the abalone species, after an abrupt temperature increase from 20°C to 25°C.

In Table 3.8 the oxygen consumptions at 25°C of standard sized, 20°C acclimated, animals of each species are given.

As with the animals at 20°C, the cardiac outputs of these animals can be simply computed by the formula

$$\text{C.O. ml/min} = \frac{\text{oxygen consumption ml/hr} \times 100}{60 \times \text{A-V difference vol \%}}$$

i.e. (1) *H. roei*

$$\text{C.O. ml/min} = \frac{3.91 \times 100}{60 \times .599}$$

$$= 10.87 \text{ ml/min for a standard animal}$$

$$= 120.8 \text{ ml/min/kgm}$$

(2) *H. ruber*

$$\text{C.O. ml/min} = \frac{3.29 \times 100}{60 \times .47}$$

$$= 11.7 \text{ ml/min for a standard animal}$$

$$= 129.8 \text{ ml/min/kgm}$$

(3) H. laevigata

$$C.O.\ ml/min = \frac{3.17 \times 100}{60 \times .52}$$

$$= 10.2\ ml/min \text{ for a standard animal}$$

$$= 112.8\ ml/min/kgm$$

Table 3.10 summarises oxygen consumption, A-V difference, and cardiac output data for the three abalone species at 20°C, and after an abrupt temperature increase to 25°C.

It can be seen that the cardiac outputs of all three species have increased quite markedly compared to the 20°C values.

Table 3.10

Summary of oxygen consumption, A-V difference in vol % of oxygen, and cardiac output data for three species of abalone at 20°C, and after an abrupt temperature increase from 20°C-25°C.

	Temperature		% difference between 20°C and 25°C value + = increase ± = decrease
H. roei	20°C	25°C	
Oxygen consumption ml/hr	3.1	3.91	+26%
A-V difference in vol % of oxygen	.92	.599	-35%
Cardiac output ml/min	5.6	10.87	+94%
H. laevigata	20°C	25°C	
Oxygen consumption ml/hr	2.54	3.17	+25%
A-V difference in vol % of oxygen	.61	.52	-15%
Cardiac output ml/min	6.94	10.16	+46%
H. ruber	20°C	25°C	
Oxygen consumption ml/hr	2.86	3.29	+15%
A-V difference in vol % of oxygen	.562	.47	-16%
Cardiac output ml/hr	8.52	11.67	+37%

The greatest increase in cardiac output has occurred in H. roei, the value after an abrupt temperature increase being almost twice that at 20°C (194%). This increase in cardiac output is brought about by two factors (1) a large (26%) increase in oxygen consumption, coupled with (2) a considerable drop (35%) in the A-V difference.

H. laevigata also shows a similar increase (25%) in oxygen consumption from 20°-25°C. However the relative decrease in A-V difference is not as large as in H. roei. Therefore, although the cardiac output increases considerably, it is not as marked an increase as observed in H. roei.

H. ruber shows the smallest increase in oxygen consumption from 20°-25°C (15%). This, coupled with a drop in haemocyanin efficiency comparable to that of H. laevigata means that the relative increase of cardiac output from 20°-25°C is smallest in this species. It should be noted that despite the fact the H. roei's cardiac output undergoes the greatest relative change from 20°-25°C the final value of this parameter is much the same for this species as it is for the other two species. Moreover this similar cardiac output is maintaining a higher oxygen consumption in H. roei. Therefore when looked at in terms of a given cardiac output for a given oxygen consumption, H. roei is still the most efficient species at 25°C. This line of reasoning is discussed further in part II of this chapter in the light of information about the role of the heart in circulation in the abalone.

Although the cardiac output values for all species at 25°C are well within the range of cardiac output values recorded for other invertebrates, it is possible that the increase from the 20°C value is large enough to stress the circulatory mechanisms of the animals. The appearance of the animals themselves when subjected to a sudden temperature increase certainly suggests that they are physically stressed.

In part II of this chapter, the heartrates of the abalone are measured, and the stroke volumes calculated. The changes which must occur in these parameters in order to cope with the increased cardiac output after an abrupt temperature change are described.

Chapter 3

Part II

3.3 Heart Function

3.3.1 Introduction

As with oxygen consumption (Chapter 3 Part I) there are numerous factors which may modify the heart rate of various invertebrates. Such modifying factors include: oxygen tension of the environment (Larimer, 1962), salinity of the environment (Spaargaren, 1973), activity of the organism (Johansen and Martin, 1962), exposure or submergence in intertidal organisms (Pickens, 1965; Trueman and Lowe, 1971), nutritional state of the organism (Pickens, 1965; Widdows, 1973), thermal history of the organism (Segal, 1956), size of the organism (Burger and Smythe, 1953; Segal, 1956; Schwartzkopff, 1955; Maynard, 1960; Pickens, 1965; Ahsanullah and Newell, 1970), and temperature (Schwartzkopff, 1955; Maynard, 1960; Segal, 1956, 1962; Pickens, 1965; Ahsanullah and Newell, 1971; Trueman and Lowe, 1971; Widdows, 1975).

It is the influence on the heart rate of the abalone of these last two modifying factors, size and temperature, with which this chapter is concerned. As far as possible the other factors, such as O_2 tension of the environment, salinity of the environment, activity of the organism, and previous thermal experience, are kept constant.

3.3.11 Influence of size on the heart rate of invertebrate organisms

A considerable amount of work has been done on the influence of size on the heart rates of crustacea.

Burger and Smythe (1953) found no correlation between heart rate and body size either for the crayfish Homarus americanus or the crab Cancer irroratus.

Schwartzkopff (1955), however, investigated the relationship between heart rate, and body size for a variety of crustaceans, obtaining an exponential relationship between the factors, the general expression of which is given by the equation $F = ax^b$. This equation can also be expressed in the form $F = \log a + b \log x$, where F = the frequency of beats per minute, a is a constant denoting the intercept on the Y axis where x is zero, x is the body size, and b is the slope of the log plot of F against x. Schwartzkopff found values of 160 for a and -.12 for b. Several other workers have obtained results which generally support this work. Maynard (1960) investigated the effect of bodysize on the heart rate of the spiny lobster Panulirus, and again found that the relationship between heart rate and weight could be expressed in the form $X = aW^b$ (in this case X = heart rate, W = weight). Maynard's value for b was -.11 which is very similar to the general value of -.12 obtained by Schwartzkopff.

Ahsanullah and Newell (1970) arrived at the equation $F = 449.4\ x^{-.5644}$ as a general description of the heart rate-size relationship for the crab Carcinus maenus. The slope of the log plot (-.5644) indicates that the heart rate of this crab is markedly dependent on body size. In this case however the size (X) of the crabs was taken as the carapace length, and Ahsanullah and Newell acknowledge that had weight been used as an indication of size the value of b would have been approximately -.24 - much closer to that determined by previous workers. Even so it is apparent that weight has a larger affect on the heart rate of Carcinus than on many other crustaceans.

In some crustaceans, as found by Burger and Smythe (1953), the relationship between size and heart rate is less obvious. For example, although Larimer (1962) was not primarily concerned with the influence of size on the heart rate of the crayfish, some of his data allows comparisons of heart rates of animals of varying sizes to be made. Considering figure 2 of his paper, the largest animal shown has a body weight approximately $1\frac{1}{2}$ times that of the smallest shown. Although this size range is small, from Schwartzkopff's general formula, some negative regression might be expected between heart rate and weight over this range. In fact by taking heart rate values from figure 2 of Larimer's paper, at the common O_2 concentration of 5.5 ml/L, a positive regression ($b = +.43$) can be calculated. Again, in measuring the heart rates of three species of shrimps, Spaargaren (1973) found that individual variation was so great that although a negative regression between heart rate and weight was apparent, no significant correlation between these factors was observed. In Spaargaren's sample the weight of largest animals was only greater than the smallest by a factor of two. This means by Schwartzkopff's formula the heart rate of the largest animal would ideally have been 8% lower than that of the smallest. It is perhaps understandable that such a relatively small change might have been swamped by individual variation. Both in the case of Larimer (1962), and Spaargaren (1973), a larger size range may have revealed a significant weight-heart rate correlation.

Although a considerable amount of work has been done on molluscan hearts, much of it has been of a pharmacological nature (see Hill and Welsh (1966) for references) and relatively little data about the influences of such factors as size on the heart rates of molluscs has accumulated.

Segal (1956) examined the relationship between heart rate and wet weight of the aspidobranch gastropod Acmaea limatula. Data presented on figure 1 of his paper suggests that there is a significant negative regression between the logarithmic expressions of the two factors. However, the lines fitted to the plots are fitted by eye. Moreover, as the raw data is not given, no check as to the significance of this relationship can be made, nor can the value of b be calculated.

The heart rate-weight relationship of another species of mollusc is more exactly described by Pickens (1965). Pickens found that in the mussel Mytilus edulis the heart rate decreased with increasing size, with b between -.1 and -.24 for all populations. This value of b thus compares very closely with that determined for many crustaceans (Schwartzkopff, 1955; Ahsanullah and Newell, 1970).

Johansen (1965) failed to find any such relationship between weight and heart rate in the cephalopod Octopus dofleini. In fact he states that the heart rate of this octopus "showed a value of 16 beats per minute, which was the average value for all animals regardless of size and state of activity".

Although the size range of animals in Johansen's experiments was 5.4-15.2 kgms, the largest animal for which the heart rate is actually recorded is 10.9 kgm. Although it might be argued that over such a relatively small size range a negative regression might be obscured by individual variation, the constancy of the heart rate recordings at 16 beats/minute for nearly all individuals suggests that this might be a characteristic heart rate for this animal over a wide range of sizes.

Even in those cases where a significant correlation has been shown to exist between heart rate and size for a given species, this relationship may vary depending on other modifying factors such as those mentioned at the beginning of this chapter. Significant with regard

to this project is the possible variation of the heart rate weight relationship with temperature. As with the metabolic rate-weight relationship discussed in the introduction to Chapter 3, the slope b of a log heart-rate, log weight plot may vary with temperature. This was shown to be the case for the crab Carcinus maenas by Ahsanullah and Newell (1971). Although these authors record the mean b value over all temperatures to be -.5644, they show that b in fact varied in magnitude between -.453 (at 30°C) and -.6527 (at 25°C). However, no consistent pattern in the way in which b varied with increasing temperature from 5-30°C could be discerned.

3.3.12 Influence of temperature on the heart rates of invertebrates, particularly arthropods and molluscs

Maynard (1960) reviewed the work on the influence of temperature on the heart rate of crustaceans. Generally it has been found that the heart rate of crustaceans is profoundly influenced by acute temperature changes, the Q_{10} being of the order of 2.0 between 10°C and 25°C

$$Q_{10} = \frac{R_1}{R_2}^{10/(t_1-t_2)}$$

where R_1 = heart rate at lower temperature,
R_2 = heart rate at higher temperature.

More recent work on crustaceans has supported these findings. In Carcinus maenas, the shore crab, the heart rate increases with temperature up to about 25°C and the Q_{10} between 10 and 20°C is 1.7 (Ahsanullah and Newell, 1970). The Q_{10} of the heart rate of the osmoconforming shrimp Lysmata seticaudata was found to be 2.15 in hypersaline seawater, and that of the osmoregulating shrimp Palaemon serratus varied between 1.54 and 1.80 depending on the salinity of the seawater (60% "normal" seawater, and 18% "normal" seawater respectively (Spaargaren, 1973)).

In molluscs also it is apparent that temperature has a profound affect on the heart rate. In all reported studies it has been shown that the heart rates of molluscs are "slowed by cooling and speeded by heating within the physiological range" (Hill and Welsh, 1966).

For example, Segal (1956) measured the effect of temperature on the heart rate of the aspidobranch gastropod mollusc Acmaea limatula. He found that the heart rates of animals acclimated to cold conditions (low level intertidal animals) were higher at any given temperature than those acclimated to warmer conditions (high level intertidal animals). However, all animals showed marked response to temperature increase. Table 3.11 below is an extract from Segal (1956, Table 1) giving the Q_{10}'s of heart rates of Acmaea collected 27.7.53.

Table 3.11

Q_{10} values of heart rates of Acmaea, (after Segal, 1956, Table 1)

Temperature °C	Q_{10} High level Acmaea (warmer acclimated)	Q_{10} Low level Acmaea (cooler acclimated)
9-14	3.40	3.14*
14-19	2.56	2.53*
19-24	1.99	1.88

It can be seen that the Q_{10}'s appear to be generally larger than those recorded for most crustaceans, emphasising the temperature sensitivity of the molluscan heart.

The cold acclimated animals, as well as having higher heart rate than the warm acclimated animals also demonstrated Q_{10}'s, between 9 and 19°C, which were consistently lower than the latter population.

The heart rates of the colder acclimated animals were less temperature dependent. Segal points out that this is perhaps a little surprising in view of the fact that the high intertidal animals, although acclimated to warmer conditions experience a more fluctuating temperature environment than the low intertidal animals. Segal (1962) demonstrated that the heart rate of Acmaea subjected to an abrupt temperature increase exhibited an overshoot response - the "overshoot" being defined as the difference between the fastest (or slowest) rate achieved by an organism's heart immediately after the temperature change, and the stabilised rate reached sometime after initial stimulation. From figure 1 Segal (1962) it can be seen that in Acmaea the heart rate is approaching the stable rate as soon as 10 minutes after initial stimulation, and after 60-90 minutes could be regarded as stable at the new temperature.

More recent work has further emphasised the apparent sensitivity of molluscan hearts to temperature change. Trueman and Lowe (1971) measured the heart rate of the bivalve mollusc Isognomum alatus during warming from 27-35°C over a short period, and determined the Q_{10} over this temperature increase to be 2.0. Widdows (1973) measured the effect of abrupt temperature increase on the heart rate of the mussel Mytilus edulis. His Q_{10} values for this bivalve are very similar to those obtained by Segal (1956) over the same temperature ranges (Table 5.1), for the gastropod Acmaea. Table 5.12 below gives Widdow's Q_{10} values obtained by measuring the heart rates of well fed Mytilus acclimated to 15°C, and subjected to acute temperature changes.

Table 5.12

Q_{10} values of heart rates of Mytilus acclimated to 15°C, and subjected to acute temperature change (after Widdows, (1973))

	Temperature range °C			
	5 - 10	10 - 15	15 - 20	20 - 25
Q_{10}	4.21	3.41	2.71	2.36

Widdows found that over the lower temperature ranges starvation did not greatly affect the temperature sensitivity of Mytilus, however the Q_{10} from 20-25°C of starved animals was 3.89, indicating that the heart rate of these animals was much more affected by higher temperatures.

Moreover, Widdows found no acclimation affect, when measuring the heart rate of animals acclimated to each experimental temperature as opposed to the heart rates of those animals acclimated to 15°C, and then subjected to an abrupt temperature change to the experimental temperature. He attributed the slight clockwise relation of the heart rate-temperature curve of the acclimated animals compared to the curve of the 15°C animals to the fact that some interference due to an overshoot response was causing a slight error in the heart rate determinations of the latter animals.

In this brief introduction to the effect of temperature on the heart rate of some invertebrate organisms it has been mentioned that various factors (salinity, previous thermal experience, nutritional state) may cause variations in the Q_{10} of the heart rate of a given species over a given temperature range. The importance of one further factor on the Q_{10} of heart rate must be considered. In section 3.3.11 of this chapter it was mentioned that the temperature at which the heart rate-weight relationship is determined may lead to some variation in the value of b, the slope of the regression line relating log heart rate to log weight. Obviously if b varies between temperature, the Q_{10}'s of different sized animals must also vary.

In this section of chapter 3 an experiment is designed with the idea of describing the influence of these two interacting factors (1) size and (2) temperature on the heart rate of each of the three species of abalone.

It was anticipated that a model would be developed by which, knowing the species, size, and temperature of an animal a prediction of its heart rate could be made. This predictive model could then be used

to obtain heart rate data to relate to the previously calculated cardiac-output data (section 3.2.6) enabling calculations of stroke-volumes of the hearts of each of the three abalone species under specified temperature conditions.

3.3.2 Method and Materials

3.3.21 Animals

All abalone used in the heart rate experiments were "summer" animals (cf. oxygen consumption experiments, Chapter 4, part I), collected at West Island during November when mean water temperatures were 18°C. The animals were transferred to the laboratory and maintained for ten days at 20°C ± 1.5°C before heart-rate experiments commenced. The animals were considered to be 20°C acclimated. Animals were not fed during maintenance in the laboratory.

3.3.22 Preparation for heart rate experiments

One day before the heart rate experiments, the specimens were briefly removed from the aquaria, and the shells were clipped to expose the heart, (cf. Fig. 3.2). Care was taken to remove as little of the shell as possible so that the respiratory chamber through which water circulates over the ctenidia was not disturbed. Any animal injured during shell clipping was discarded. After shell clipping the animal's exposed heart could be discerned, beating strongly. The animals were then replaced in the 20°C aquaria for use in heart rate experiments the next day.

3.3.23 Experimental procedure

The heart rate of a given specimen was determined by measuring the time taken for 30 heart beats, using a stopwatch (cf. Segal, 1956). For any heart rate determination three separate measurements of the time required for 30 heart beats were taken. In all cases these times were very similar, often exactly the same. The mean of these three determinations was accepted for calculating the heart rate of the animal

in beats/minute.

If, during a heart rate determination the animal showed signs of movement, the determination was discarded: the heart rate was then redetermined when the animal had "settled down".

The experiment was designed with the idea of determining the effects of (1) species, (2) temperature and (3) size, on the heart rate of a given abalone.

The heart rate of the abalone were determined at three experimental temperatures (1) the acclimation temperature 20°C, (2) 25°C, and (3) 15°C. For each species at each temperature the heart rates of eleven (11) specimens of varying sizes were determined. The heart rate of any given specimen was determined at only one temperature. This avoids the possibility that previous thermal experience other than acclimation to 20°C may influence the heart rate of an abalone at the experimental temperature.

Where heart rates were to be determined at temperatures other than the acclimation temperature (20°C) the specimens were transferred directly to well aerated aquaria of filtered aged, seawater accurately maintained at the new temperature (15°C or 25°C). Because Segal (1962) has shown that the heart rate of gastropod molluscs may demonstrate an overshoot response on abrupt temperature change, the heart rate determinations were not made until 60-90 minutes after transfer to the new temperature. When the heart rate of a given specimen had been determined, its wet body weight, minus the shell, was carefully determined (cf. oxygen consumption section 3.2.3).

3.3.3 Results and Discussion

3.3.31 Effect of the abrupt temperature changes on the behaviour and appearance of the abalone

As with the oxygen consumption experiments, all species seemed to tolerate the abrupt temperature decrease to 15°C without any signs of

stress. The hearts of all specimens could be discerned beating strongly and steadily 60-90 minutes after transfer, when the heart rate readings were taken.

All species, when transferred abruptly from 20-25°C showed considerable initial activity and typical escape responses. 60-90 minutes after transfer, when the heart rate readings were taken, all specimens were settled. The heartbeats of all specimens were obvious, although, in H. laevigata in particular, the degree of expansion and contraction of the heart at each beat seemed irregular. Despite the irregular appearance of the heartbeat of this species the number of beats/given time varied very little.

At the time of heart rate measurement at 25°C only H. roei specimens retained a firm grip on the substrate. H. ruber and H. laevigata specimens maintained only a weak grip on the substrate. The shells of some specimens of the latter two species were lifted away from the epipodia, and the epipodia exhibited in some cases the typical buckled appearance noted in oxygen stress experiments (figure 3.5). When removed from the substrate at the completion of the heart rate experiments at 25°C all specimens had flaccid foot muscles as previously described.

3.3.32 Heart rate measurement results

Table 3.13 gives the results of the heart rate measurements of H. roei, H. ruber and H. laevigata at 15, 20 and 25°C. The logarithmic expressions (ln) of the weights of the specimens and the observed heart rates are given later in Tables 3.17 -3.19). Figures 3.15-3.17 give the plots of this observed data: lines drawn through the data are fitted regression lines. It can be seen that the regression lines have the general form described by the equation

$\ln H.R. = a + \beta \ln W$ where

H.R. = heart rate (beats/min)

W = weight gms

a is the intercept on the Y axis where $\ln W = 0$, and

β is the slope of the regression line relating ln H.R. to ln W.

The analysis carried out on the data was the same as that used to interpret the oxygen consumption-weight data. An analysis of covariance was carried out to see (1) if there was any inherent species difference in heart rate, (2) to see whether the heart rate of any given species was significantly affected by the abrupt temperature change, and (3) to see whether or not the weight of an individual animal significantly affected its heart rate with respect to other individuals of the same species at the same temperature.

The first step in the analysis was to determine whether the affect of weight on the heart rate of the abalone could be considered to be the same for all species under all conditions. The slopes of the regression lines shown on Figures 3.15, 3.16 and 3.17 were calculated for each species at each temperature. Table 3.14 below gives the individual β values calculated for each species-temperature combination, that is, for each cell of Table 3.13. The variances of the β values are also given in the table.

These individual values were then subjected to an analysis of variance to see if there were any significant differences either between treatments for a single species, or between species, with regard to the influence of weight on heart rate. The results of this analysis are given in Table 3.15 below. As a result of this analysis it can be seen that there is no significant difference between the β's of the various treatments, in other words a common slope β, that is a common weight coefficient, can be accepted.

Table 3.13

Heart rates of H. roei, H. laevigata and H. ruber at 15°C, 20°C and 25°C

	H. roei		H. ruber		H. laevigata	
	Weight gms	Heart rate beats/min	Weight gms	Heart rate beats/min	Weight gms	Heart rate beats/min
1	57.8	27.9	202.5	26.2	179.5	26.2
2	19.8	32.4	76.5	37.9	117.3	34.0
3	42.0	27.6	97.1	34.3	113.9	22.0
4	71.8	21.4	72.4	34.8	82.2	26.1
15°C 5	52.3	19.4	254.8	25.0	109.8	27.3
6	84.9	22.4	108.6	30.6	52.4	28.5
7	30.7	30.0	148.6	27.9	226.8	30.5
8	24.4	32.8	169.5	28.5	29.1	28.6
9	10.9	41.7	9 27.2	33.8	9 98.0	25.0
10	39.0	35.8	10 43.2	32.9	10 208.7	22.1
11	51.5	33.3	11 83.0	30.0	11 80.6	30.9
1	76.7	46.2	1 257.3	31.5	1 193.2	38.9
2	38.7	54.6	2 148.6	39.0	2 99.6	42.9
3	76.4	35.8	3 49.2	40.9	3 149.3	41.4
4	84.4	35.1	4 82.0	33.6	4 42.0	50.0
20°C 5	46.4	45.3	5 139.2	38.3	5 50.7	45.8
6	45.5	46.8	6 170.4	33.0	6 37.6	51.4
7	19.6	54.1	7 49.3	50.0	7 65.6	44.4
8	22.0	50.0	8 131.7	43.0	8 295.6	31.6
9	61.8	37.5	9 99.9	38.0	9 113.8	38.8
10	40.6	48.8	10 70.4	46.7	10 25.7	49.2
11	89.6	37.3	11 64.2	45.9	11 218.7	30.8
1	79.2	52.2	1 291.2	43.8	1 255.3	35.3
2	41.4	80.0	2 59.9	38.1	2 20.6	60.0
3	27.6	70.6	3 110.4	34.6	3 60.0	48.4
4	53.1	62.0	4 84.0	38.1	4 78.9	60.0
25°C 5	46.5	60.0	5 124.6	49.3	5 84.5	55.3
6	40.1	68.6	6 146.6	38.1	6 111.6	45.6
7	25.9	80.0	7 135.0	32.9	7 131.0	50.8
8	40.3	75.0	8 178.2	46.0	8 17.5	54.6
9	58.0	60.0	9 134.1	34.6	9 145.3	41.7
10	33.5	66.7	10 188.0	42.0	10 122.5	45.3
11	8.7	78.2	11 50.0	48.0	11 35.9	55.0

Figures 3.15, 3.16 and 3.17

Plots of observed ln heart rate data against ln weight for *Haliotis roei*, *H. ruber* and *H. laevigata* at 15°C, 20°C (the acclimation temperature) and 25°C.

Lines are fitted regression lines.

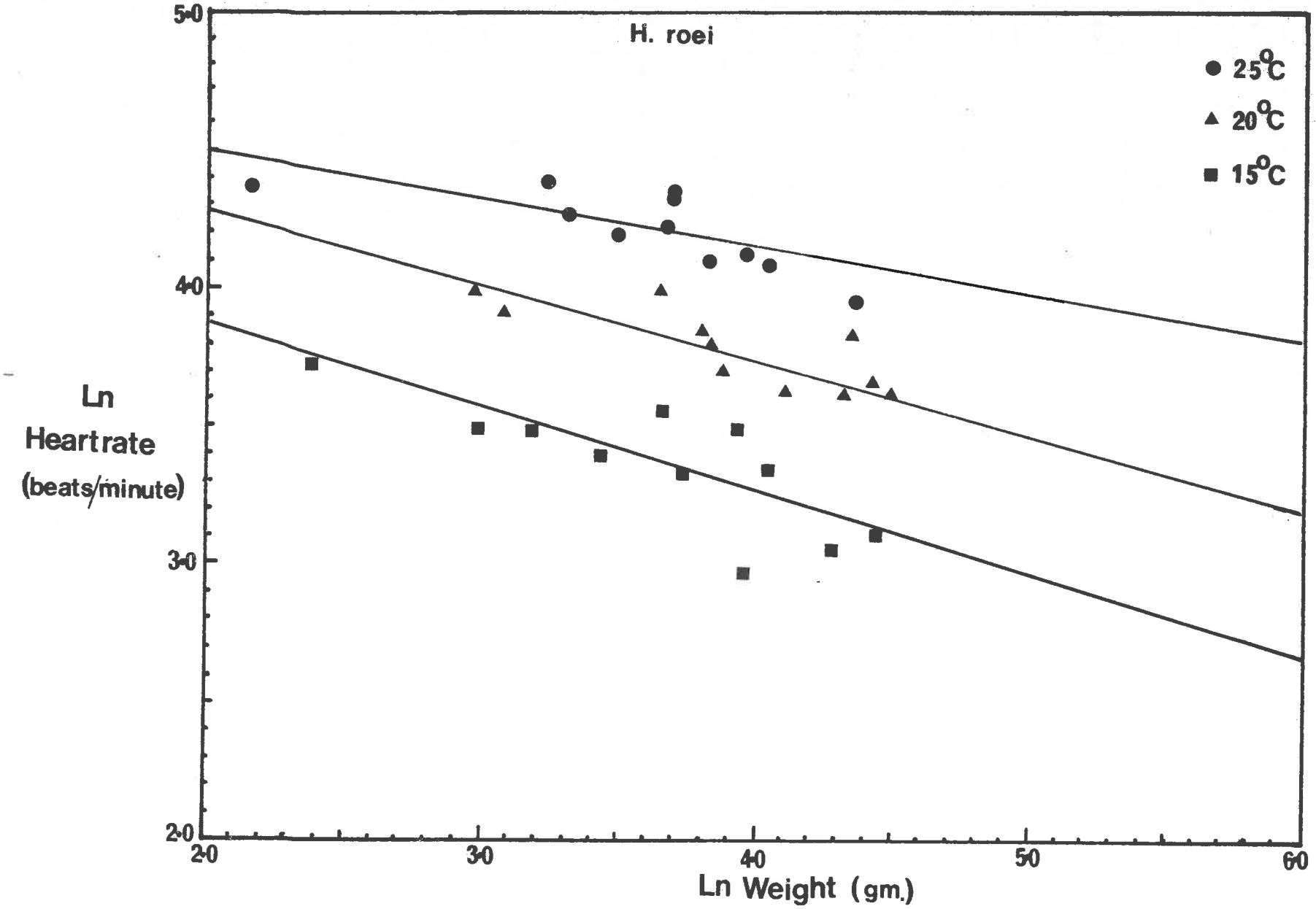
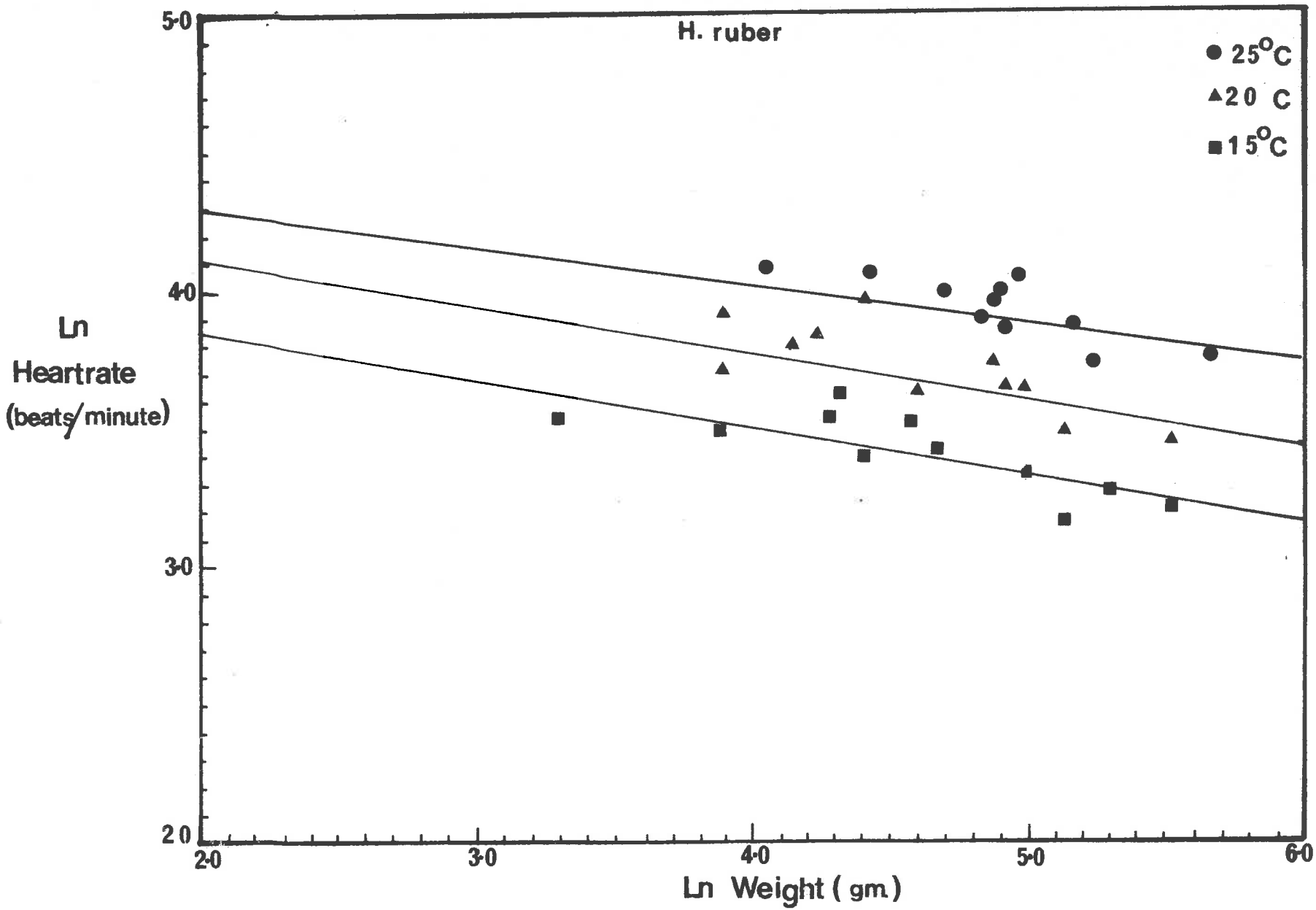

H. roei
25°C
20°C
15°C
Ln
Heartrate
(beats/minute)
5·0
4·0
3·0
2·0
2·0
3·0
4·0
5·0
6·0
Ln Weight (gm.)

Fig.3.15


H. ruber
● 25°C
▲20 C
■15°C
5·0
4·0
3·0
2·0
Ln
Heartrate
(beats/minute)
2·0
3·0
4·0
5·0
6·0
Ln Weight (gm.)

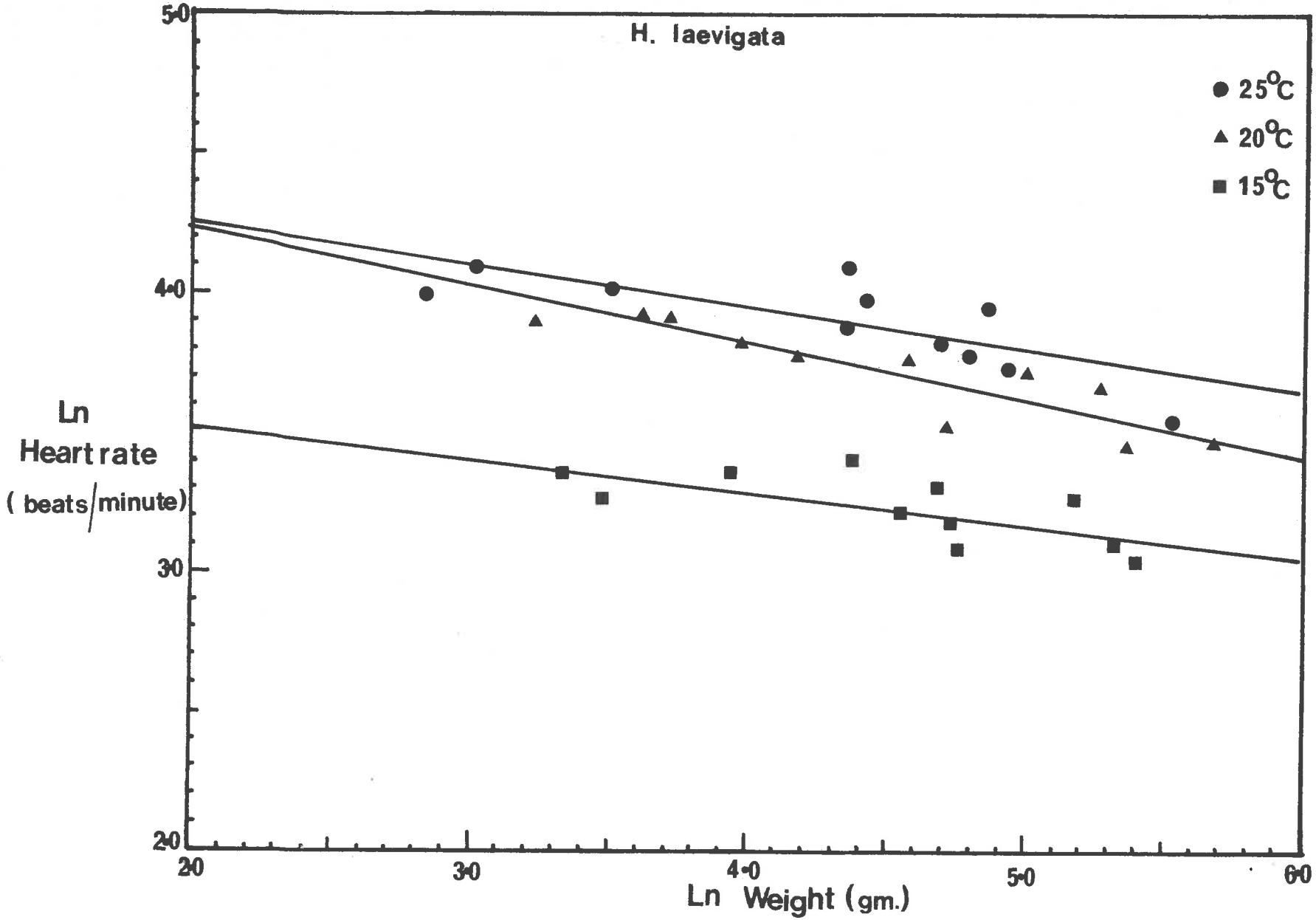

H. laevigata
● 25°C
▲ 20°C
■ 15°C
Ln
Heart rate
(beats/minute)
5·0
4·0
3·0
2·0
2·0
3·0
4·0
5·0
6·0
Ln Weight (gm.)

Fig 3.17

Table 3.14

Individual values calculated for each species-temperature combination

Cell	Species	Temperature °C		Variance of β
1	H. roei	15	-.2981	.0034
2	H. roei	20	-.2686	.0046
3	H. roei	25	-.1699	.0057
4	H. ruber	15	-.1780	.0029
5	H. ruber	20	-.2567	.0045
6	H. ruber	25	-.1160	.0048
7	H. laevigata	15	-.1206	.0026
8	H. laevigata	20	-.2015	.0019
9	H. laevigata	25	-.1472	.0017

Table 3.15

Coefficients of weight covariate (data converted to logarithms)

	Sums of Squares	Degrees of Freedom	Mean Squares	Variance Ratio	
Common	.1181	8	.0148	1.32	N.S.
Residual	1.0036	90	.0112		
Total	1.1217	98			

Having accepted a common weight coefficient for each cell, an analysis of covariance can then be carried out (as for the oxygen consumption-weight analysis) to determine which factors of (1) species, (2) temperature and (3) weight, significantly influence the heart rate of the abalone. The model investigated by the analysis of covariance is expressed by the general equation

$$E\ (\ln HR_{ijk}) = u + s_i + t_j + (st)_{ij} + \beta\left(\ln W_{ijk} - \ln \overline{W}_{ij}\right)$$

where β = the common slope of the heart rate - weight regression lines.

u = that part of the heart rate which is common to each cell, the general mean.

s_i = the factor representing that part of the heart rate determined by the fact that an animal belongs to the "i^{th}" species.

t_j = the factor representing the influence of the "j^{th}" temperature on the heart rate of the animal.

$(st)_{ij}$ = the interaction factor which accounts for any specific influence that the particular species-temperature combination may have on heart rate.

W_{ijk} = the weight of the "k^{th}" animal in the ij cell

and HR_{ijk} = the observed heart rate of the animal W_{ijk}.

As in the oxygen consumption weight analysis, if only a single cell is being considered the terms s_i, t_j and $\beta \ln W_{ij}$ are included with u, reducing the equation to

$$E\ (\ln H.R. = u + \beta \ln W)$$

which is the general expression relating heart rate to weight proposed by Schwartzkopff (1955), Ahsanullah and Newell (1970) and others (5.5.11). The results of the analysis of covariance are given below in Table 3.16.

Table 3.16

Analysis of covariance: model described by

$$E\ (\ln H.R._{ijk}) = u + s_i + t_j + (st)_{ij} + \beta\ (\ln W_{ijk} - \ln \bar{W}_{ij})$$

Source	Sums of Squares	Degrees of Freedom	Mean Squares	Variance Ratio
Grand mean	1553.82	1		
Species	.48	2	.24	19.07 (Sig)
Temperature	7.79	2	3.89	309.12 (Sig)
Species and Temperature	.40	4	.10	7.95 (Sig)
Regression on ln weight	1.31	1	1.31	104.10 (Sig)
Residual	1.12	89		
Total	1364.93	99		

Therefore all factors are significant

From this analysis it can be seen that :

(1) Temperature has a significant affect on the heart rate of an abalone, regardless of species.

(2) Over the range of experimental temperatures 15, 20, 25°C, there is a significant difference in the heart rate response of the three species.

(3) There is a significant species-temperature interaction which has an influence on the heart rate of a given abalone specimen.

(4) The weight of an animal significantly influences its heart rate, regardless of species and temperature (common β significantly different from 0).

From the analysis of covariance carried out on the observed log heart-rate and log weight data, values of u, s_i, t_j, $(st)_{ij}$ and were calculated for each cell. These values are presented in Table 3.17, 3.18, and 3.19. These values in turn were used to estimate the theoretical heart rates HR_{ijk} of the animals and in the experiments, and the 95% confidence limits about these theoretical heart rates. These theoretical heart rates and 95% confidence limits are also given in Tables 3.17, 3.18 and 3.19.

Figures 3.18, 3.19 and 3.20 are the plots of calculated heart rates against ln weights for each species at each temperature. The 95% confidence limits are plotted about the regression lines drawn through these plots. All lines drawn through the plotted points must obviously have the common slope, β = -.187.

Using the calculated values for the parameters u, s_i, t_j, $(st)_{ij}$ for each cell, and knowing the value of the common β it can be seen that the theoretical heart rate for any abalone of known weight can be calculated, provided its species, and the temperature (15, 20 or 25°C) are specified. Such calculated heart rate values will obviously lie on

Table 3.17 H. roei

Logarithmic expressions of measured and calculated values for the parameters in the model describing heartrate in the abalone.

T°C	Animal No.	u general mean	s_i species factor	t_j temperature factor	$(st)_{ij}$ species-temperature interaction factor	common slope of heartrate - weight regression	$\bar{w}$ mean weights of animals in cell	V_{ijk} weights of animal	HR_{ijk} observed heart rates	$\hat{HR}_{ijk}$ calculated heartrates	95% confidence limits about $\hat{H}_{ijk}$ lower	upper
	1	3.698	.089	-.363	-.063	-.187	3.642	4.057	3.329	3.283	3.214	3.353
	2	3.698	.089	-.363	-.063	-.187	3.642	2.986	3.478	3.483	3.411	3.555
	3	3.698	.089	-.363	-.063	-.187	3.642	3.738	3.318	3.343	3.275	3.411
	4	3.698	.089	-.363	-.063	-.187	3.642	4.274	3.063	3.243	3.171	3.314
15	5	3.698	.089	-.363	-.063	-.187	3.642	3.957	2.965	3.302	3.233	3.371
	6	3.698	.089	-.363	-.063	-.187	3.642	4.441	3.109	3.212	3.138	3.285
	7	3.698	.089	-.363	-.063	-.187	3.642	3.424	3.401	3.401	3.333	3.470
	8	3.698	.089	-.363	-.063	-.187	3.642	3.195	3.490	3.444	3.375	3.514
	9	3.698	.089	-.363	-.063	-.187	3.642	2.389	3.731	3.595	3.513	3.676
	10	3.698	.089	-.363	-.063	-.187	3.642	3.664	3.578	3.357	3.289	3.424
	11	3.698	.089	-.363	-.063	-.187	3.642	3.942	3.506	3.305	3.236	3.373
	1	3.698	.089	.043	-.047	-.187	3.892	4.340	3.833	3.699	3.629	3.768
	2	3.698	.089	.043	-.047	-.187	3.892	3.656	4.000	3.826	3.758	3.895
	3	3.698	.089	.043	-.047	-.187	3.892	4.536	3.52-	3.699	3.630	3.769
	4	3.698	.089	.043	-.047	-.187	3.892	4.436	3.558	3.681	3.610	3.751
20	5	3.698	.089	.043	-.047	-.187	3.892	3.837	3.813	3.792	3.725	3.860
	6	3.698	.089	.043	-.047	-.187	3.892	3.818	3.846	3.796	3.728	3.864
	7	3.698	.089	.043	-.047	-.187	3.892	2.976	3.991	3.953	3.878	4.029
	8	3.698	.089	.043	-.047	-.187	3.892	3.091	3.912	3.932	3.858	4.005
	9	3.698	.089	.043	-.047	-.187	3.892	4.124	3.624	3.739	3.671	3.807
	10	3.698	.089	.043	-.047	-.187	3.892	3.704	3.688	3.817	3.749	3.885
	11	3.698	.089	.043	-.047	-.187	3.892	4.495	3.619	3.670	3.598	3.741
	1	3.698	.089	.320	.111	-.187	3.600	4.372	3.955	4.074	4.001	4.147
	2	3.698	.089	.320	.111	-.187	3.600	3.723	4.382	4.195	4.127	4.263
	3	3.698	.089	.320	.111	-.187	3.600	3.518	4.257	4.271	4.202	4.339
	4	3.698	.089	.320	.111	-.187	3.600	3.972	4.127	4.148	4.079	4.218
25	5	3.698	.089	.320	.111	-.187	3.600	3.839	4.094	4.175	4.105	4.241
	6	3.698	.089	.320	.111	-.187	3.600	3.691	4.226	4.201	4.133	4.269
	7	3.698	.089	.320	.111	-.187	3.600	3.250	4.382	4.283	4.214	4.352
	8	3.698	.089	.320	.111	-.187	3.600	3.696	4.317	4.200	4.132	4.268
	9	3.698	.089	.320	.111	-.187	3.600	4.060	4.094	4.132	4.062	4.202
	10	3.698	.089	.320	.111	-.187	3.600	3.512	4.200	4.234	4.167	4.302
	11	3.698	.089	.320	.111	-.187	3.600	3.163	4.359	4.386	4.400	4.572

Table 3.18 H. ruber

Logarithmic expressions of measured and calculated values for the parameters in the model describing heartrate in the abalone.

T°C	Animal No.	u general mean	s_i species factor	t_j temperature factor	$(st)_{ij}$ species-temperature interaction factor	common slope of heartrate - weight regression	$\bar{w}$ mean weights of animals in cell	W_{ijk} weights of animal	HR_{ijk} observed heart rates	$\hat{HR}_{ijk}$ calculated heartrates	95% confidence limits about $\hat{H}_{ijk}$ lower	upper
	1	3.698	-.008	-.365	.085	-.187	4.588	5.311	3.266	3.277	3.205	3.350
	2	3.698	-.008	-.365	.085	-.187	4.588	4.337	3.635	3.459	3.390	3.527
	3	3.698	-.008	-.365	.085	-.187	4.588	4.576	3.535	3.414	3.347	3.482
	4	3.698	-.008	-.363	.085	-.187	4.588	4.282	3.550	3.469	3.400	3.538
15	5	3.698	-.008	-.365	.085	-.187	4.588	5.540	3.219	3.234	3.158	3.310
	6	3.698	-.008	-.363	.085	-.187	4.588	4.608	3.428	3.393	3.326	3.461
	7	3.698	-.008	-.365	.085	-.187	4.588	5.001	3.329	3.335	3.266	3.404
	8	3.698	-.008	-.363	.085	-.187	4.588	5.133	3.157	3.310	3.240	3.381
	9	3.698	-.008	-.365	.085	-.187	4.588	5.503	3.520	3.652	3.569	3.734
	10	3.698	-.008	-.365	.085	-.187	4.588	5.875	3.493	3.545	3.472	3.617
	11	3.698	-.008	-.365	.085	-.187	4.588	4.419	3.401	3.444	3.376	3.512
	1	3.698	-.008	.043	-.012	-.187	4.612	5.350	3.450	3.546	3.470	3.622
	2	3.698	-.008	.043	-.012	-.187	4.612	5.001	3.664	3.648	3.579	3.717
	3	3.698	-.008	.043	-.012	-.187	4.612	5.706	3.711	3.854	3.782	3.927
	4	3.698	-.008	.043	-.012	-.187	4.612	4.407	3.982	3.759	3.691	3.827
20	5	3.698	-.008	.043	-.012	-.187	4.612	4.956	3.645	3.660	3.592	3.729
	6	3.698	-.008	.043	-.012	-.187	4.612	5.188	3.497	3.623	3.552	3.692
	7	3.698	-.008	.043	-.012	-.187	4.612	5.588	3.912	3.854	3.781	3.927
	8	3.698	-.008	.043	-.012	-.187	4.612	4.831	3.761	3.671	3.602	3.739
	9	3.698	-.008	.043	-.012	-.187	4.612	4.604	3.658	3.722	3.655	3.790
	10	3.698	-.008	.043	-.012	-.187	4.612	4.854	3.844	3.786	3.719	3.856
	11	3.698	-.008	.043	-.012	-.187	4.612	4.168	3.828	3.805	3.735	3.874
	1	3.698	-.008	.320	-.073	-.187	4.804	5.674	3.780	3.775	3.700	3.850
	2	3.698	-.008	.320	-.073	-.187	4.804	4.093	4.062	4.070	3.993	4.145
	3	3.698	-.008	.320	-.073	-.187	4.804	4.704	4.000	3.956	3.888	4.024
	4	3.698	-.008	.320	-.073	-.187	4.804	4.451	4.062	4.007	3.938	4.076
25	5	3.698	-.008	.320	-.073	-.187	4.804	4.625	3.990	3.935	3.866	4.001
	6	3.698	-.008	.320	-.073	-.187	4.804	4.980	4.062	3.903	3.835	3.971
	7	3.698	-.008	.320	-.073	-.187	4.804	4.735	3.969	3.930	3.855	3.988
	8	3.698	-.008	.320	-.073	-.187	4.804	5.176	3.871	3.867	3.798	3.936
	9	3.698	-.008	.320	-.073	-.187	4.804	4.909	4.000	3.920	3.852	3.988
	10	3.698	-.008	.320	-.073	-.187	4.804	5.236	3.786	3.857	3.787	3.926
	11	3.698	-.008	.320	-.073	-.187	4.804	5.314	3.871	4.104	4.029	4.179

Table 3.19 *H. laevigata*

Logarithmic expressions of measured and calculated values for the parameters in the model describing heartrate in the abalone

T°C	Animal No.	u general mean	s_i species factor	t_j temperature factor	$(st)_{ij}$ species-temperature interaction factor	common slope of heartrate - weight regression	$\bar{w}$ mean weights animals in cell	W_{ijk} weights of animal	HR_{ijk} observed heart rates	$\hat{HR}_{ijk}$ calculated heartrates	95% confidence limits about $\hat{R}_{ijk}$ lower	upper
	1	3.698	-.081	-.363	-.022	-.187	4.546	5.190	3.266	3.112	3.040	3.184
	2	3.698	-.081	-.363	-.022	-.187	4.546	4.765	3.178	3.192	3.125	3.260
	3	3.698	-.081	-.363	-.022	-.187	4.546	4.778	3.091	3.189	3.121	3.257
	4	3.698	-.081	-.363	-.022	-.187	4.546	3.505	3.262	3.427	3.349	3.505
15	5	3.698	-.081	-.363	-.022	-.187	4.546	4.699	3.307	3.204	3.136	3.272
	6	3.698	-.081	-.363	-.022	-.187	4.546	5.959	3.350	3.342	3.271	3.413
	7	3.698	-.081	-.363	-.022	-.187	4.546	5.424	3.020	3.069	2.994	3.143
	8	3.698	-.081	-.363	-.022	-.187	4.546	3.371	3.353	3.452	3.371	3.532
	9	3.698	-.081	-.363	-.022	-.187	4.546	4.585	3.219	3.225	3.157	3.293
	10	3.698	-.081	-.363	-.022	-.187	4.546	5.541	3.096	3.084	3.010	3.158
	11	3.698	-.081	-.363	-.022	-.187	4.546	4.389	3.414	3.262	3.194	3.329
	1	3.698	-.081	.043	.059	-.187	4.495	5.299	3.361	3.571	3.497	3.644
	2	3.698	-.081	.043	.059	-.187	4.495	4.601	3.759	3.699	3.631	3.767
	3	3.698	-.081	.043	.059	-.187	4.495	5.006	3.723	3.625	3.553	3.694
	4	3.698	-.081	.043	.059	-.187	4.495	5.738	3.913	3.860	3.787	3.933
20	5	3.698	-.081	.043	.059	-.187	4.495	3.926	3.824	3.825	3.754	3.896
	6	3.698	-.081	.043	.059	-.187	4.495	3.627	3.940	3.881	3.806	3.955
	7	3.698	-.081	.043	.059	-.187	4.493	4.184	3.763	3.777	3.708	3.845
	8	3.698	-.081	.043	.059	-.187	4.493	5.699	3.453	3.496	3.415	3.577
	9	3.698	-.081	.043	.059	-.187	4.493	4.734	3.520	3.674	3.606	3.742
	10	3.698	-.081	.043	.059	-.187	4.493	3.246	3.996	3.952	3.870	4.033
	11	3.698	-.081	.043	.059	-.187	4.495	5.388	3.428	3.552	3.477	3.627
	1	3.698	-.081	.320	-.038	-.187	4.292	5.542	3.564	3.666	3.594	3.747
	2	3.698	-.081	.320	-.038	-.187	4.292	3.025	4.094	4.135	4.053	4.217
	3	3.698	-.081	.320	-.038	-.187	4.292	4.094	3.879	3.956	3.868	4.004
	4	3.698	-.081	.320	-.038	-.187	4.292	4.368	4.094	3.885	3.817	3.953
25	5	3.698	-.081	.320	-.038	-.187	4.292	4.437	3.976	3.872	3.804	3.940
	6	3.698	-.081	.320	-.038	-.187	4.292	4.715	3.880	3.820	3.751	3.890
	7	3.698	-.081	.320	-.038	-.187	4.292	4.875	3.955	3.790	3.719	3.861
	8	3.698	-.081	.320	-.038	-.187	4.292	3.982	4.090	4.166	4.080	4.251
	9	3.698	-.081	.320	-.038	-.187	4.292	4.965	3.731	3.774	3.702	3.846
	10	3.698	-.081	.320	-.038	-.187	4.292	4.906	3.768	3.803	3.733	3.873
	11	3.698	-.081	.320	-.038	-.187	4.292	3.523	4.007	4.042	3.969	4.116

Figures 5.18, 5.19 and 5.20

Regression lines relating calculated ln heart rate ($\hat{HR}_{ijk}$) and ln W for each species at each experimental temperature (15°C, 20°C and 25°C). The 95% confidence limits about these regression lines are plotted on the figures.

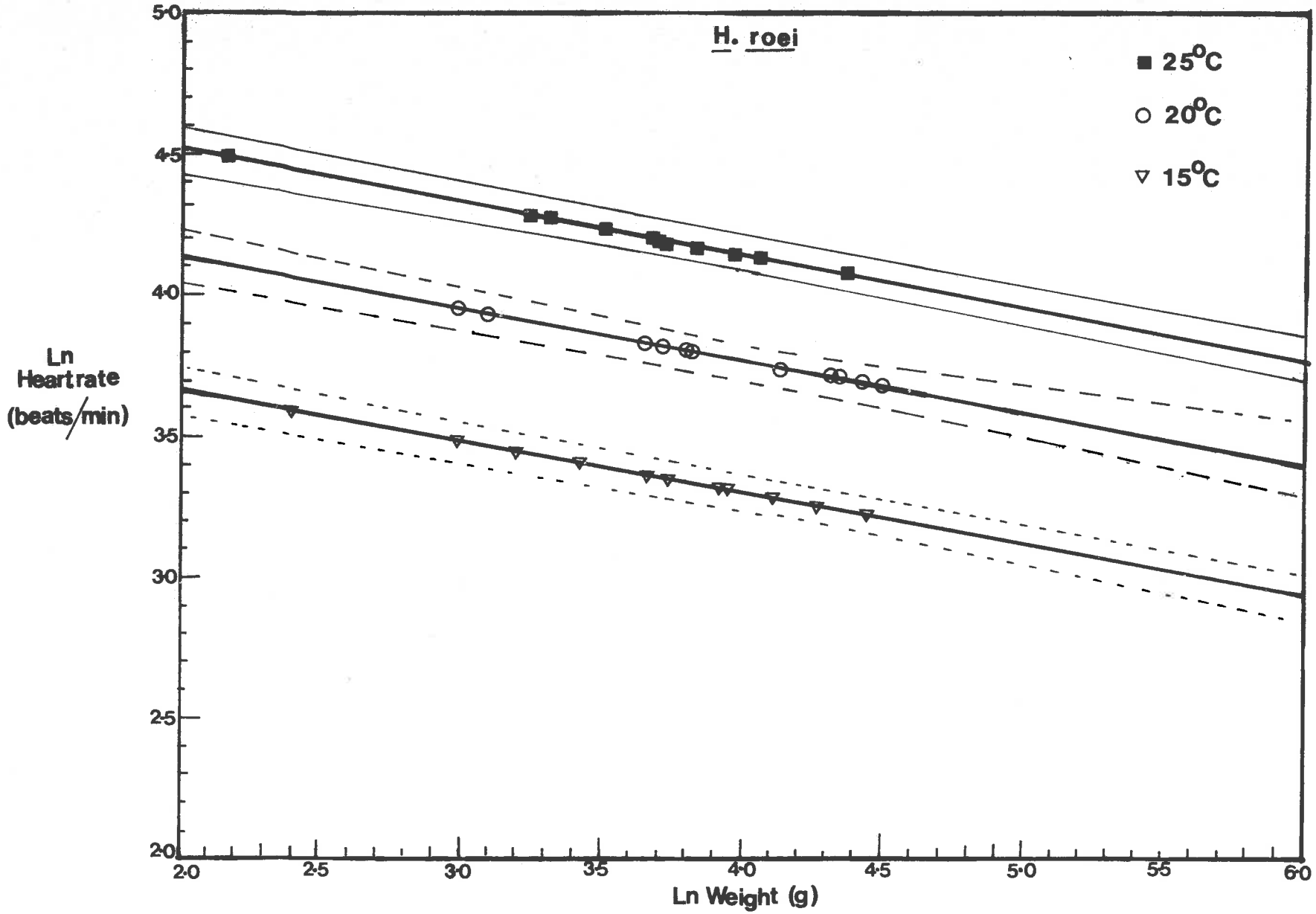

H. roei
■ 25°C
○ 20°C
▽ 15°C
Ln Heartrate (beats/min)
5·0
4·5
4·0
3·5
3·0
2·5
2·0
2·0
2·5
3·0
3·5
4·0
4·5
5·0
5·5
6·0
Ln Weight (g)

Fig-3.18

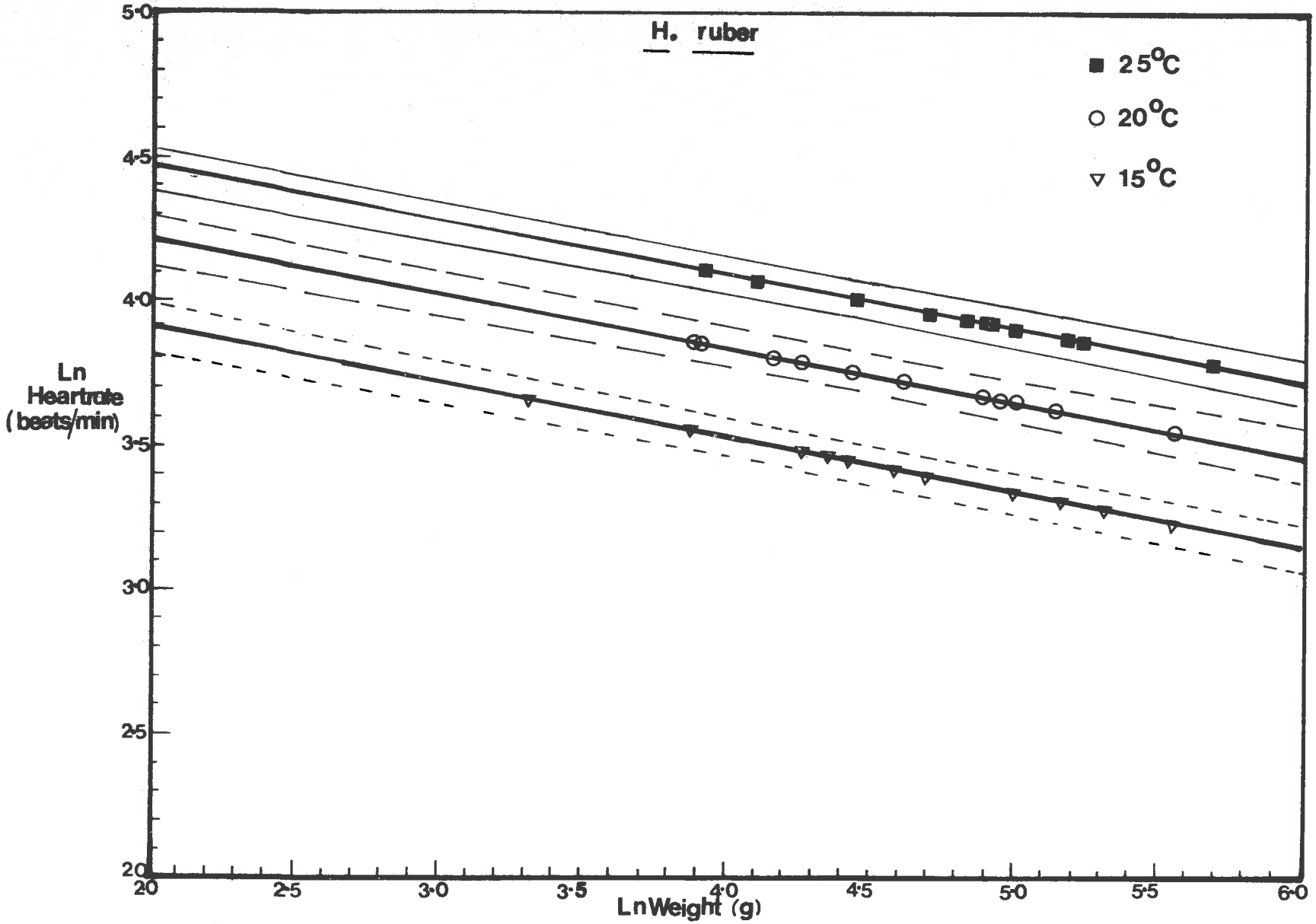

H. ruber
25°C
20°C
15°C
Ln Heartrate (beats/min)
5·0
4·5
4·0
3·5
3·0
2·5
2·0
2·0
2·5
3·0
3·5
4·0
4·5
5·0
5·5
6·0
LnWeight (g)

Fig. 3.19

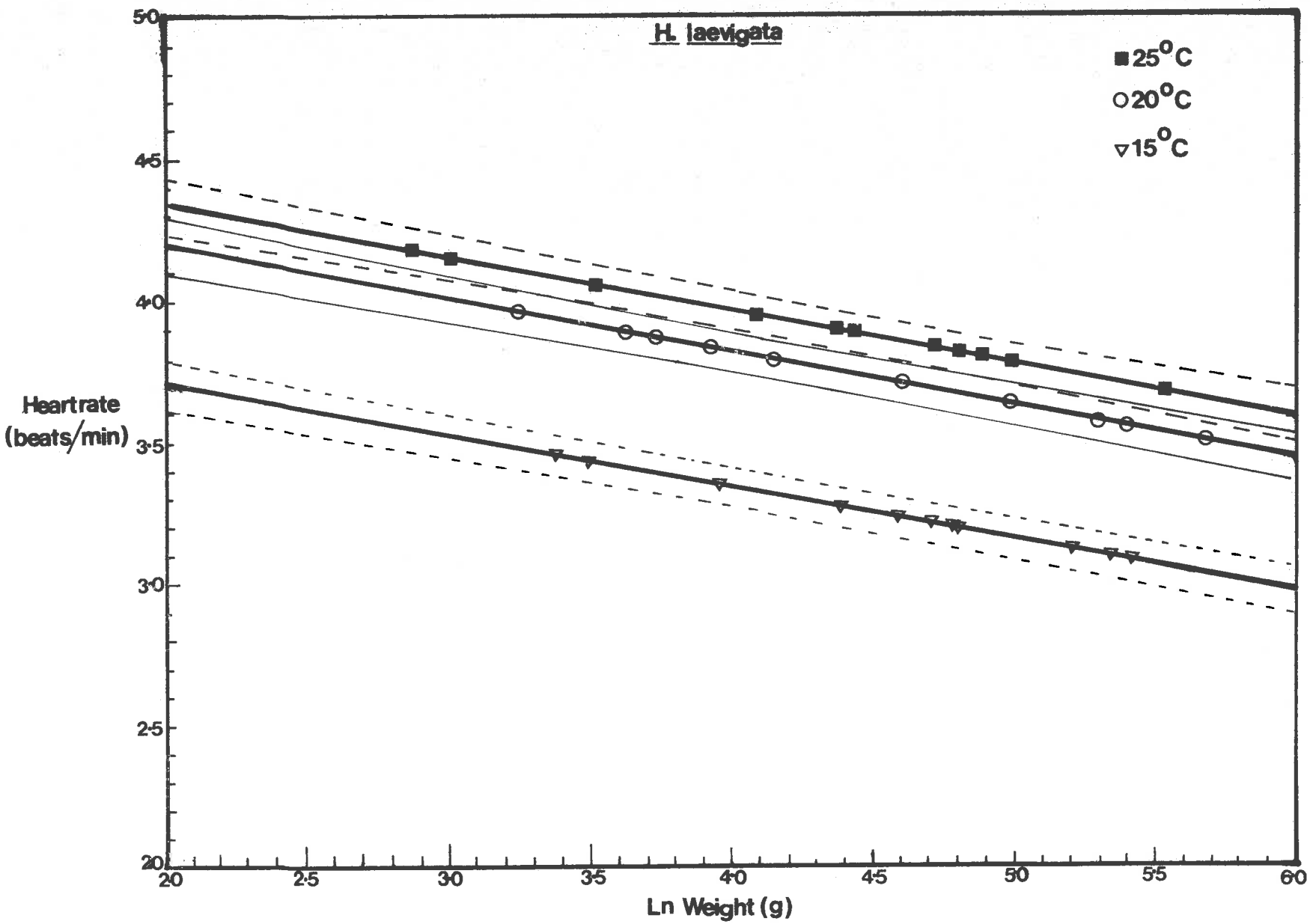

H. laevigata
25°C
20°C
15°C
Heartrate (beats/min)
5·0
4·5
4·0
3·5
3·0
2·5
2·0
2·0
2·5
3·0
3·5
4·0
4·5
5·0
5·5
6·0
Ln Weight (g)

Fig. 3.20

the regression line relating ln heart rate to ln weight for the particular cell being considered (Figs. 3.18-3.20). The intercept a on the Y axis of the general equation relating ln heart rate to ln weight will be defined when ln W = 0 for each cell. For example, consider the regression line of H. roei at 15°C. The general equation describing this line is

$$E\ (\ln H.R.) = u + s_i + t_j + (st)_{ij} + \beta\ (\ln W_{ijk} - \ln \overline{W}_{ij})$$

Substituting the known values for the parameters u, s_i, etc. at ln W = 0 the equation becomes

$$E\ (\ln H.R.) = 3.698 + .089 + .563 - .063 - .187\ (-3.642)$$

$$= 3.561 + .681$$

$$= 4.042$$

The value of a is therefore 4.042

Table 3.20 below gives the equations describing the ln heart rate - ln weight relationships for each cell; the equations are expressed in terms of the calculated values of a for each cell, and the common slope β.

Table 3.20

Equations describing ln heart rate, ln weight relationships for individual cells

Cell	ln H.R. = lna + β ln W
1	ln H.R. = 4.040 - .187 ln W
2	ln H.R. = 4.508 - .187 ln W
3	ln H.R. = 4.390 - .187 ln W
4	ln H.R. = 4.268 - .187 ln W
5	ln H.R. = 4.581 - .187 ln W
6	ln H.R. = 4.834 - .187 ln W
7	ln H.R. = 4.080 - .187 ln W
8	ln H.R. = 4.557 - .187 ln W
9	ln H.R. = 4.700 - .187 ln W

3.3.33 Effect of weight on the heart rate of the abalone

The fact that there is a common β value within a given species is not surprising in view of the fact that it has been shown that such common regression slopes may relate metabolism to size over a wide temperature range in a variety of organisms (Davis, 1966; Barnes and

Barnes, 1969; and others). Further, in this study the ln oxygen consumption - ln weight relationships for a given species had a common slope at all experimental temperatures. Moreover the slopes b of the ln oxygen consumption - ln weight plots were common between species. Once again therefore it is not surprising that there is a common value for all species.

The value of the common β found for the abalone in this study is quite comparable with other values of this parameter reported in the literature for molluscs and arthropods (Schwartzkopff, 1955; Pickens, 1965; Ahsanullah and Newell, 1970).

3.3.34 Effect of temperature on the heart rate of abalone

The way in which temperature effects the heart rate of the abalone is perhaps best illustrated by determining the Q_{10}'s of heart rates over the experimental temperature. As the regression lines have common slopes at all temperatures, for any species, the Q_{10} of heart rate of an animal of a given size will be the same as the Q_{10} of heart rate of an animal of any other size.

For the purposes of calculating the Q_{10} of heart rates, the "standard sized" animal 90.02 gms (ln 90.02 = 4.5) was selected for each species.

The heart rates of the standard animals of each species at 15, 20 and 25°C are given on Table 3.21 and plotted on figure 3.20. The Q_{10} values are also tested on Table 3.21.

Generally, it can be seen that the Q_{10}'s of the heart rates of each of the abalone species are similar to Q_{10}'s of heart rates of other invertebrates reported in the literature (see introduction to this chapter). The Q_{10}'s of the heart rates of the abalone are particularly similar to those reported for the gastropod mollusc Acmaea over the same temperature range (Segal, 1956), and to those reported for the bivalve mollusc Mytilus over the same temperature range (Widdows, 1973).

Figure 3.20

Heartrates of standard weight (90.02 g) specimens of *Haliotis roei*, *H. ruber* and *H. laevigata* at 15°C, 20°C (the acclimation temperature) and at 25°C.

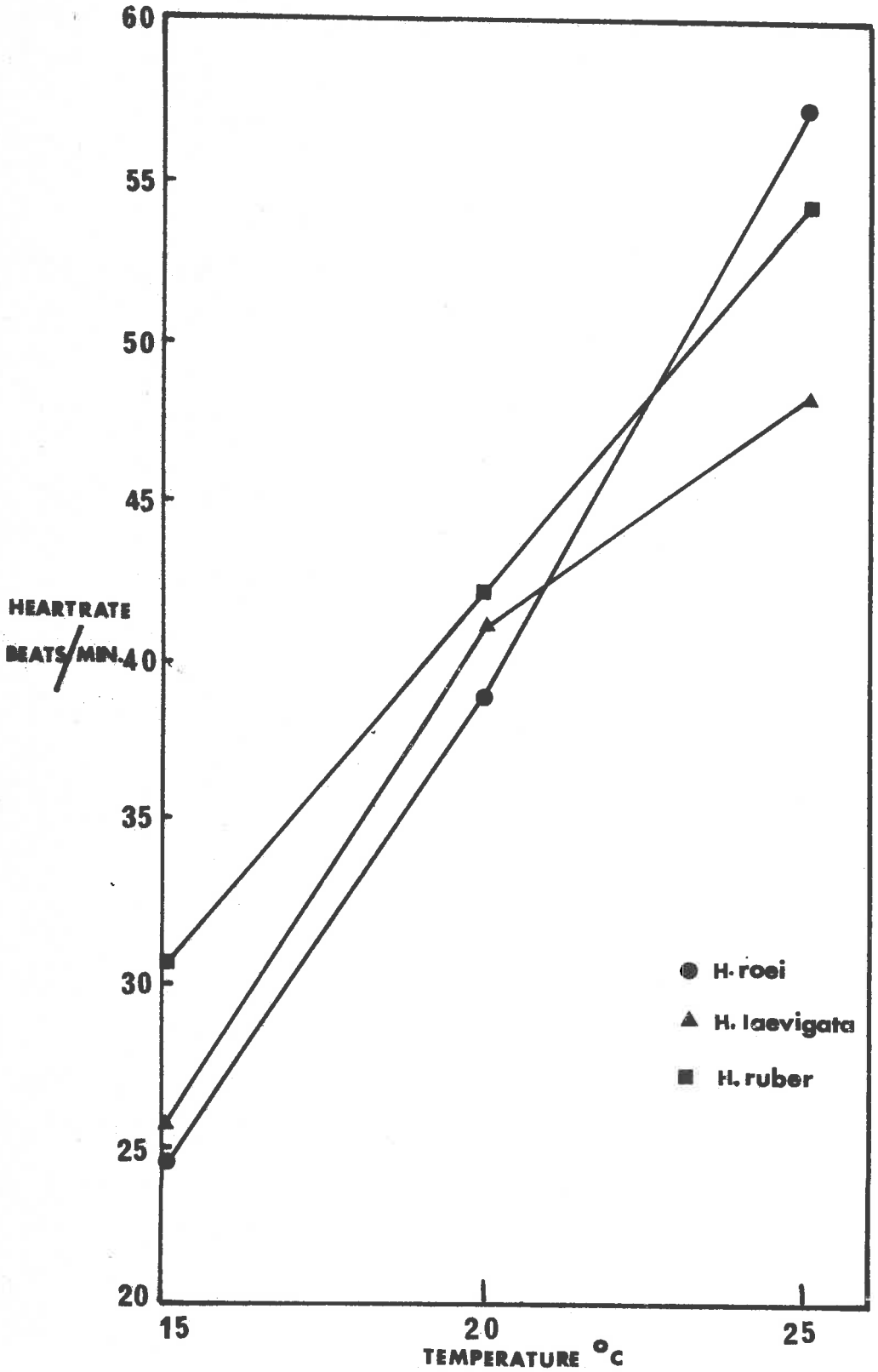

60
55
50
45
40
35
30
25
20
HEARTRATE
BEATS/MIN.
15
20
25
TEMPERATURE °C
H. roei
H. laevigata
H. ruber

Table 3.21

Heart rates (beats/min) standard animals 90.09 g

Temperature °C	H. roei	H. ruber	H. laevigata
15	24.5	30.8	25.5
20	39.1	42.1	41.1
25	57.3	54.2	47.4
	Q_{10} values for heart rates of abalone		
15°C - 20°C	2.55	1.87	2.59
20°C - 25°C	2.15	1.66	1.53

3.3.35 Comparison of the effects of temperature on the heart rates of the three species, and discussion of the significance of increased heart rate in the light of other data

As the analysis of covariance has shown, over the range of temperatures used here the response of the heart rates of the three species to abrupt temperature change differ significantly. At 15°C the heart rates of H. roei, and H. laevigata do not appear to differ greatly, and it can be reasonably assumed that there is no significant difference between them at this temperature. (Any statistical test comparing just these two species at 15°C is invalidated by the fact that the slope of the common regression line was obtained through a consideration of all species at all temperatures). The Q_{10} values indicated that between 15 and 20°C the heart rates of these two species are very similar in their sensitivity to temperature change. Above 20°C, though, the heart rate of H. roei continues to increase rapidly with temperature increases whereas the Q_{10} of H. laevigata is considerably reduced. The result of this is that at 25°C the heart rate of H. roei is considerably higher than that of H. laevigata. At 15°C, and to a lesser extent at 20°C (the acclimation temperature) H. ruber has a higher heart rate than either of the other two species. Like H. laevigata, however, from 20-25°C the Q_{10} of H. ruber heart rate decreases compared to that between 15 and 20°C, resulting in H. ruber's final heart rate at 25°C being

considerably lower than that of H. roei, but still higher than that of H. laevigata (cf. oxygen consumption data Fig. 3.13 chapter 3, part I.)

The significance of the way in which the heart rates of the three species respond to temperature change can best be seen in the light of the way in which the factors oxygen consumption and A-V difference in O_2 content (and hence cardiac output), change with abrupt temperature change.

From 20-25°C the oxygen consumptions of all species show a considerable increase; the increase being most marked in H. roei (see chapter 3 part I). This increase in oxygen consumption after an abrupt temperature increase is accompanied by a decrease in the amount of oxygen delivered by the haemocyanin in all species (Chapter 2). Hence to maintain the necessary oxygen supply the cardiac output increases (Table 3.10). Cardiac output (C.O.) is determined by two factors, (1) the heart rate (H.R.) whose response to temperature change has just been described, and (2) the stroke volume (S.V.). Values for the latter parameter can be calculated.

3.3.36 Calculation of stroke volumes

All parameters considered are those of standard 90.02 gm animals of each species.

In Chapter 3 part I, the C.O. of a standard 90.02 gm specimen of H. roei at the acclimation temperature (20°C) was found to be 5.6 ml/minute. The H.R. of such a standard H. roei at 20°C is 39.1 beats/min. (Table 3.21, Fig. 3.20). C.O. ml/min - H.R. beats/min. x S.V. ml/beat. Therefore for H. roei the stroke volume of the standard sized animal at 20°C can be simply calculated S.V. ml/beat = $\frac{5.6}{39.1}$ = .14 ml/beat.

After an abrupt transfer of this species from 20°C to 25°C, the cardiac output of the standard specimen rises considerably, almost doubling to become 10.8 ml/minute. The heart rate also increases quite markedly (Q_{10} between 20 and 25°C = 2.15) to 57.5 beats/min. Hence at

25°C the S.V. ml/beat = $\frac{10.8}{57.3}$ = .19 ml/beat.

In the standard specimen of H. ruber at 20°C the cardiac output is 8.5 ml/minute. Again, knowing the heart rate of such a specimen, the stroke volume can be computed to be .2 ml/beat. After an abrupt temperature change from 20-25°C the cardiac output of a standard H. ruber specimen increases to 11.7 ml/minute, while the heart rate increases from 42.1 to 54.2 beats/min. The S.V. at 25°C is therefore .22 ml/beat.

In the third species H. laevigata, at 20°C the cardiac output is 6.9 ml/minute for a standard 90.02 gm specimen. The heart rate of such an animal is 41.1 beats/minute. The stroke volume can therefore be calculated to be .17 ml/beat. The cardiac output for a standard animal of this species after an abrupt temperature increase to 25°C is 10.1 ml/minute: the heart rate is 47.4 beats/minute. The stroke volume must therefore increase to .21 ml/beat to maintain the calculated cardiac output.

Table 3.22 below lists the cardiac outputs, the heart rates, and the stroke volumes of the standard specimens of the three abalone species at 20°C, and after an abrupt temperature increase to 25°C.

Table 3.22

Cardiac output, heart rate, and stroke volume data for abalone at 20°C, and after an abrupt temperature increase to 25°C.

T°C	C.O. (ml/min)	H.R. beats/min	S.V. ml/beat
		H. roei	
20	5.6	39.1	.14
25	10.8	57.3	.19
		H. ruber	
20	8.5	42.1	.20
25	11.7	54.2	.22
		H. laevigata	
20	6.9	41.1	.17
25	10.1	47.4	.21

In terms of absolute increases of these parameters, it can be seen from Table 3.22 that the cardiac output of H. roei undergoes by far the greatest increase; both the heart rate, and the stroke volume increases of this species are substantially larger than those of the other species. The heart rate increases by 47% of its 20° value, and the stroke volume by 36% of its original value. These figures for (1) H. ruber, and (2) H. laevigata are (1) 29%, and 10% and (2) 15% and 24% for heart rate and stroke volume respectively.

The response of the abalone to such an abrupt temperature transfer indicates that these species, particularly H. laevigata, and H. ruber are stressed by this treatment. The irregularity of the heart beat observed in H. laevigata at 25°C may indicate that the heart function is reaching a limit at this temperature. It is even possible to hypothesise that due to the decreased contribution of haemocyanin to the A-V difference in O_2 content, and to the fact that the heart is reaching a plateau in its performance the O_2 supplied to the abalone might be insufficient to maintain metabolism after the abrupt temperature increase. This could to some extent explain the apparent distress of the specimens, particularly in H. laevigata after abrupt increase. Further it is possible that under these circumstances the aerobic metabolism may be to a greater or lesser extent supplemented by anaerobic respiration. This possibility is discussed in more detail in chapter 4 of this thesis.

3.4 Discussion of all parameters involved in oxygen transport in abalone

A summary of the essential parameters describing oxygen exchange in the three species of abalone at the acclimation temperature 20°C, and after an abrupt temperature increase to 25°C is presented in Table 3.23.

The first thing which is apparent from an examination of this table is the general similarity in the oxygen exchange systems of the three species. However a close examination of the data reveals that there are some differences between the species.

I will consider first the abalone at the acclimation temperature. I have already stated that in one sense, as a whole organism, because of its higher standard metabolism H. roei is less efficient than the other two species. H. ruber in turn is less efficient in this sense than H. laevigata which has the lowest standard metabolism at 20°C.

However, it can be seen that because of the higher oxygen capacity of the blood, and the lower venous % saturation of the haemocyanin of H. roei the A-V difference in vol % of oxygen is considerably greater in this species than in H. ruber and H. laevigata, resulting in a much smaller cardiac output at 20°C. Both the heart rate and the stroke volume have lower values than the same parameters in the other species. It is evident that because of the high A-V difference in vol % oxygen the energy expended to maintain the higher oxygen consumption of H. roei will be less than in the other two species. When viewed in this manner then the oxygen supply system of H. roei is more efficient than those of the other two species.

At 20°C heart rate, stroke volume and hence cardiac output of H. laevigata are smaller than the same parameters in H. ruber. The lower cardiac output is a consequence of a slightly greater A-V

Table 3.23

Summary of the essential parameters describing oxygen exchange in the South Australian abalone H. roei, H. ruber and H. laevigata (mean values)

Species	Arterial P_{O_2}	Arterial P_{CO_2}	Venous P_{O_2}	Venous P_{CO_2}	O_2 capacity of haemocyanin vol %	Arterial P_{50} mm Hg	Venous P_{50} mm Hg	Arterial % saturation	Venous % saturation	A-V difference % saturation	A-V difference vol % of oxygen	% of total O_2 delivered by haemocyanin	Standard 90.02 g oxygen consumption ul/g/hr	Calculated cardiac output ml/min	Heart rate of standard 90.02g animal beats/min	Calculated stroke volume of standard animal ml/beat
Animals acclimated to 20°C																
H. roei	36.7	1.13	9.6	4.3	1.44	24.0	13.5	81.0	23.0	58.0	.92	91	54.5	5.6	39.1	.14
H. ruber	38.8	1.20	11.5	6.0	1.01	28.5	13.5	84.0	36.7	47.3	.56	86	31.8	8.5	42.1	.20
H. laevigata	37.4	1.21	13.8	3.5	1.04	26.5	16.0	85.5	33.8	51.7	.61	90	28.2	6.9	41.1	.17
Animals after an abrupt temperature increase from 20°C-25°C																
H. roei	34.4	1.20	9.6	4.25	1.44	36.0	25.0	48.5	12.0	36.5	.60	88	43.4	10.8	57.3	.19
H. ruber	45.0	1.20	15.8	4.30	1.01	41.5	27.0	60.0	21.0	39.0	.47	84	36.6	11.7	54.2	.22
H. laevigata	39.4	1.30	12.4	3.2	1.04	35.0	26.0	62.0	18.8	43.2	.52	86	35.2	10.1	47.4	.21

difference and a markedly lower standard rate of oxygen consumption in H. laevigata.

Effect on oxygen transport system of an abrupt temperature increase to 25°C

In the field H. roei experiences more variable temperatures, and, at times, higher temperatures than either H. ruber or H. laevigata. A priori therefore it was expected that the O_2 transport system of H. roei might adjust more readily to cope with an abrupt temperature increase than the systems of either H. ruber or H. laevigata.

As can be seen from Table 3.23, a sudden increase in temperature to 25°C caused a reduction in the amount of oxygen delivered to the tissues by the haemocyanin of all three species. In all species the reduction in the amount of O_2 delivered after an abrupt temperature increase stemmed largely from the fact that while the oxygen affinity of the haemocyanins decreased with temperature increase there was no compensatory increase in in vivo P_{O_2} resulting in a considerable reduction in the % saturation of the arterial haemocyanins.

This was most marked in H. roei where the arterial haemocyanin was only 48.5% saturated after the temperature increase, compared to 81% saturation at 20°C. Although the % saturations of the venous haemocyanins were also lowered, again most notably in H. roei, this did not compensate for the large change in arterial % saturation. Due to the greater reduction in venous % saturation in H. roei the overall difference in % saturations between arterial and venous haemocyanin in this species is similar to that of the other species; 36.5%, 42.2% and 39.0% for H. roei, H. laevigata, and H. ruber respectively at 25°C. It is clear however that arterial-venous difference in % saturation has undergone the greatest reduction compared to its value at 20°C in H. roei. In a single circulation of the blood at 25°C, H. roei's haemocyanin delivers only about 65% of the oxygen delivered at 20°C

whereas the haemocyanins of the other two species both deliver about 85% of the amount of oxygen delivered at 20°C. In this sense then, it appears that H. roei's oxygen delivery system is not coping with the abrupt temperature change any better than, or in fact as well as, the systems of H. ruber, and H. laevigata. However, despite this large reduction in efficiency of H. roei haemocyanin, when the larger O_2 capacity of the haemocyanin of this species is taken into account the arterial-venous differences in vol % of oxygen is still larger than that of the other two species.

After an increase from 20-25°C, the oxygen consumption of H. roei increases by 28% of its 20°C value, compared to 15% and 25% increases for H. ruber, and H. laevigata respectively (Table 3.10). As H. roei's 20°C oxygen consumption was larger than that of the other two species, this means that at 25°C this species maintains a considerably higher oxygen consumption than the other two species. Hence, with the increased oxygen consumption, but considerably reduced A-V difference, the cardiac output of H. roei must increase quite drastically, almost doubling to become 10.8 cc/min. Likewise with the reduced efficiency of their haemocyanins, in terms of A-V difference in vol % of oxygen, and increased oxygen consumptions the cardiac outputs of the other two species must also increase - (11.7 cc/min and 10.1 cc/min for H. ruber and H. laevigata respectively).

Thus it can be seen although the change in the cardiac output of H. roei from 20-25°C is considerably greater than that of the other two species, the final size of the cardiac output is quite comparable to that of H. ruber, and H. laevigata.

It is evident in H. roei, that although the stroke volume at 25°C is maintained at a smaller value than for the "standard" animals of the other two species, the relative increase in this parameter compared to the 20°C stroke volume is largest in this species. Although the cardiac output of H. roei at 25°C is similar to that of the other two species, it

is maintaining, through the characteristics of the blood, a higher standard metabolism. Therefore, when viewed in the absolute terms of how much blood the heart must pump to maintain a given oxygen consumption at 25°C the oxygen supply system of H. roei would still appear to be more efficient than those of the other two species.

However, it must be remembered that the characteristic of the blood which allows this relatively low cardiac output to supply enough oxygen to maintain the greater metabolic needs of H. roei is the oxygen capacity of the haemocyanin. This, as has been shown in chapter 2, stems directly from the greater haemocyanin concentration in the haemolymph of this species. It is logical to assume that H. roei therefore uses more energy than the other species in the synthesis of haemocyanin. The question then arises as to why this should be so? It is only possible to speculate on answers to this question. It could simply be proposed that as H. roei is an active species and moves around to feed, whilst the other species have to rely on drift algae, the more regular food supply enables the former species to maintain the higher haemocyanin concentration. However, Pilson (1965) has shown that haemocyanin concentration apparently does not vary with nutritional state in the abalone. Nor could the variability of abalone haemocyanins in this study be correlated with any physiological or environmental variations (see Chapter 5, on field sampling of abalone haemocyanin). In view of these facts I propose that the consistantly higher haemocyanin concentration in H. roei as compared to H. ruber and H. laevigata is a genetic trait which confers a selective advantage in the particular niche of this species.

H. roei has a significantly higher standard oxygen consumption than the other species investigated. It is possible that it is less costly in terms of the overall energy budget of the species to supply its increased oxygen needs by means of a higher haemocyanin concentration than by an increased cardiac output.

H. roei is by far the most active of the species studied during this project. It may be then, that the high oxygen capacity of the haemocyanin of this species acts as a potential reserve for the O_2 transport during periods of intense activity. At least in two groups of haemocyanin bearing organisms it has been demonstrated or suggested that during times of activity, the in vivo P_{O_2} values adjust in such a way that more oxygen is delivered to the tissues of the organism than when the animal is at rest. Specifically, Johansen et al. (1970) have shown that activity in the crab Cancer magister leads to a drop in the arterial and venous P_{O_2}. Because of the shape of the oxygen equilibrium curve of the haemocyanins of this animal, this leads to a small change in the % saturation of the arterial haemocyanin, but a large drop in the % saturation of the venous haemocyanin, substantially increasing the amount of oxygen delivered to the tissues by the blood during activity. In the large cephalopod Octopus dofleini however, Johansen and Lenfant (1966) propose that activity may be accompanied by an increased saturation of the arterial haemocyanin, perhaps due to increased P_{O_2} of inhaled water due to increased ventilation. Without a marked increase in venous % saturation this will also lead to increased O_2 supply to the tissues during activity.

Assume in the abalone species H. roei that during activity both arterial and venous P_{O_2} drop about 7 mm Hg. The arterial haemocyanin would then become about 70% saturated, but virtually all of the O_2 in the venous haemocyanin would have been delivered to the tissues (fig. 2.12). This means, that the haemocyanin alone of H. roei, without considering the dissolved O_2, would deliver 7.01 vol % of oxygen or an increase of 0.17 vol % of oxygen (20%) per circulation compared to the resting value delivered. If, in addition, the arterial P_{O_2} was maintained at the original value, perhaps by increased ventilation, the haemocyanin would deliver about 1.15 vol % per circulation or an increase of .31 vol % (37%) of the amount of O_2 delivered when the animal is in the resting state.

It can be seen that due to the high oxygen capacity of H. roei haemocyanin this increase in oxygen delivered per circulation (.31 vol %) is, in absolute terms, a significant amount. Similar changes in arterial and venous % saturations in either H. ruber or H. laevigata would result in considerably smaller increases in the absolute amounts of oxygen being delivered, simply because the O_2 capacities of the haemocyanins of these species are smaller. Presuming also an increased circulation during activity (cf. Johansen and Lenfant, 1966) it is possible to see that a much higher metabolism might be maintained in the active H. roei than in the other species.

H. roei lives in the shallow, barely subtidal waters of West Island, and during summer months experiences considerable variation in water temperature depending, for example, on how much mixing occurs between warm surface waters and deep cooler waters. The high haemocyanin concentration of H. roei (hence the high O_2 capacity) might allow H. roei to survive a relatively abrupt increase in temperature without undue circulatory, and hence respiratory stress.

Consider hypothetically that H. roei has the same mean haemocyanin concentration as H. ruber. In animals acclimated to 20°C this will mean that .59 vol % of oxygen (haemocyanin) + .079 vol % of oxygen (solution) = .66 vol % of oxygen will be delivered by the haemolymph of this animal per circulation. With the standard 20°C H. roei oxygen consumption of 3.1 ml/hr, this means that a cardiac output of 7.8 ml/minute must be maintained at this temperature. This cardiac output is quite comparable to those of the other species measured at this temperature, and would seem entirely reasonable for such an invertebrate (see section 3.2.61). However, after an abrupt temperature change, assuming H. roei haemocyanin's own oxygen equilibrium characteristics, but a mean haemocyanin concentration like that of H. ruber, the oxygen delivered per circulation becomes .37 vol % of oxygen (haemocyanin) + .069 vol % of oxygen (in solution = .44 vol %

of oxygen. With the increased oxygen consumption of H. roei after an abrupt temperature increase the cardiac output becomes

$$\frac{3.91 \text{ ml/hr} \times 100}{.44 \text{ vol \%} \times 60}$$

= 14.81 ml/min (standard 90.02 gm animal)

= 164.52 ml/min/kgm

As can be seen from the previous discussion on available cardiac output data for other invertebrates (section 3.2.61) this is quite a large value and might be supposed to considerably stress the circulatory system of the abalone.

No information is available on the function of the haemocyanin of H. roei when adapted to the higher temperature 25°C. However, it would seem logical that after a period of acclimation to this temperature the in vivo P_{O_2} would adjust such that the A-V difference in % saturation approaches the 20°C value (cf. Falkowski, 1973). It can be seen that H. roei with its higher haemocyanin concentration (and hence O_2 capacity) blood will survive this acclimation period with less stress to its circulatory system than it would with a lower concentration of haemocyanin in the blood.

Chapter 4

Anaerobic Respiration

4.1 Introduction

In this study it has been shown that in the abalone the haemocyanin contributes significantly to the transport of oxygen to the tissues, both at the temperature to which the animal is acclimated and also at a higher temperature after the animal has been subjected to an abrupt temperature increase. However there are numerous examples in the literature where anaerobic metabolism has been shown to supplement aerobic metabolism in organisms subjected to stress either by relative or absolute oxygen shortage (e.g. Von Brand, 1946; Von Brand et al. 1950; Von Brand & Mehlman 1955; Blazka, 1958; Teal et al. 1967; Newell, 1970; Bennet, 1971). Moreover, for a wide range of haemocyanin bearing invertebrates species, it has been suggested that anaerobic respiration may supplement aerobic respiration at times when haemocyanin can no longer maintain the necessary oxygen supply (Johansen, 1966; Falkowski, 1973; Spoek, 1974).

Therefore, I feel that it is important to know if when the abalone are stressed as by an abrupt increase in temperature, the entire metabolism is still maintained by aerobic means, or if under such circumstances anaerobic metabolism plays a part in maintaining the metabolic demands of the animal. The importance of the contribution of haemocyanin to the maintenance of metabolism in the animal under any set of circumstances can then be judged when the overall metabolism is considered.

In this chapter then, I will consider the likelihood of anaerobic metabolism supplementing the observed aerobic metabolism under conditions of oxygen stress in the abalone.

4.2 Experimental Approach

The investigation of the possibility that anaerobic respiration might supplement aerobic respiration in the abalone during times of stress, was approached in two different ways:

1. The first approach consisted of a brief investigation to see whether the abalone demonstrated an oxygen debt response after being stressed in anaerobic conditions. It was anticipated that if such an oxygen debt response could be demonstrated in the abalone, this would be a simple, straight-forward way of determining that anaerobic respiration occurred in these animals.

2. The second, and more detailed approach consisted of an investigation of the effect of oxygen stress brought about by (a) absolute oxygen shortage (anoxia), and (b) stress on the oxygen transport system as a result of abrupt temperature increase, on the high energy phosphate store of the abalone foot muscle.

4.2.1 Oxygen debt determination

4.2.11 Method

The oxygen consumption measurements were made in the closed respirometer system with the Titron oxygen electrode, as described in Chapter 4 part I. The oxygen consumption measurements were made at the temperature at which the animals were collected and maintained in the laboratory (15°C).

The pre anaerobic oxygen consumption of an animal was measured over the range of oxygen tensions for which previous work had shown a relatively constant O_2 uptake by the three species of abalone (Chapter 3 part I). The O_2 tension in the chamber was then allowed to drop until virtually no oxygen could be detected by the Titron O_2 electrode. At this point the abalone was obviously stressed (see previous description of abalone in low O_2 tension in section 3.2.41. The abalone was allowed to remain in this low O_2 tension for one hour. After this time the

animal could no longer retain a grip on the glass wall of the respiratory chamber. The animal was then transferred to an identical chamber containing fully oxygenated water, and the oxygen consumption was again determined, over the range of O_2 tensions for which a constant O_2 uptake might be expected on the basis of previous results.

4.2.12 Results and Discussion

In Table 4.1 are the results of oxygen consumption measurements of specimens of the three species of abalone before, and after, being subjected to anerobic conditions. During the course of this study, S.J. Roper, working in the Zoology Department, Adelaide University, used the same technique to measure pre and post anaerobic oxygen consumption of two abalone species H. laevigata and H. roei. With permission his unpublished results are also included in this table (4.1).

From this table it can be seen that there is no evidence in any of the abalone species that there is an "oxygen debt" response after a period in anoxic conditions. This indicates that there is no accumulation of an end product of anaerobic metabolism in these animals.

In the subtidal abalone however, it is possible that any end product of anaerobic respiration may have been expelled into the environment. This negative result therefore does not necessarily indicate that no anaerobic respiration is taking place (although the presence of an oxygen debt would have indicated that anaerobic respiration was taking place).

As was discussed in the introduction of this chapter, the end products of anaerobic respiration in invertebrates are quite variable. Instead of attempting to identify any of the various possible end products and measure loss to the environment, another method of investigating the response of the metabolism of abalone to anoxic conditions was devised.

Table 4.1

Oxygen consumptions of abalone before, and after subjection to anoxic conditions.

Species	Animal No.	Oxygen consumption ul/gm/hr Before anoxia	After anoxia	
H. laevigata	1	33.4	30.2	
	2	31.0	25.2	
	3	36.8	39.9	
	4	31.3	32.9	
	5	38.7	35.0	*
	6	31.7	32.1	*
	Means	33.8	32.55	
	t = .52 c̄ 10 af. i.e. N.S.D.		p = <.1	
H. roei	1	36.4	33.2	
	2	32.9	32.6	
	3	33.7	39.3	
	4	39.7	45.3	
	5	19.3	18.7	*
	6	38.5	40.1	*
	Means	33.4	34.9	
	t = .30 c̄ 10 af. i.e. N.S.D.		p = <.1	
H. ruber	1	23.5	23.4	
	2	26.7	25.7	
	3	29.8	28.0	
	4	25.7	28.8	
	Means	26.4	27.7	
	t = .88 c̄ 6 af. i.e. N.S.D.		p = <.1	

* B.J. Roper, 1973.

4.2.2 Measurement of effect of anaerobiosis on the high energy phosphate store

In molluscan muscle most "high energy" phosphoryl groups are stored in the form of phosphoarginine (Virden and Watts, 1964; Watts, 1971). This can be used to phosphorylate ADP to ATP which is the ubiquitous energy source in cellular metabolism:

$$Arg\ P_{O_4} + ADP \longleftrightarrow ATP + Arg$$

$$ATP \longleftrightarrow ADP + P$$

If ATP is not regenerated either through oxidative phosphorylation or by anaerobic substrate level phosphorylations the overall result of the above reactions will be a decrease in arginine phosphate and an increase in inorganic phosphate. Measurement of inorganic and total phosphate before and after oxygen stress will therefore indicate the extent to which aerobic metabolism is able to maintain the "high energy" phosphate store. In these experiments the high phosphate store consists of phosphoarginine plus any other organic phosphates. Phosphate is released after heating at 100°C for 2 minutes - these are henceforth referred to collectively as organic phosphates.

4.2.21 Method

(a) Treatment of animals

The animals used in these experiments were collected at West Island from sea water at about 15°C, and maintained in the laboratory at this temperature.

1. "Normal" organic phosphate levels in the foot muscle of the abalone H. roei, H. ruber and H. laevigata were determined on animals removed directly from the oxygenated aquaria at 15°C.

2. Organic phosphate levels were also determined on animals which had been subjected to anaerobic conditions for 200 minutes. The specimens were sealed in small glass respirometers in which the sea water had been degassed under vacuum, and then had pure nitrogen passed through it for one hour. At the end of 200 minutes all animals showed obvious signs of respiratory stress. In some cases the animals showed no reflex muscle contraction at all when touched on the foot muscle, or near the head region. In these cases the animals were taken to be dead, and no phosphate analysis was carried out.

(b) Inorganic and organic phosphate measurement

The phosphate analysis was derived from Virden and Watt's (1964) assay for guanidine kinases. The procedure was as follows:

1. The extract for phosphate analysis was prepared by crushing approximately .5 g of abalone foot muscle tissue with acid washed sand and frozen sodium chloroacetate-perchloric acid buffer pH 2.5 in a -20°C NaCl ice bath. The homogenizer and sand were weighed immediately before the addition of the tissue, and again after crushing to obtain the exact weight of the foot muscle sample.

2. The homogenate was centrifuged at 3,200 R.P.M. at 0°C for 25 minutes.

3. The supernatant from step 2 was diluted 1:1 by the addition of perchloric acid (16 mls 60% + 34 mls H_2O).

4. The resultant solution was halved, one half boiled for 2 minutes to release the phosphate from the organic phosphates (see Virden and Watts, 1964), and the other half kept at room temperature.

5. The boiled and non-boiled extracts were diluted 10x, i.e. .1 mls of the extract were diluted by .9 mls sodium chloracetate-perchloric acid buffer pH 2.5. (This step was found to be necessary in preliminary experiments as the phosphate concentration was otherwise too high for measurement).

6. To one ml of the diluted extract, 1 ml of 2% ammonium molybolate and .1 ml of Rhodol was added. The reaction was timed with a stopwatch and the absorbance at 660 mμ was read 6 minutes after the addition of Rhodol to each tube. In fact, after this time little change in the absorbance was noted for several minutes.

7. A standard phosphate curve had been previously constructed (Appendix 3). The standards for the standard curve were prepared from a solution of 100 mgm/100 ml phosphate (King and Wooton, 1956). The same volumes and dilutions of the standards as the extract were used in all determinations.

The blank used was 1 ml sodium chloracetate-perchloric acid buffer, pH 2.5, 1 ml ammonium molybdate solution, and .1 ml Rhodol.

Inorganic phosphate was calculated from the standard curve as mg P_{O_4} /gm of tissue from the non boiled samples. Arginine phosphates

plus other organic phosphates were calculated as mg P_{O_4}/gm tissue by subtraction of non boiled samples (inorganic phosphate) from boiled samples (total phosphate).

4.2.22 Organic phosphates of Cellana ariel

As a comparison with the abalone, similar organic phosphate determinations were made on the foot muscle of Cellana ariel, which is a large intertidal limpet found at the study area where the abalone are collected. This animal has a shell which fits extremely closely to the substrate, presumably as an adaptation to enable it to survive long periods of emergence without dessication. It was considered a priori that it was probable that under these conditions the animal would have to tolerate anoxic conditions. It was decided therefore that the organic phosphate store of this other archaeogastropod under anoxic conditions would be compared to that of the abalone. This is quite an arbitrary comparison, as too little physiological work has been done on any South Australian gastropod molluscs to allow a comparison of the abalone phosphate stores with an organism known to survive anoxic conditions. As the results show however, it became evident during the course of the experiment that Cellana ariel could survive for a considerable time in anoxic conditions and still appear healthy, maintaining a strong grip on the substrate.

4.2.3 Effect of temperature change on organic phosphate

One of the factors which suggested that it was important to know what sort of role anaerobic metabolism might play in the abalone was the observed reduction in oxygen delivering capacity of the blood after an abrupt temperature increase. Although it is possible that the circulatory system "makes up" for this reduced effectiveness of the blood, it is also possible that the abalone might experience some oxygen shortage after such an abrupt temperature increase.

It was decided therefore that the effect of such a temperature change on the percentage of organic phosphate in foot muscle would be determined, in the light of the knowledge of the effect of anoxia on this parameter.

4.2.31 Method

The animals were taken from their 15°C acclimation temperature and replaced in aquaria of oxygenated sea water at 25°C. The phosphate analyses were carried out twelve hours after transfer.

The effect of both anaerobic conditions and abrupt temperature rise was also examined in a few specimens.

4.3 Results and Discussion

4.3.1 Effect of 200 minutes in anoxic conditions on the physical appearance of the animals

After 200 minutes in anoxic conditions, animals of all species showed obvious signs of physical distress. Many specimens had lost grip on the substrate, and were lying immobile on the bottom of the container.

The remaining animals retained only a weak grip on the substrate, and exhibited a typical buckled appearance of the epipodium (Fig. 3.1), with the shell hanging away from the foot. On removal of the containers animals showed little signs of life, although muscle contraction could be elicited by stimulation by touch.

4.3.2 Effect on anoxia on phosphate levels in abalone foot muscle

The results of the determinations of phosphate levels in abalone foot muscle before and after subjection to anaerobic conditions are presented in Table 4.2.

Before discussing this data further it should be noted that later analysis of variance of total phosphate levels of all animals from this, and the temperature change experiments revealed that there was no

significant difference between the species with regard to the total phosphate/gm of foot muscle ($F = 4.9$, 2 and 32 diff. - N.S.D. 5% level - Table 4.5). Unless specifically mentioned then the species will be regarded as a single population.

From Table 4.2 it can be seen that for animals taken from normal, oxygenated conditions there is a consistent difference between the total phosphate per gram of tissue before and after treatment to release organic phosphate. The calculations based on the boiled aliquots reveal a significantly higher mean phosphate content (mg p/g tissue) than those based on the non boiled aliquots (students t test $P < .1$). This indicates that a proportion of the total phosphate in the muscle sample is being stored as phosphoarginine and other organic phosphates.

As can be seen from Table 4.2 the mean % of the total phosphate stored as "high energy" phosphate is about 33%, or about one third of the total phosphate of the foot muscle. Considering the species separately the mean % stored phosphate values are 27.4%, 36.7% and 54.4% for H. roei, H. ruber, and H. laevigata respectively. (It is possible that a more effective freezing technique could have resulted in higher values for stored phosphates.) However, analysis of covariance revealed that there was no significant difference between the three species with regard to the percentage of organic phosphate under normal conditions ($F = .685$ $\bar{c}$ 2 and 3 degrees of freedom, F not significant at 5% level). Naturally, a degree of caution must be used in interpreting these results as the sample sizes are small.

After anaerobiosis for 200 minutes however, the percentage of organic phosphate has been drastically depleted. As can be seen from Table 4.2 there is now no significant difference between the amount of free phosphorus detected in the muscle before and after treatment to release phosphate from organic phosphate.

It is apparent that a period of anaerobiosis leads to a change

Table 4.2

Organic and inorganic phosphates in abalone foot muscle, before and after subjection to anoxic conditions (15°C animals)

Species	Boiled sample, i.e. Total phosphate mg p/g tissue	Nonboiled sample, i.e. Inorganic phosphate mg p/g tissue	Organic phosphate as % of Total phosphate
	Oxygenated		
H. roei	.68	.58	14.7
"	.54	.34	37.0
"	.46	.32	30.4
H. ruber	.52	.32	38.5
"	.73	.45	38.4
"	.63	.42	33.3
H. laevigata	.64	.49	23.4
"	.57	.45	21.1
"	.56	.33	41.1
"	.70	.43	38.6
"	.86	.45	47.7
means (all species)	.65	.42	35.1
	$P < .1$ (t test, $t = 5.25$ with 20 d.f.) therefore means significantly different		
	Anoxic conditions for 200 minutes		
H. roei	.44	.44	0
"	.61	.62	0 -
"	.57	.54	5.3
H. ruber	.60	.57	5
"	.78	.78	0
"	.68	.67	1
H. laevigata	.51	.50	2
"	.55	.56	0 -
"	.50	.50	0
means	.58	.58	less than 1.5% stored
	therefore no difference between means.		

in the organic/inorganic phosphate balance in the abalone foot muscle.

Phosphagens generally are considered as reserves for the quick resynthesis of ATP in, for example, working muscle (e.g. Watts, 1971); however, some workers (e.g. Zielinski, 1937; Harrison, 1965) have shown that such phosphagens can act as a phosphate store to maintain ATP levels during anoxia. It is apparent that this is what must be

occurring in the abalone foot muscle; during the 200 minutes of anaerobic stress, the organic phosphate in the abalone foot muscle is being dephosphorylated. This is virtually identical to the situation reported by Harrison (1965) in amphibian embryos subjected to anaerobic conditions. A further comparison can be drawn with Harrison's (1965) results. After 200 minutes of anoxia in both frog embryos and abalone the organic phosphate has been completely dephosphorylated. This fact, together with the abalones' evident physical distress after such a period without oxygen suggests that the dephosphorylation of the phosphoarginine is only a temporary measure to enable short term survival of anaerobic conditions.

4.3.3 Organic phosphates in Cellana ariel foot muscle

In Table 4.3 the results of the phosphate of Cellana ariel foot muscle are given. In aerobic conditions it can be seen that there is a considerable difference in the phosphate/g of tissues determined before and after treatment to hydrolyse organic phosphate. In fact the mean percentage of organic phosphate from the two determinations on Cellana ariel from oxygenated conditions indicates that about 32.1% of the phosphate is in the form of phosphoarginine and/or other organic phosphates. This is virtually identical to the value obtained for abalone from oxygenated conditions.

However, there is an obvious difference between the total phosphate present in the foot muscle of the limpet and that of the abalone. In fact, considering the mean total phosphate determined for all abalone species (.614 mg P/g tissue), and the mean total phosphate of all the limpets examined (.194 mg P/g tissue) it can be seen that the abalone have about three times as much phosphate per gram of foot muscle than the limpets. This in turn means that there is about three times as much organic phosphate per gram of foot muscle in the abalone.

Table 4.3

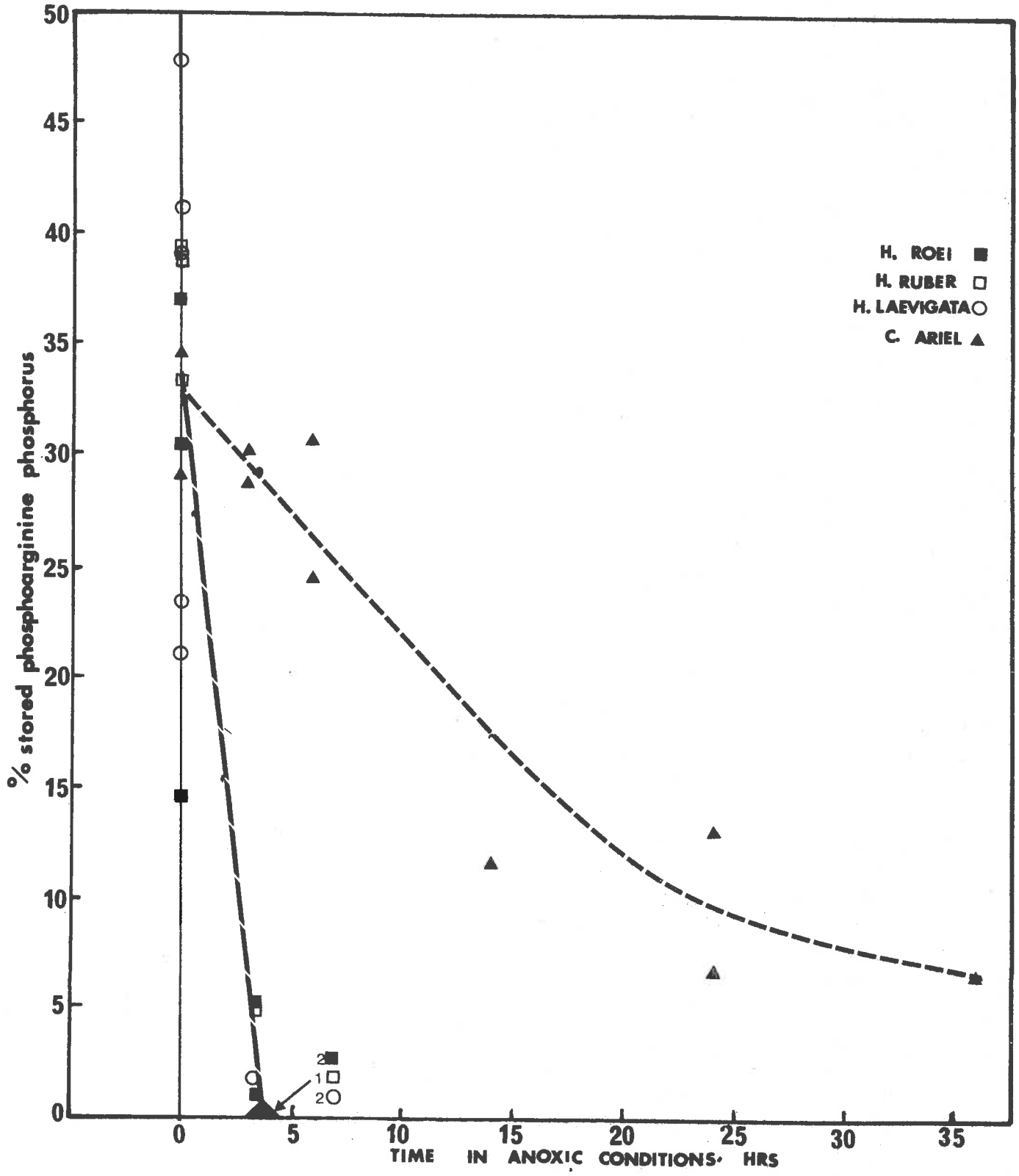
Organic and inorganic phosphate in Cellana ariel subjected to various periods of anoxia

Time in anoxic conditions (hours)	Boiled sample, i.e. Total phosphate mg P/g tissue	Nonboiled sample, i.e. Inorganic phosphate mg P/g tissue	Organic phosphate as % of Total phosphate
0	.162	.115	29
0	.185	.121	34.6
3	.162	.113	30.2
3	.264	.188	28.8
6	.185	.140	24.4
6	.163	.113	30.7
14	.232	.205	11.6
15	dead	found to have injured foot	
24	.205	.191	6.8
24	.228	.198	13
36	.212	.198	6.61
48	dead	-	-
48	dead	-	-
Animals at 60, 72 and 90 hours were dead and decomposing when removed from 'respiratory' chamber.			
9 days exposed to air	.178	.14	21.3
7 days exposed to air	.149	.116	22.1%

Despite the fact that the initial store of organic phosphate in Cellana ariel is smaller than that of the abalone species, when this intertidal limpet is subjected to anaerobic conditions, the rate at which the percentage of organic phosphate decreases is much slower than in the abalone. This can be seen from the data in Table 4.3, and is illustrated in Figure 4.1. In each of the abalone species, 200 minutes after commencement of anaerobic conditions virtually all the phosphoarginine store has disappeared; however, after three hours in anaerobic conditions the percentage of organic phosphate in Cellana ariel foot muscle has decreased relatively little.

Figure 4.1

Graph showing decrease in percentage of organic phosphate in abalone and limpet foot muscle with time in anoxic conditions.


% stored phosphoarginine phosphorus
TIME IN ANOXIC CONDITIONS· HRS
H. ROEI
H. RUBER
H. LAEVIGATA
C. ARIEL
2
1
2
means of all abalone species
plot for C. ariel fitted by eye

In fact this species of intertidal limpet was found to survive up to 36 hours in the complete absence of oxygen. Moreover it is apparent that even when the limpet is reaching the end of its survival period in anaerobic conditions it still has not "tapped" the last of its high energy phosphate store. It would seem that this species, like other facultative anerobes, must have some alternative path of producing ATP which does not depend on the phosphoarginine store alone. Nevertheless, the fact that the high energy phosphate store is reduced to less than $\frac{1}{5}$th of its original value after about 36 hours in anoxic conditions indicates that in the limpet, as in the abalone, this store plays some part in supplying energy to maintain metabolism in anerobic conditions.

It is interesting to note that after a considerable period (> 150 hours) of emergence, the phosphoarginine store of these limpets although slightly reduced compared to that in well aerated water is still much higher than after 36 hours in anoxic conditions. This would indicate that even though the limpets are tightly clamped to the substrate during emergence, some aerobic respiration is taking place.

Although this brief comparison of the abalone species with the intertidal limpet indicates that there are some similarities in the way in which the high energy phosphate store is depleted when the animals are subjected to anoxic conditions it also serves to emphasise the fact that unlike the limpet the abalone appears to lack any further mechanisms to survive prolonged anoxia.

4.3.4 Effect of abrupt temperature increase on the appearance of the animals

The animals subjected to the abrupt temperature increase from 15 to 25°C showed obvious signs of being stressed by the higher temperature twelve hours after transfer. All animals of all species had weak grips on the substrate, or in some cases had completely lost their grips on the substrate. When removed from the aquaria the foot muscles were observed to be flaccid, with little sign of life. One

specimen each of H. ruber and H. laevigata was dead after twelve hours at 25°C.

4.3.5 Effect of abrupt temperature increase on organic phosphate in abalone foot muscle

In Table 4.4 the results of the analysis to determine the amount of organic phosphate in the foot muscle of the abalone after the animals are subjected to an abrupt temperature change are given.

Table 4.5 lists the total phosphate/g tissue from all specimens of all species in all experiments (from Table 4.3 and Table 4.4).

Table 4.4

Organic and inorganic phosphate levels 12 hours after to 25°C from 15°C

Species	Boiled sample, i.e. Total phosphate mg P/g tissue	Nonboiled sample, i.e. Inorganic phosphate mg P/g tissue	Organic phosphate as % of total phosphate
	(a) Oxygenated conditions		
H. roei	.59	.50	15.3
"	.56	.53	5.4
"	.63	.57	9.5
"	.60	.53	11.7
"	.54	.38	29.6
H. ruber	.34	.84	0
"	.72	.70	2.8
"	dead	-	-
"	.67	.65	6
H. laevigata	dead	-	-
"	.74	.72	2.7
"	.75	.72	4.0
"	.49	.42	14.3
"	.38	.27	28.9
	means (all species) .63	.57	10.85%
	t = .9901 with 22 df, therefore N.S.D. P < .1		
	(b) Deoxygenated conditions		
H. roei	.47	.47	0
H. ruber	.79	.79	0
H. laevigata	.58	.57	1.7
	means .61	.61	.57 %

Table 4.5

Total phosphate/g tissue from all specimens of all species (from Table 4.3 and 4.4)

M. roei Total mg P/g tissue	*M. ruber* Total mg P/g tissue	*M. laevigata* Total mg P/g tissue
.68	.52	.64
.54	.73	.57
.46	.65	.56
.44	.60	.70
.61	.78	.86
.57	.68	.51
.59	.84	.55
.56	.72	.50
.63	.67	.74
.60	.79	.75
.54		.49
.47		.38
		.58
means .56	.70	.60

Mean of all species = .61 mg p/g tissue

Table 4.6

Analysis of variance: total phosphate/g tissue data on Table 4.5

Source of Variation	Sums of Squares (S.S.)	Degrees of Freedom (d.f.)	Mean square (M.S.)	Variance ratio (F)
Between species	.1070	2	.0535	49
Residual	.346	32	.0108	
Total	.453	34		

F = 4.9 with 2, 32 d.f., therefore H.S. at 1% level.

An analysis of variance carried out on this data revealed that at the 1% and .1% levels the null hypothesis that there was no significant difference between the means of the three species could be accepted. Table 4.6 gives the results of this analysis. As a result of this analysis, in this temperature change experiment, and in the first experiment reported in this chapter, unless otherwise stipulated all species are considered together.

The results in Table 4.4 reveal that unlike the animals analysed after living at normal acclimation temperature (Table 4.2) those animals subjected to an abrupt temperature change show very reduced organic phosphate twelve hours after initial temperature increase. In fact considering all species there was no significant difference between the mean amounts of free phosphate detectable before and after treatment to release organic phosphate (students t test - $t = .9901$ with 22 d.f. - $30 > P < 40$). An analysis of variance comparing the percentage of organic phosphate of each of the species revealed no significant difference between them ($F = 1.45$ with 2, and 9 degrees of freedom, NOT significant at 5% level).

Table 4.7, gives the results of determinations of the percentage of organic phosphate at 15°C, and after 12 hours at 25°C for all specimens, considered as a single population.

Although it must be admitted that variation at 25°C is considerably greater it is probably still safe to say that there is a significant difference between the means of the percentage of organic phosphate at the two temperatures (students t test, comparison of means of samples of unequal sizes ($t = 5.06$ with 21 d.f., $.1 < P < 1$).

In figure 4.2 for each species the organic phosphate determinations at 15°C and after 12 hours at 25°C are plotted. This figure shows clearly how in all species the organic phosphate is quite drastically depleted 12 hours after temperature increase.

Table 4.7

Organic phosphate in abalone foot muscle at 15°C, and after 12 hours at 25°C.

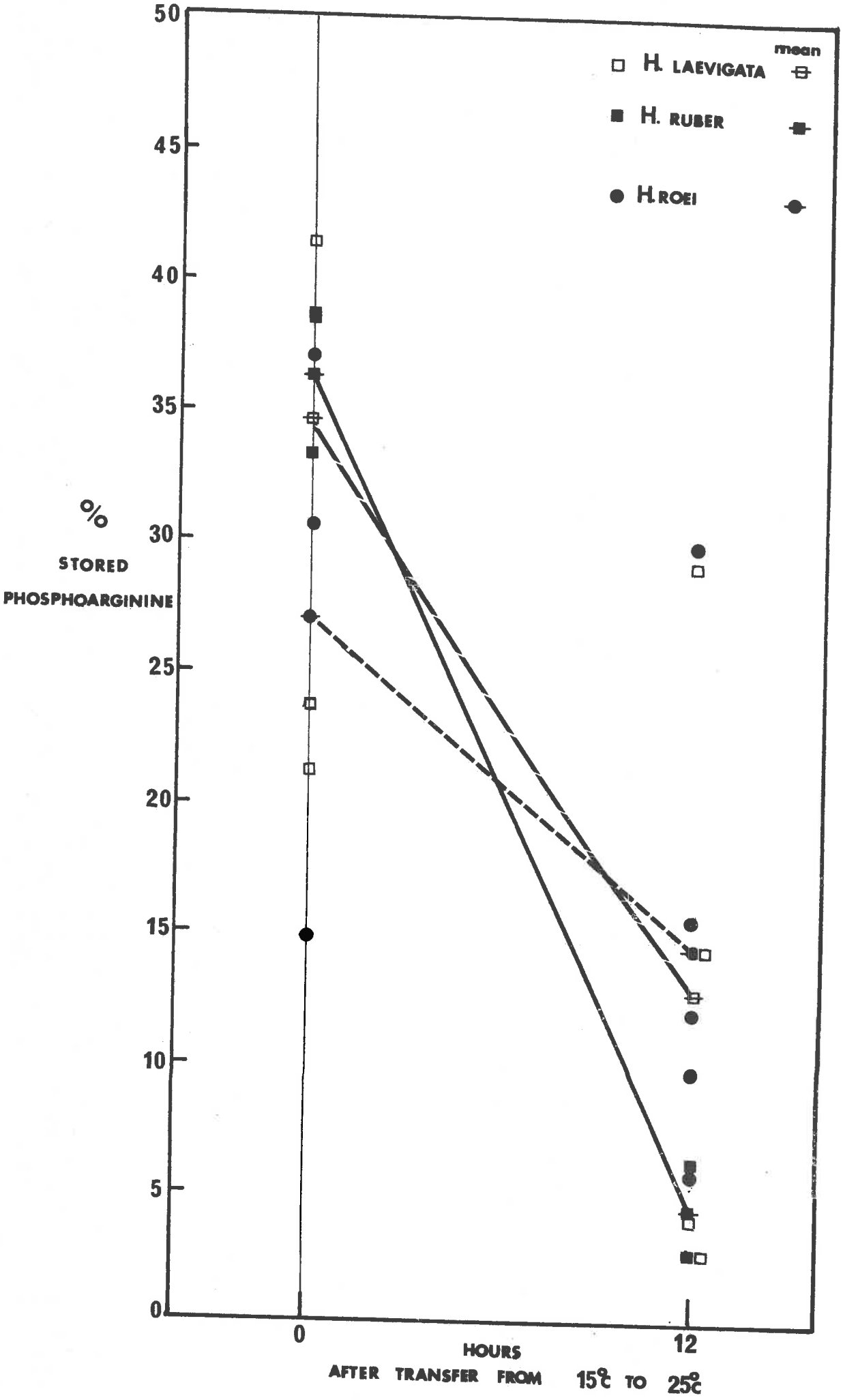
Organic phosphate as a % of total phosphate aerated conditions 15°C	Organic phosphate as a % of total phosphate aerated conditions 25°C
14.7	15.3
37.0	5.4
30.4	9.4
38.5	11.7
36.4	29.6
35.5	0
23.4	2.8
21.1	6.0
41.1	2.7
38.6	4.0
47.7	14.3
	28.9
mean 29.6	mean 10.9

At the time of sampling, and for some hours previously the animals had shown no signs of movement; it can therefore be fairly safely assumed that the lowering of the organic phosphate in the foot muscle is not a result of muscular activity. It therefore appears that the lowering of the organic phosphate in the abalone foot muscle after an abrupt temperature increase closely resembles the response to total anoxic conditions. However the depletion of the organic phosphate is neither quite so rapid nor quite as drastic as in the latter case.

I propose that the partial depletion of the organic phosphate is, in this case, brought about by the fact that with the temperature increase the respiratory and circulatory systems of the abalone cannot maintain aerobic respiration at a high enough level to completely satisfy the animal's energy needs. As the animals used in these experiments were subjected to a 10°C temperature rise it is probable that the effect is exaggerated. Nevertheless, in the A-V difference, oxygen-consumption, and heart function experiments reported earlier in this study there was evidence that even after an abrupt 5°C temperature change the oxygen

Figure 4.2

Graph showing decrease in the percentage of organic phosphates in the foot muscle of 15°C acclimated abalone, 12 hours after an abrupt temperature increase to 25°C.


50
45
40
35
30
25
20
15
10
5
0
%
STORED
PHOSPHOARGININE
H. LAEVIGATA
H. RUBER
H. ROEI
mean
0
12
HOURS
AFTER TRANSFER FROM 15°C TO 25°C

transport system was reaching a limit to its ability to supply oxygen for aerobic respiration.

It can be seen that the Q_{10} of oxygen consumption of H. ruber decreases from 20-25°C compared with that from 15-20°C (Fig. 3.13). Moreover, the cardiac output of this species is the largest of the three abalone species. It might be hypothesised then, that in this species the lowered Q_{10} of oxygen consumption from 20-25°C is to some extent brought about by the fact that the circulatory system is reaching a mechanical limit in maintaining the oxygen supply, given the in vivo characteristics of the blood. On the basis of this hypothesis, it is interesting to note that H. ruber seems to be affected most, in terms of the amount of the organic phosphate used after an abrupt temperature increase (Fig. 4.2). Conversely, H. roei whose large oxygen consumption is maintained most efficiently after an abrupt temperature increase, shows least change in organic phosphate after an increase in temperature (Fig. 4.2). However although this is apparent from figure 4.2, because the sample sizes are so small, statistically there are no differences between the organic phosphates of the three species after temperature increase and more specimens would have to be examined before the above hypothesis could be verified.

In the field H. roei in particular is likely to be exposed to abrupt temperature changes, particularly in the shallow water during hot summer weather.

Chapter 5

Variations of haemocyanin concentrations in the haemolymph of H. ruber, M. roei and H. laevigata in the field at West Island

5.1 Introduction

In several species of haemocyanin bearing arthropods it has been well documented that the concentration of haemocyanin in the haemolymph varies considerably between individuals.

Zuckerkandle (1960) described the variation of haemocyanin of the crab Maia squinado, reporting that at times some individuals showed a complete absence of haemocyanin in the haemolymph. He was able to correlate this absence of haemocyanin with stages of the moult cycle, and it has been suggested that the haemocyanin may be catabolized to maintain energy supply at this time (Djangmah, 1970).

The use of haemocyanin as an energy reserve during forced starvation has also been suggested for other arthropods - for example the woodlouse Porcellio laevis; however in this organism no change in haemocyanin concentration during the moult cycle was observed (Alikan, 1971). Horn and Kerr (1963) have presented extensive data on the serum protein and serum copper concentration (and hence by inference haemocyanin concentration) in the blood of the blue crab Callinectes sapidus. They found a considerable range (18-fold) in the haemocyanin concentrations of this species. They found that female crabs had significantly higher haemocyanin concentrations than male crabs, but these authors could offer no other explanations for the observed variation.

He investigated the concentration of haemocyanin in the blood of several species of California Haliotis, and found huge variation (65 fold). These concentrations ranged from .03 gm/100 ml to 1.89 gm/100 ml with a mean of .54 gm/100 ml, and he found that this large variation was unrelated to weight, sex, reproductive activity, nutritional state, the depth of water at which the animals were collected,

or the time of year. As has been discussed (1.1.45) Pilson questioned the compatability of such large variations in haemocyanin concentration with any of the proposed physiological functions of haemocyanin. He asked the question: why, if one animal could survive, seemingly without stress, with a given haemocyanin concentration should another animal have a much greater concentration?

Weischer (1965) also found pronounced differences between individual concentrations of copper (hence haemocyanin) in the land snail Helix pomatia. However, he was able to find that despite this large individual variation there was an overall seasonal variation in haemocyanin concentration in these animals. In this case, when the animals were hibernating the haemocyanin concentration was greatest, and when they were most active the concentration was lowest. Betzer and Pilson (1974) found a different situation in the conch Busycon canaliculatum. Again they found considerable individual variation in the haemocyanin concentration between individuals, but they were also able to discern an overall seasonal fluctuation. The haemocyanin concentration rose sharply at the time of emergence from hibernation in May and June to reach a mean maximum of about 4x the winter level. In other words, when these animals are most active their haemocyanin concentrations are highest. Betzer and Pilson emphasise that from their data, and that of workers such as Weischer, no conclusions can be drawn as to the primary function of haemocyanin in the snails. One fact which is evident from such data however, is that Ghirreti's (1966) statement that in molluscs, "during the life span of an animal the concentrations of haemocyanin seems to remain fairly constant under normal physiological conditions", is not generally correct.

In this study it has already been demonstrated that even at what appear to be extremely low haemocyanin concentrations the haemocyanin contributes the major part of the oxygen needed for respiration in the three species of abalone. Moreover, it is suggested that in abalones

and cephalopods the oxygen capacity of haemocyanin is related to the concentration of haemocyanin in the haemolymph in a way which will considerably minimize the variation in the amount of O_2 delivered to the tissues by haemolymphs containing varying concentrations of haemocyanins.

Concurrently with the physiological studies of this project, samples of blood taken from abalone in the field at various times of the year were analysed for haemocyanin concentration to determine whether or not there occurs the sort of variation recorded by Pilson (1965) for California Haliotis.

5.2 Methods and Materials

All analyses for haemocyanin concentrations were carried out on animals taken from the Abalone Cove region of West Island, South Australia (Map 2). Throughout the year, random samples of each species were examined for haemocyanin concentration of the blood. When possible approximately ten specimens of each species were examined for this parameter on each visit to the island.

The animals were collected and quickly transferred to the boat where they were placed in 12 gallon plastic bins of seawater. A blood sample was taken by inserting a 20 g needle into the region of the pedal sinus and withdrawing 1 ml of blood. The samples were stored on ice. Some of the animals were tagged by wiring Dymotape labels through the respiratory pores, or by attaching a Dymotape label to the shell using Vepox underwater putty (Vessey Chemicals Limited) (see Chapter 1, Plate 1). The animals were then returned, as nearly as possible, to the positions from which they were taken, having been exposed to the air only for a brief period in which the blood sample was removed and the tag applied to the shell. Laboratory tests indicated that animals bled in this way survived, without any ill effects, for as long as animals which were not bled. However observations in the field showed that the animals were more susceptible to predators when first returned to their home site. On one occasion when I was returning tagged

H. laevigata to their home sites, I was followed by a large stingray which wrenched the replaced abalone from the substrate and ate them. The tag and shell fragments of one abalone were seen being discarded by the predator. Subsequent checking revealed that all tagged animals replaced that dive had been taken by the predator. The affect of predator action will be discussed later in considering the results.

The blood samples collected were returned on ice to the laboratory, and analysed the next day for haemocyanin concentration in mg protein/ml haemolymph.

5.2.1 Method for determining haemocyanin concentration (mg protein/ml haemolymph)

Using the Buiret reaction, and a series of solutions of pure bovine serum albumin (B.S.A.), a standard curve of protein concentration mg/ml, against absorbance at 550 nm was constructed (Fig. 5.1). All absorbances were measured using a Perkin Elmer Model 124, double beam grating spectrophotometer. A series of serial dilutions of abalone haemolymphs in .025M Tris-HCl .5M NaCl, pH 7.25 were then made. After treatment by the Buiret reaction the protein concentrations of aliquots of these haemocyanin solutions could be read from the standard curve constructed using known concentrations of B.S.A. Further aliquots of the same haemocyanin solutions were well shaken in air to ensure oxygenation and the absorbances of the samples at 346 nm were then determined. Standard curve of absorbance of the haemocyanin solutions against protein concentration (mg/ml) were then constructed (e.g. Fig. 5.1 b). For analysis the field blood samples were centrifuged at 2000 R.P.M. in a BH.9 bench centrifuge for five minutes to remove all debris.

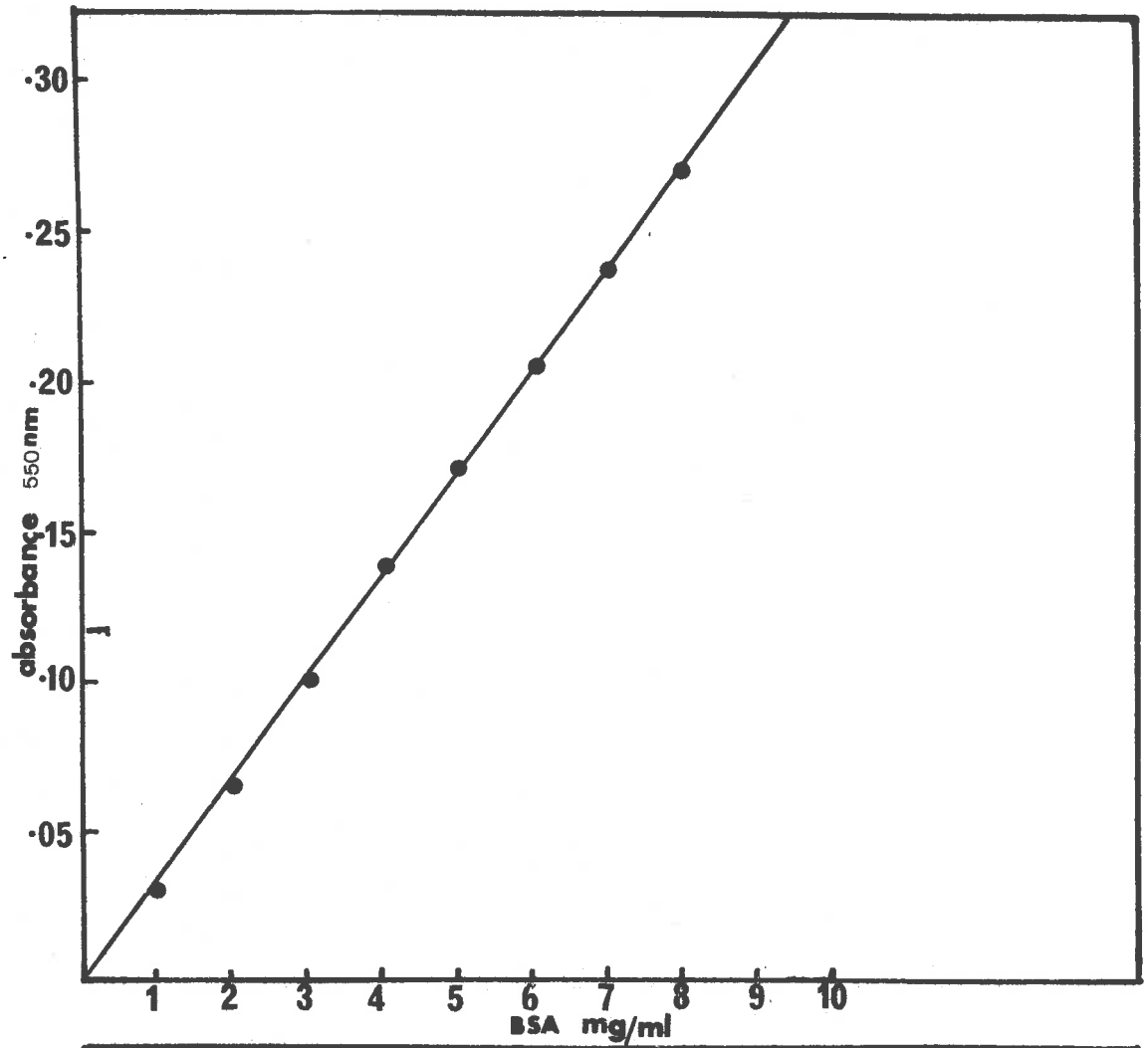
The supernatant was then diluted with .025M Tris-HCl .5M NaCl, pH 7.25. To ensure "on scale" readings the dilution ratio was usually .5 ml haemolymph: 1.5 ml buffer, but this depended on the concentration of the fresh haemolymph. After dilution the sample was shaken to

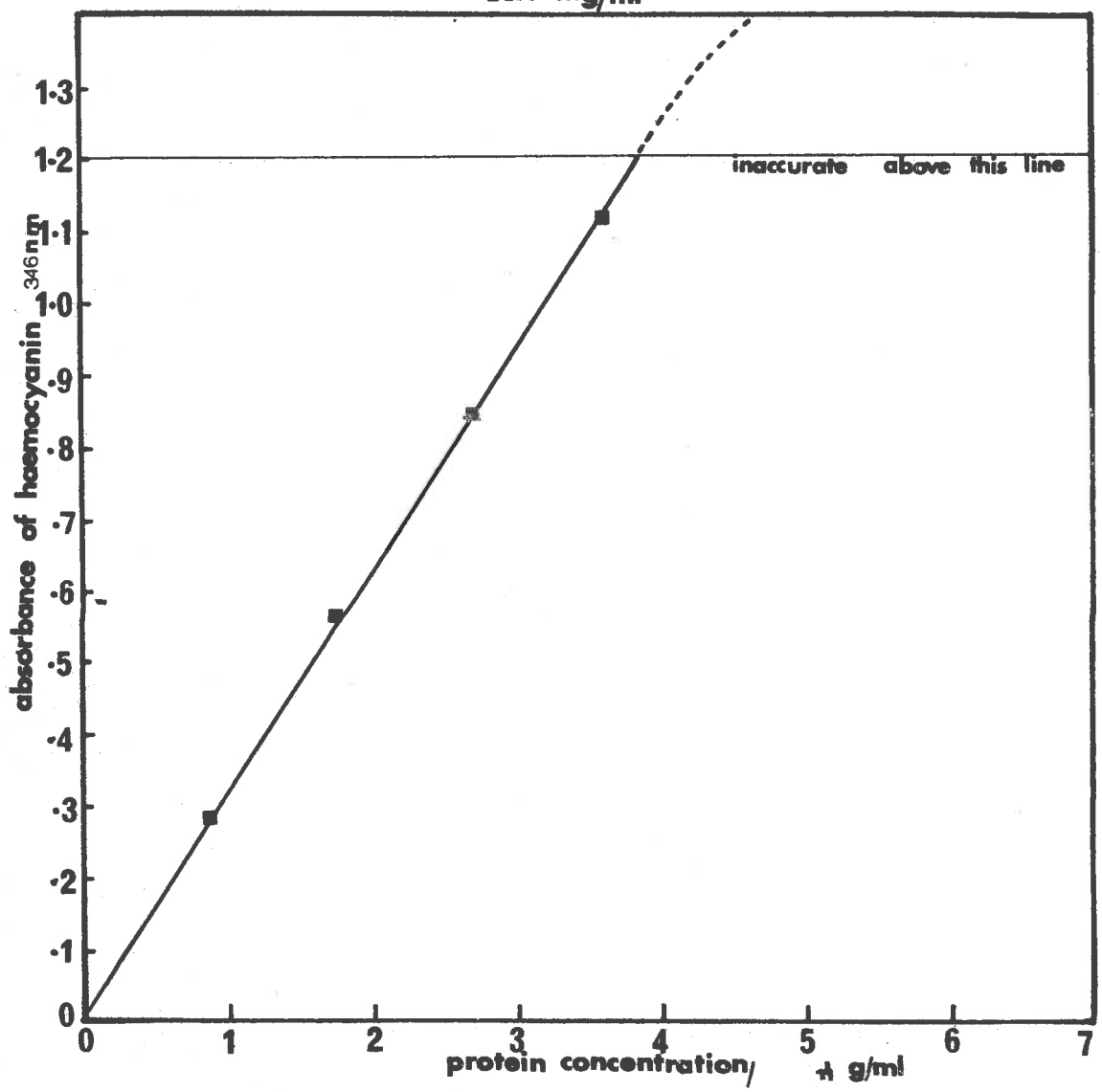
Figure 5.1 (a)

Standard curve; protein concentration (BSA mg/ml) against absorbance at 550 nm.

Figure 5.1 (b)

Standard curve: protein concentration (mg/ml) against absorbance of haemocyanin at 346 nm.


·30
·25
·20
·15
·10
·05
absorbance 550nm
1
2
3
4
5
6
7
8
9
10
BSA mg/ml


1·3
1·2
1·1
1·0
·9
·8
·7
·6
·5
·4
·3
·2
·1
0
absorbance of haemocyanin 346nm
inaccurate above this line
0
1
2
3
4
5
6
7
protein concentration g/ml

ensure oxygenation, and the absorbance at 346 nm was determined. From the standard graph Fig. 5.1 (b) the protein concentration of the sample could then be determined.

5.3 Results and Discussion

5.3.1 Seasonal samples

Table 5.1 gives the results of the analysis of haemocyanin samples taken from individuals of H. roei, H. laevigata and H. ruber in the field at West Island. These results, means ± standard errors of the means are plotted on Figure 5.2. For all samples of each species, Bartlett's test of homogeneity of variance was carried out to see whether the variances of the monthly samples could be considered homogeneous, allowing a valid analysis of variance to be carried out. In the case of H. roei there was found to be significant differences between the variances of the different monthly samples at the 1% level. However the differences were only just significant. In the cases of H. ruber and H. laevigata there were no significant differences between the variances within either of their monthly samples, although the data for H. laevigata was approaching a point $.05 < P < .1$ where care would be needed in interpreting any subsequent analysis of variance.

Analyses of variances were however carried out for the data of each species. For H. roei the F value 6.15 c 12 and 110 degrees of freedom clearly indicated that at the .1% level there is a significant difference between the means recorded throughout the year, this is not unexpected when the plots on Figure 5.2 are examined, the means of December 1973, and January 1974 being considerably lower than those about a year earlier, with no overlap of the standard errors about the means.

An analysis of variance of the data of H. laevigata also shows significant differences between the means of various monthly samples at the .1% level ($F = 6.15$, 12 and 110 df.), and again, there are significant differences between the means of the monthly samples of H. ruber at .1% level ($F = 3.24$ c 12 and 110 df.).

Table 5.1

Haemocyanin concentrations mg/ml of abalone from West Island (means ± S.E. of means)

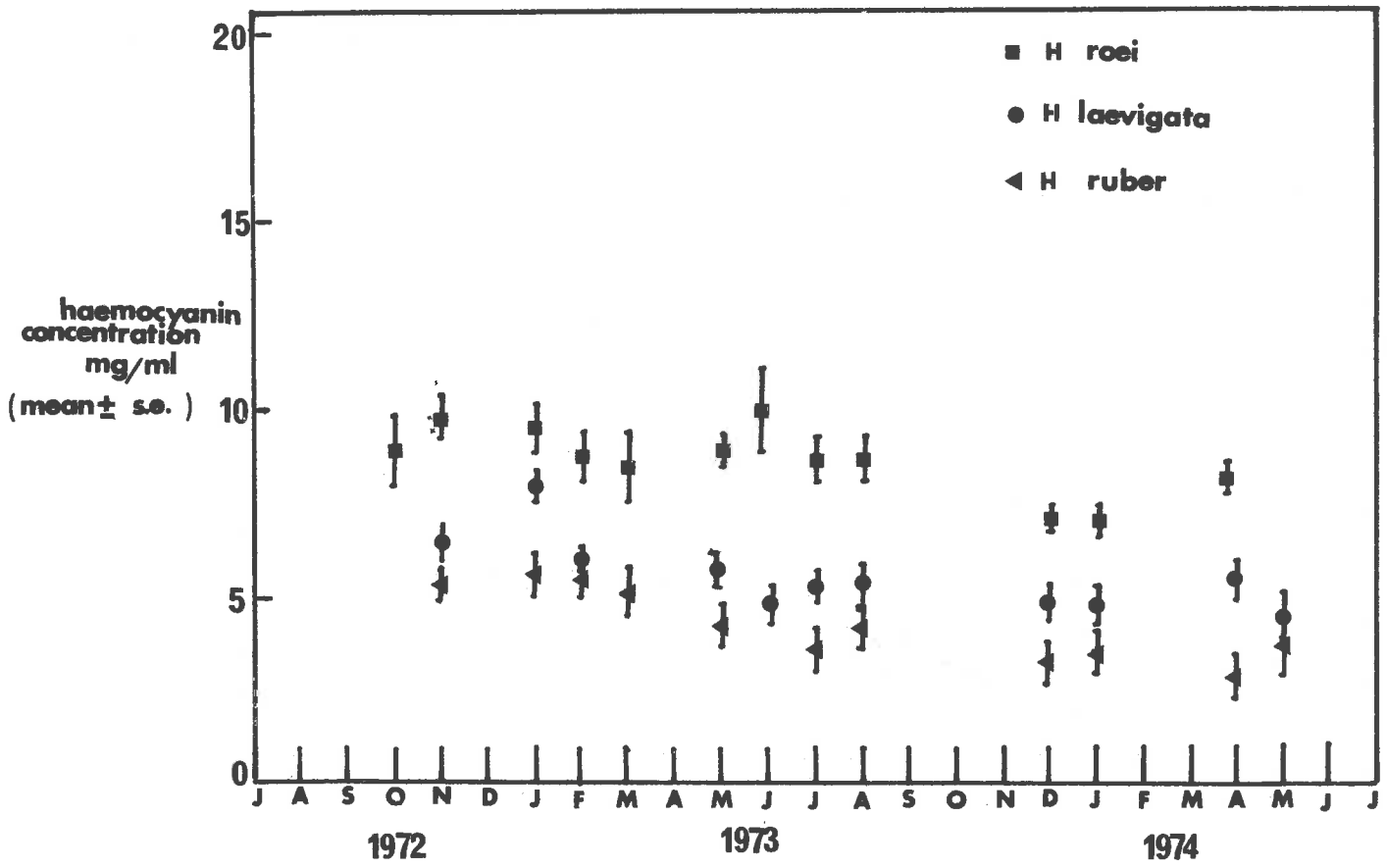
* at bottom of column for any month denotes H. roei significantly higher than H. laevigata
\+ " " " " H. roei " " H. ruber
x " " " " H. laevigata " " H. ruber

(Mann-Whitney u test - Willcoxon U statistic 5% level of significance, 95% confidence limits)
no. in brackets = sample size.

Month	July	A	S	O	N	D	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M	A	M
	1972						1973												1974				
H. roei	7.8± .73 (10)			9.0± 1.0 (10)	9.9± .62 (11)		9.6± .63 (10)	8.8± .63 (9)			9.0± .43 (9)	10.0 ±1.14 (10)	8.6± 6.0 (10)	8.7± .57 (10)				7.2± .29 (8)	7.1± .37 (9)		8.0± .32 (10)		
H. laevigata	6.8± .55 (12)				6.5± .51 (10)		8.1± .44 (10)	6.1± .24 (9)	8.5± 1.1 (8)		5.8± .38 (10)	4.8± .49 (10)	5.3± .47 (9)	5.3± .61 (7)				4.8± .54 (10)	4.8± .47 (10)		5.4± .54 (10)	4.5± .43 (8)	
H. ruber					5.5± .46 (8)		5.7± .62 (9)	5.6± .64 (7)	5.3± .63 (9)		4.3± .5 (9)	4.9± .53 (10)	3.6± .61 (10)	4.3± .54 (8)				3.3± .4 (9)	5.5± .47 (9)		2.94 ±.60 (9)	3.7± .6 (9)	
					*			*			*	*	*	*				*	*		*		
					\+		\+	\+			\+	\+	\+	\+				\+	\+		\+		
							x		x		x										x		

Figure 5.2

Haemocyanin concentrations of monthly blood samples taken from abalone at West Island. Plots are means ± S.E. of the means.


20
15
10
5
0
haemocyanin concentration mg/ml (mean ± s.e.)
H roei
H laevigata
H ruber
J A S O N D J F M A M J J A S O N D J F M A M J J
1972
1973
1974

In each species then, there are a wide range of haemocyanin concentrations for any given month. Further, at least in H. roei, these haemocyanin samples exhibit a different amount of variability in some months compared to others.

In all species the mean concentrations of the haemocyanins varied significantly throughout the sampling period. This variation is apparent in Figure 1. However, no pattern could be established in the fluctuations of the mean which could be correlated with those factors described by Shepherd (1973), Shepherd and Laws (1974) (feeding patterns, breeding seasons, seasonal temperature fluctuations etc.).

From Figure 5.2 it appears that the mean concentrations of haemocyanin samples taken from the end of 1973, and the beginning of 1974 are lower than those taken from the same period a year previously in all three species. It might be hypothesised a priori that some of the samples taken at the start of the third year could be from animals which have been disturbed by previous sampling, causing a lowered mean haemocyanin concentration through the technique of replacing sampled animals in the field. However, the serial sampling experiments reported later in this chapter indicate that previous sampling should not necessarily cause a drop in haemocyanin concentration. I can offer no explanation for this observation.

The data obtained for haemocyanin concentration of abalone in the field in this study are very similar to those obtained by Pilson (1964) during his study of California abalone. He also observed large variation in haemocyanin concentration between individuals, and could not correlate his observations with any factor. Pilson plotted total organic nitrogen/100 ml of blood against month of sample collection (Fig. 8, Pilson, 1965). His data shows the same variable characteristics as that observed in this study. The fluctuations of the mean organic nitrogen values are large and apparently random, and although no figures are given it is quite obvious from the plots that

samples taken from month to month have significantly different variances.

Ignoring the fact that the monthly samples from any given species must be considered as coming from different "populations", and considering only the wide range of haemocyanin concentrations observed for each of the three species of abalone, the following figures were obtained during this study: H. roei haemocyanin concentrations averaged .86 g/600 ml, and ranged from .36-1.51 g/100 ml; H. laevigata haemocyanin concentrations averaged .59 g/100 ml, and ranged from .24-1.42 g/100 ml; and H. ruber haemocyanin concentrations averaged .41 g/100 ml and ranged from .10-9.9 g/100ml. Again, these results compare closely with those observed by Pilson (1965) in California abalone: H. fulgens average .54 g/100 ml, range .03 to 1.89 g/100 ml; H. corrugata, average .15 g/100 ml, range .0017 to 1.53 g/100 ml; and H. cracherodii, average .38 g/100 ml and .210 to 2.03 g/100 ml. The degree of variability between the highest and lowest concentrations observed in each South Australian species was most similar to that of H. cracherodii where a ten fold range was observed. No examples of the extremely high (900 fold) variability as observed in H. corrugata was recorded in this study.

Considering the results of chapter 2 on the respiratory function of haemocyanin in the abalone, it can be seen that even at the lowest haemocyanin concentration observed in the field analyses (1 mg/ml) the haemocyanin will contribute considerably more O_2 to the tissues than will the O_2 carried in solution.

Referring to molluscs, Ghiretti (1966) stated:

"Closely related species do not present great differences in the concentration of haemocyanin in their blood".

An examination of Figure 5.2 however suggests that H. roei nearly always has a higher mean haemocyanin concentration than either H. ruber or H. laevigata from West Island. Evidence from electrophoretic and

immunological work presented in chapter 6 of this study suggests that H. laevigata, H. ruber and H. roei are relatively closely related. In order to check whether or not the apparently larger mean concentrations of H. roei were significantly greater than those of the other two species, the Mann-Whitney procedure was used to compare the three species in each month where they were sampled at the same time. This procedure was used in view of the fact that the analyses of variances had shown that none of the species could be considered a single population over the whole time period of sampling. The symbols *, +, and x presented in Table 5.1 indicate where the values of the Wilcoxon U-statistic obtained by the Mann-Whitney procedure indicated that the means of two species were significantly different at the 5% level of significance with 95% confidence limits. It can be seen that for every coinciding sample H. roei haemocyanins concentrations are significantly higher than those of H. ruber, and that in 9 of 11 coinciding monthly samples H. roei haemocyanin concentrations are significantly higher than those of H. laevigata. In the other two cases there was no significant difference between H. roei and H. laevigata. Generally then, H. roei has a significantly higher haemocyanin concentration than either of the other two species at West Island. Moreover, in four of twelve coinciding samples H. laevigata has a significantly higher haemocyanin concentration than H. ruber; whereas in no case has H. ruber a significantly higher concentration than H. laevigata.

5.3.2 Tagging Experiments

Of 70 animals which had a blood sample taken and tag fixed to the shell before being replaced in the field only 12 were successfully recovered and sampled again; three were recovered more than once. No H. roei were included in the recoveries.

Animals bled in the laboratory were observed to survive as long in captivity as those which were not bled. However, observations in the field suggested that those animals bled, and replaced in the field stood a much greater chance of being taken by predators than other animals. A Stingray was observed to select freshly bled animals (see introduction), and the crab Plagusia, known to be a predator of abalone (Shepherd, 1973) was observed on several occasions to vigorously attack freshly replaced H. roei, necessitating the immediate removal of the abalone to another site.

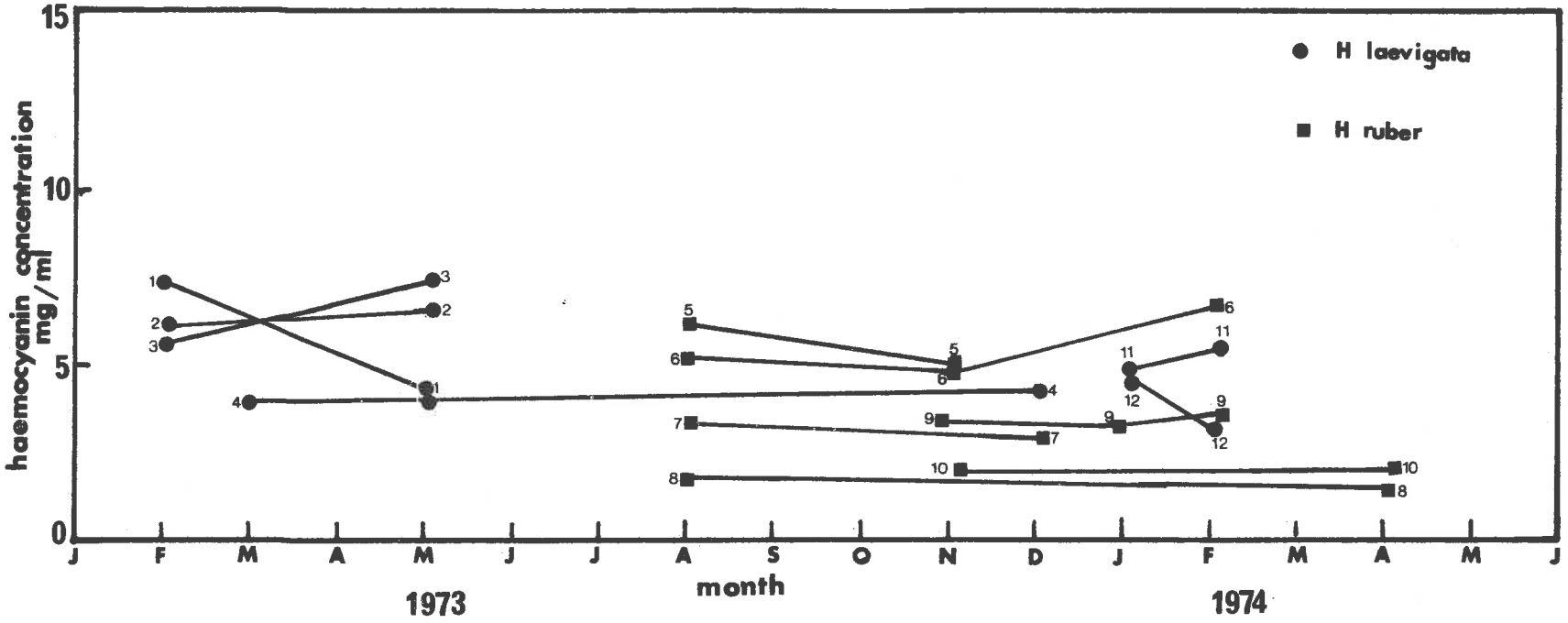
I believe it is largely the fact that predators seem actively to select the freshly bled and tagged animals which led to the relatively low recovery of tagged animals in this experiment. Figure 5.3 shows the haemocyanin concentrations of animals which were tagged, bled, and recovered and bled a second or third time. In some individuals (H. laevigata 1 and 12; H. ruber 5) the haemocyanin concentration dropped to a greater or lesser extent between samplings. In other individuals (H. laevigata 2,4; H. ruber 7,8 and 10) there was little change in the haemocyanin concentrations between samplings, and in still others (H. laevigata 3; H. ruber 6) the haemocyanin concentration increased, in the case of the last animal after having been stable for at least five months previously.

The fact that all three possible "variations" in haemocyanin concentration (fall, stable, rise) occurred suggests that the possible sampling of previously sampled animals did not bias the determinations.

It would seem then, that the haemocyanin concentration within a given individual is capable of fluctuating over a period of time. Although the observed changes in haemocyanin concentration were not always very large the evidence nevertheless suggests that Ghiretti's

Figure 5.5

Haemocyanin concentrations of animals sampled two or more times in the field.


15
10
5
0
haemocyanin concentration mg/ml
H laevigata
H ruber
J F M A M J J A S O N D J F M A M J
1973
month
1974

(1966)statement that "during the life span of an animal the concentrations of haemocyanin seems to remain fairly constant under normal physiological conditions" is not true for abalone, any more than it is for those other molluscs which have been described as having a seasonal fluctuation in haemocyanin concentration. However, in the individual sampling of the abalone, as with the population samples, the data suggest no pattern of fluctuation which might be related to other factors.

Chapter 6

Comparison of physical characteristics of haemocyanins of H. roei, H. laevigata and H. ruber

6.1 Introduction

This study is primarily involved with the description of the physiological role of haemocyanin in three species of abalone. However, in this final chapter a brief description of some of the physical properties of haemocyanins is given. The examination of these properties was carried out with two main reasons in mind:

1. Variation in physical characteristics within a species might be the basis of variation in the function of the protein, making interpretation of comparative physiological data difficult. Some variation in the physical characters of the haemocyanin of a single species has been reported in the literature. Manwell and Baker(1963) using electrophoretic techniques found both qualitative, and considerable quantitative variation in the sera of various individuals of each of the crab species Callinectes sapidus, Emerita talpoida, and Uca pugilator. It was therefore deemed necessary to determine whether such variation was likely to occur within a given abalone species at a certain locality, and also between populations of the species at different localities.

2. It was also anticipated that an examination of the physical characteristics of the haemocyanin might enable a judgement to be made about the degree of relatedness of the molecular species. A priori, it was thought that physical characteristics might be correlated with the physiological characteristics of the haemocyanins.

6.2 Purification and Identification of the Haemocyanins

Haemocyanin has been found to be by far the most important, if not the only, protein in the blood of haemocyanin bearing molluscs. In the cephalopod Octopus haemocyanin accounts for 98% of the protein in the blood, while in the gastropods Helix, and Busycon it accounts for

95%, and 90% of the protein respectively. A small percentage of low molecular weight proteins have also been found in the sera of some molluscs (Ghiretti, 1966).

The haemolymphs of the three species of abalone were therefore investigated by gel chromatography to determine if haemocyanin was the only protein present. Moreover, it was thought that it might be possible to determine if multiple forms of the haemocyanin occur at physiological pH, as was reported by Aboderin and Kareem (1971) for the land snail Achatina fulica.

6.2.1 Methods

Pure haemolymph was removed from the abalone by making a deep incision along the median line of the foot, just posterior to the head. The haemolymph welled up in this incision, and was collected in a pasteur pipette. The fresh haemolymph was then centrifuged at about 1400 x g for 10 minutes in a BHC bench centrifuge to remove cells. It was then ready for application to the gel column.

Sephadex G.75, and Sephadex G200 which have fractionation ranges of 3,000-70,000, and 5,000-800,000 daltons respectively for peptides and globular proteins were initially chosen to separate any low molecular weight impurities and small proteins respectively, from the haemocyanin.

Using Sephadex G200 Aboderin and Kareem (1971) were able to demonstrate multiple forms of the haemocyanin of the gastropod Achatina fulica. It was anticipated that this gel might demonstrate dissociation of the abalone haemocyanin.

Various eluting buffers adjusted to various pH's were used. The eluting buffers used were:

(a) .025M Tris-HCl, .5M NaCl, pH 7.25 (which was the pH found for fresh haemolymph in preliminary investigations);

(b) Boric acid - $LiOH_2$, pH 8.0; and

(c) .025M Tris-HCl, pH 8.5, the buffer used by Aboderin and Kareem (1971). The haemocyanin was also eluted through the Sephadex G200 and Sephadex G75 gels in a 0.85% physiological saline solution.

(d) The effluent from the gel was monitored at 280 nm using a flow through cell in a Perkin Elmer Model 124 double beam grating spectrophotometer with a Perkin Elmer 165 recorder. The effluent was also collected in a fraction collector and the absorbance at 346 nm was determined for each fraction. Fraction collection was commenced from the time the haemocyanin sample first penetrated the top of the gel bed. These wavelengths were chosen because protein has a maximum absorbance of about 280 nm, and haemocyanin has been shown to have a characteristic absorbance peak at about 346 nm (e.g. Nickerson and Van Holde, 1971). Absorption spectra of the samples were also determined.

Some of the pure buffered haemocyanins obtained after elution though Sephadex G200 and G75 were concentrated by pressure dialysis, and re-run through a column of Biogel A-5M, an agarose gel with a fractionation range of 100,000 to 5,000,000 daltons, in the same buffers as previously used. This column had been previously "calibrated" with diluted indian ink in 0.1% NaCl. Indian ink has a very high molecular weight, which exceeds the exclusion limit of the gel; it was therefore used to measure the void volume of the gel. 280 nm was arbitrarily chosen as the wavelength to detect this large molecule in the collected fractions.

It is known that high pH will cause most haemocyanins to dissociate (Ghiretti, 1968). In order to simply verify that this is the case with abalone haemocyanin, the haemocyanins were chromatographed on a Biogel A-5M column in .025M Tris-HCl, pH 10.25.

6.2.2 Results and Discussion

Figure 6.1 (a) demonstrates a typical chart record of gel purification of the haemocyanin of each of the three abalone species. Two peaks were obtained, both of which were evident at both 346 nm and 280 nm (latter not shown on the figure). The first peak was much larger than the second. The second peak was obviously due to a lower molecular weight substance than the first. Absorption spectra were run on the samples which constituted both peaks.

Figure 6.1 (b) shows a typical absorbtion spectra of the samples from peak I. These samples have absorbtion peaks at 280 nm, which indicates the presence of protein, and at 346 nm and 550 nm which are typical maxima for haemocyanin (cf. Redmond, 1955; Nickerson and Van Holde, 1971). As would be expected therefore, these samples showed the typical blue colour of oxygenated haemocyanin.

Figure 6.1 (c) shows a typical absorbtion spectrum run on the samples constituting the second peak. Unlike the haemocyanin which constituted the first, and much larger peak these samples do not have absorbtion peaks at 346, and 550 nm; moreover they do not show the typical protein maximum absorbance at 280 nm. The maximum absorbances recorded for this unknown substance(s) were at 320 nm, and at slightly less than 270 nm.

Subsequent attempts to identify this unknown substance(s) failed; all that can be said is that besides haemocyanin, abalone haemolymph appears to contain one or more non haemocyanin, perhaps non protein, low molecular weight substances.

As the results show (Fig. 6.1 (a)) only a single haemocyanin peak was obtained for the haemolymphs of each of the abalone species with any of the above buffers and either Sephadex G75 or G200.

It is known that gastropod haemocyanins generally are very large. For example the molecular weight of Helix pomatia haemocyanin has been estimated at 8,910,000 daltons by sedimentation techniques,

and 6,540,000 by light scattering methods (Chiretti, 1966). The difference between these two estimations (2,570,000 daltons) shows the sort of error obtained in measuring the molecular weights of such large proteins. It was possible therefore that the abalone haemocyanin may have been dissociated, but that the subunits were still too large to be impeded by the gels used, and thus appeared as a single peak. However, when the haemocyanins of each of the species were eluted through Biogel A-5M with each of the above mentioned buffers there was still no evidence of "multiple forms" of the haemocyanin such as were observed by Aboderin and Kareem (1971) for the haemocyanin of the large land gastropod Achatina at physiological pH.

Two determinations of the void volume of the Biogel A-5M column were made. They were (1) 88.45 cc and (2) 85.1 cc with an average of 86.78 cc.

The elution volumes were calculated as the volumes eluted between the time of sample penetration and the time at which the half height of the leading edge of the solute peak was reached (Biorad Laboratories, 1971).

When the haemocyanin of H. roei was eluted through the Biogel A-5M column in .025M Tris HCl, .5M NaCl, pH 7.25 the elution volumes for three determinations were found to be (1) 94.3 cc, (2) 97.4 cc, and (3) 106.0 cc with an average of 99.2 cc. From these results it would appear that the elution volume (Ve) of the haemocyanin through a column of agarose Biogel A5-M is slightly larger than the void volume of the gel, suggesting that the haemocyanin molecule is being slightly retarded in its passage through the gel. This would indicate that the haemocyanin of the abalone in this buffer adjusted to physiological pH, has a molecular weight somewhat less than 5,000,000 daltons.

When the haemocyanin was eluted with .025M Tris HCl, pH 10.0, through this column the elution volume was greatly increased to a Ve = 150 cc (average of two determinations). It is apparent then that at high pH the abalone haemocyanin is dissociating into a smaller molecule,

Figure 6.1 (a)

A typical chart record made during chromatograph of abalone blood on a Sephadex G75 column with .025M Tris HCL, .5M NaCl, pH 7.25.

Figure 6.1 (b)

Absorbance spectrum of abalone haemocyanin; absorbance peaks at 280 nm, 346 nm and 550 nm.

Figure 6.1 (c)

Absorbance spectrum of the low molecular weight "unknown" separated from the haemocyanin during chromatography.

Fig.6.1a TYPICAL CHART RECORD, PURIFICATION OF ABALONE HAEMOCYANIN — 0·025M TRIS HCl 0·5M NaCl, pH 7·25 SEPHADEX G 75

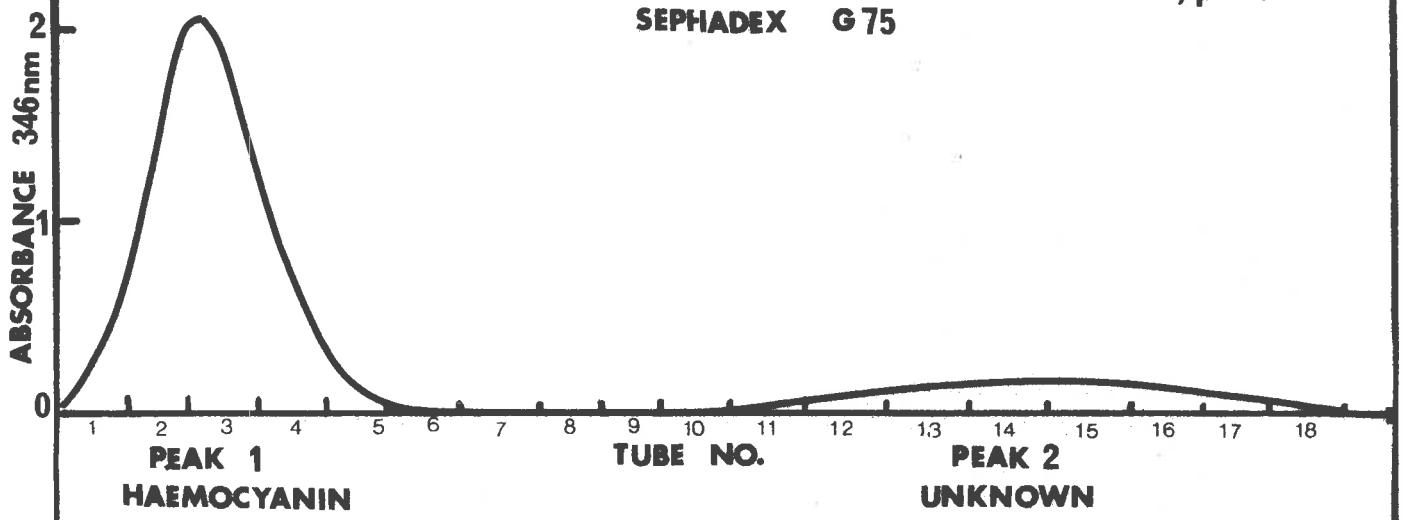
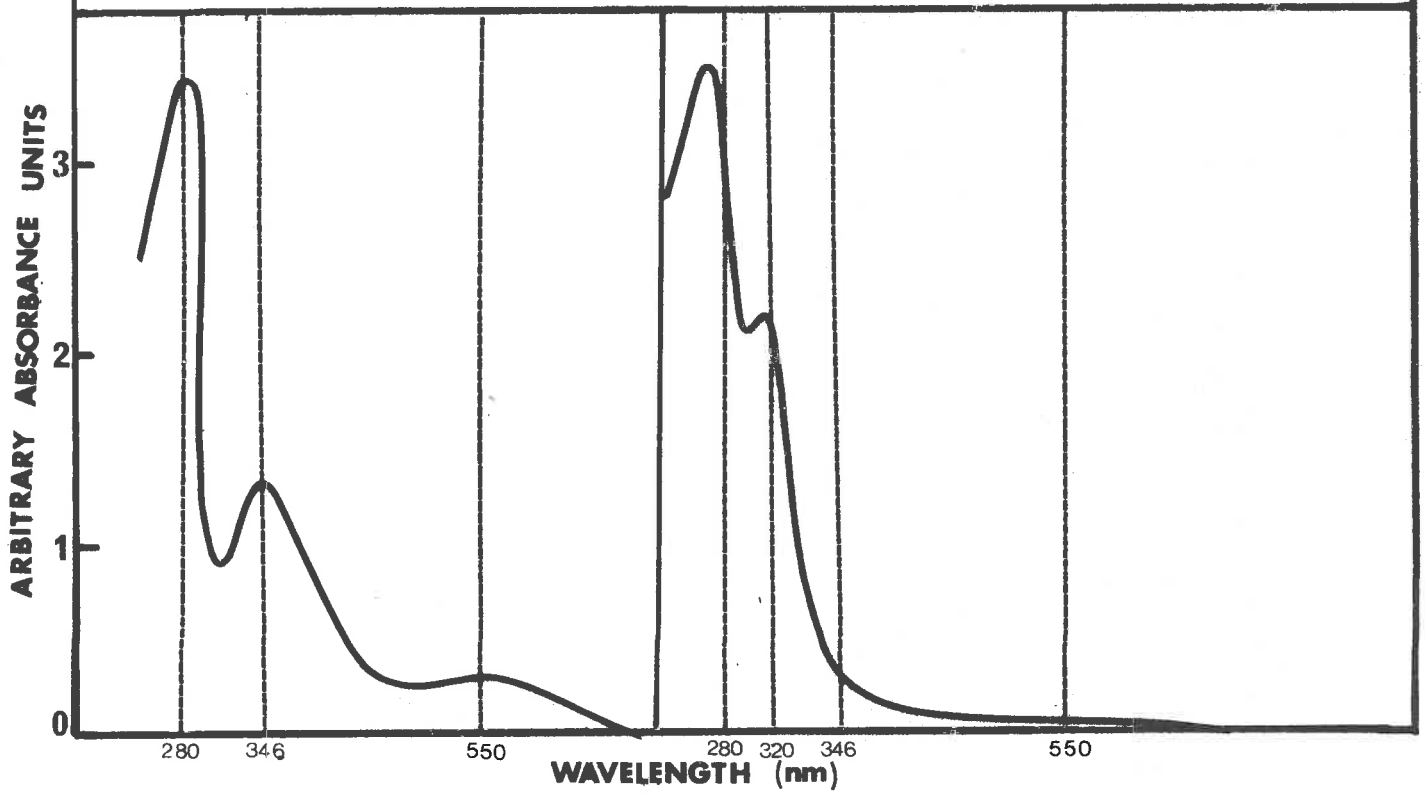


Fig.6.1b
ABSORBANCE SPECTRUM
HAEMOCYANIN

Fig.6.1c
ABSORBANCE SPECTRUM
UNKNOWN



with a subsequently slower passage through the gel column.

6.3 Starch Gel Electrophoresis of the Haemocyanins of the Three Abalone Species - Haliotis ruber, Haliotis roei, and Haliotis laevigata

Electrophoretic comparisons of blood proteins has long been used to establish relationships between and within groups of haemoglobin-bearing vertebrates (e.g. Pollitzer, 1959; Sick, 1961; Sinderman, 1963; Bertini, 1962; Gorman et al., 1966; Nalder, 1966; Newcomer, 1967; Coates, 1967; Shaughnessy, 1970). The technique has been used with more limited success with haemocyanins. Woods et al. (1958) compared the electrophoretic patterns of a wide range of invertebrate sera. They were able to demonstrate that the blood of the various species had characteristic electrophoretic patterns. They detected no individual variation within a species. Their results indicated that the haemocyanin patterns of closely related species were likely to be similar. For example, the two hermit crabs Pagurus longicarpus and Pagurus pollicaris showed very similar haemocyanin patterns, virtually indistinguishable in the Figure 1 of their paper. Again, from this figure it can be seen that the patterns of the three species of fiddler crab Uca minax, Uca pugnax and Uca pugilator are very similar. Woods et al. point out that in some cases the similarities in pattern can be extended beyond generic level to groupings of families; for example the patterns of Ozalipes and Carcinides in the family Portunidae are very similar. However, they emphasise that care must be taken in interpreting the significance of this higher order grouping, as the third Portunid examined, Callinectes "has practically no similarity to the other two" with regard to electrophoretic pattern. In fact, from figure (1) of this paper I believe it is difficult to say which of the three species most closely resembles any of the others.

Manwell and Baker (1963) electrophoretically examined the haemocyanins of several species of marine arthropods. Their results

virtually support those of Woods et al. (1959). They found species specific haemocyanin patterns for the animals they examined. However, they did detect some variation between individuals of a given species. This variation was largely quantitative, some "bands" appearing more strongly in some individuals than others. They did detect some slight qualitative differences, particularly in what they term the "dianisidine oxidase activity" (see Manwell and Baker, 1963). However, none of these differences between individuals were great enough to obscure the difference between species.

Their studies lead them to conclude that at least in the case of arthropods the "considerable species specificity of electrophoretic serum patterns holds some promise of being useful in systematic relationships...". Other workers such as Inoue et al. (1969) have examined the electrophoretic patterns of haemocyanins. Their work has supported Manwell's observations of species specific patterns of haemocyanins, without contributing in any way to the idea that similarities and/or differences in haemocyanin patterns may allow evaluations of relatedness of the species. Comparatively little has been done in the way of electrophoresis of molluscan haemocyanins from this point of view, although the technique has been used to demonstrate dissociation characteristics of particular snail haemocyanins (Elliot and Hoebeke, 1968).

In this study the haemocyanins of the three experimental abalone species H. ruber, H. roei, and H. laevigata were examined with the idea of gaining some idea of the relationship of the three protein species.

Shepherd (1973) has pointed out that there is considerable confusion in the taxonomy of the H. ruber complex, due to the variable morphological appearance of this species. Similar variability occurs in the appearance of H. roei specimens. With this in mind the haemocyanins of large numbers of H. ruber, H. roei and H. laevigata

from the main study area West Island, were examined electrophoretically, in order to see whether any polymorphisms could be detected in the haemocyanins which could perhaps be associated with recognizable morphological forms. Animals of each species from other areas were also compared to those at West Island. Electrophoretic comparisons of the two other South Australian abalone species H. emmae and H. cyclobates were made with the experimental species.

6.3.1 Method

Electrophoresis of the haemocyanin was carried out using a simplified system of starch gel electrophoresis which was based fundamentally on Smithies (1955) horizontal method, modified to improve resolution (Manwell, personal communication, 1972).

A comparatively steep voltage gradient (500 volts power supply) was used, with glass backed gel trays containing relatively thin, short gels. This decreased the running time of the gel from 8-16 hours (typical of low voltage starch gel electrophoresis) to about three hours per gel. All electrophoresis was carried out in a refrigerator in order to avoid distortion of the gels due to overheating. The Ferguson-Wallace (1961) discontinuous buffer system was used. The pH of this buffer was adjusted to 8.0, the pH of the buffers used to electrophorese haemocyanins by Manwell and Baker (1963), and Inoue et al. (1969).

It is known that highly alkaline conditions lead to the dissociation of the haemocyanins of many organisms (Elliot and Hoebeke, 1968, 1970; Gruber, 1968). Gel filtration of abalone haemocyanin in alkaline buffers have suggested that this is also true of abalone haemocyanins (section 6.2.2). It was therefore decided to run the haemocyanins of H. roei, H. ruber and H. laevigata at highly alkaline pH to see if the dissociation behaviour was the same in each case. Tris buffers have been used to demonstrate dissociation in gastropod haemocyanins (Elliot and Hoebeke, 1968, 1970), so an electrophoretic

buffer using .5M Tris-HCl, .5M NaCl, pH 10.1, was chosen as a highly alkaline buffer (Shaw and Prasad, 1970).

At the end of electrophoresis the gels were sliced horizontally. One half of the gel was stained for total protein with .3% Amido Black 10B dissolved in 1 part glacial acetic acid:5 parts methanol:5 parts distilled water. The other half of the gel was stained for haemocyanin by the method of Manwell and Baker (1963). This stain, which makes use of the dye 3.3 dimethoxy benzidine and hydrogen peroxide, is specific for haemocyanin or other suitable H_2O_2 coupled peroxidases and has been used successfully by other authors to demonstrate the presence of haemocyanin on gels (Inoue et al., 1969).

6.3.2 Results and Discussion

The number, species and locality of individuals examined for the electrophoretic mobility of their haemocyanins is as follows: 20 H. roei, 20 H. ruber, 12 H. emmae, and 20 H. laevigata from West Island, 12 H. roei, 12 H. ruber and 12 H. laevigata from Cape Jervis, 12 H. laevigata and 12 H. cyclobates from Tipara reef.

Comparisons between species and localities were made on the same gels.

H. laevigata

At this pH H. laevigata demonstrated a single haemocyanin band. This band stained with both the amido black total protein stain, and the dianisidine-H_2O_2 stain. No non haemocyanin protein was detected. Figure 6.2 (a), (b) show examples of two gels run with West Island H. laevigata haemocyanin showing the typical single haemocyanin band. The photographs are taken of the amido black stained half of the gel as the dianisidine-H_2O_2 stain gave a much lighter band. Although there was considerable variation observed in the concentration of the haemocyanin bands in various individuals from West Island, there were no differences in mobility.

Electrophoretic comparisons of haemocyanins of H. laevigata taken from Cape Jervis, Tipara reef, and West Island revealed no differences between the various localities.

H. ruber

All H. ruber from West Island had a single haemocyanin band of identical mobility on electrophoresis at pH 8.0 Fig. 6.2 (e) (f). As in the case of H. laevigata there was some variability in the concentrations of the bands. Similarly, all H. ruber from Cape Jervis had single haemocyanin bands with identical mobilities at pH 8.0. However the mobility of the haemocyanins of Cape Jervis individuals was less than that of the West Island individuals Fig. 6.2 (e). The mobility of the haemocyanins of H. ruber from West Island was identical to that of H. laevigata (Figure 6.2 (g) (h)).

H. roei

Unlike the other two experimental species H. roei consistantly had two well defined haemocyanin bands at pH 8.0 (Fig. 6.2 (c) (d)). Both bands stained with amido black total protein stain and with the dianisidine-H_2O_2 stain. This was interesting in view of the fact that only a single haemocyanin peak could be obtained using gel filtration of this haemocyanin at pH 8.0. There was no qualitative variation between individuals although differences in the concentrations of the two bands were observed (Fig. 6.2 (c) (d)). The fast band of the H. roei haemocyanin had a mobility identical to that of H. laevigata and H. ruber from West Island (Fig. 6.2 (d)).

H. cyclobates - H. emmae

Haemocyanins of individuals of the two species H. cyclobates and H. emmae were compared to the experimental species. Figure 6.2 (g) is a photograph of a gel comparing haemocyanin of H. laevigata, H. ruber and H. emmae from West Island, and H. cyclobates from Tipara reef. The mobility of H. emmae haemocyanin is identical to that of the other two

Figure 6.2

Representative starch gels showing the electrophoretic mobilities of abalone haemocyanins

gels stained for total protein

gels a - i, buffer pH 8.0

gels j and k, buffer pH 10.12

Key

R_w = West Island *H. ruber*

R_j = Cape Jervis *H. ruber*

L = *H. laevigata*

R_o = *H. roei*

E = *H. emmae*

C = *H. cyclobates*

The numbers refer to specific individual animals

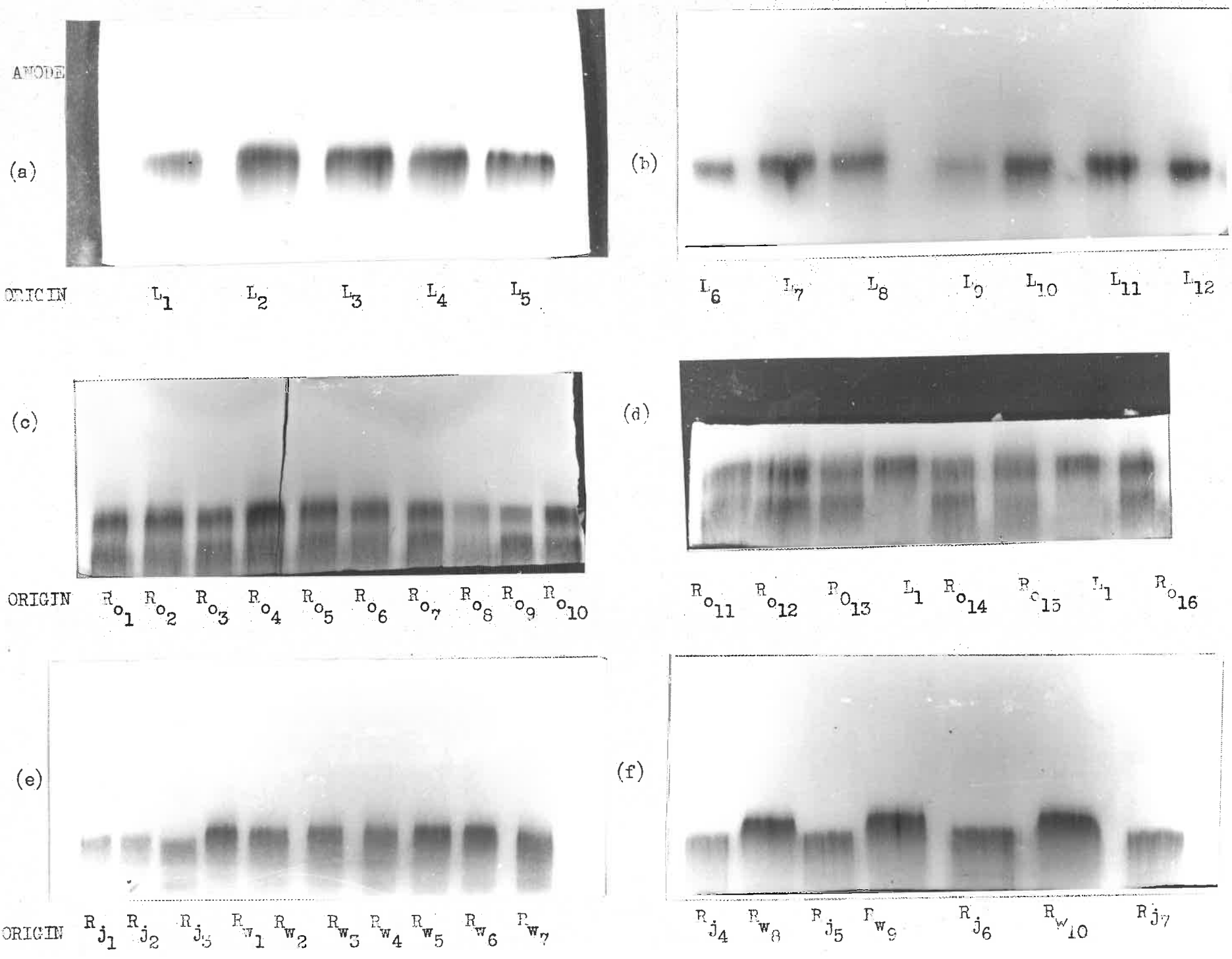

ANODE
(a)
ORIGIN
L_1 L_2 L_3 L_4 L_5
(b)
L_6 L_7 L_8 L_9 L_{10} L_{11} L_{12}
(c)
ORIGIN
R_{o_1} R_{o_2} R_{o_3} R_{o_4} R_{o_5} R_{o_6} R_{o_7} R_{o_8} R_{o_9} $R_{o_{10}}$
(d)
$R_{o_{11}}$ $R_{o_{12}}$ $R_{o_{13}}$ L_1 $R_{o_{14}}$ $R_{o_{15}}$ L_1 $R_{o_{16}}$
(e)
ORIGIN
R_{j_1} R_{j_2} R_{j_3} R_{w_1} R_{w_2} R_{w_3} R_{w_4} R_{w_5} R_{w_6} R_{w_7}
(f)
R_{j_4} R_{w_8} R_{j_5} R_{w_9} R_{j_6} $R_{w_{10}}$ R_{j_7}

Fig. 6.2

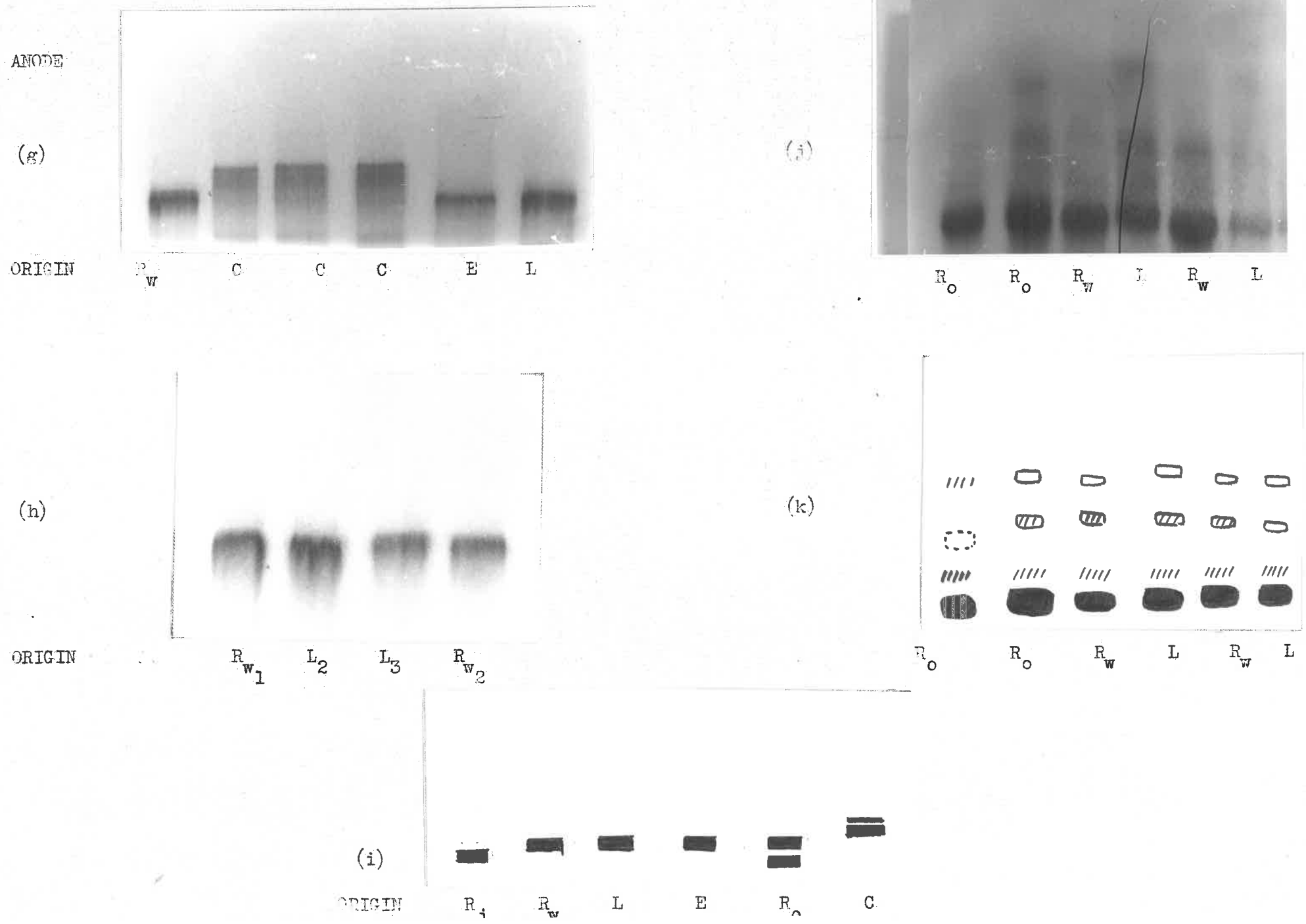
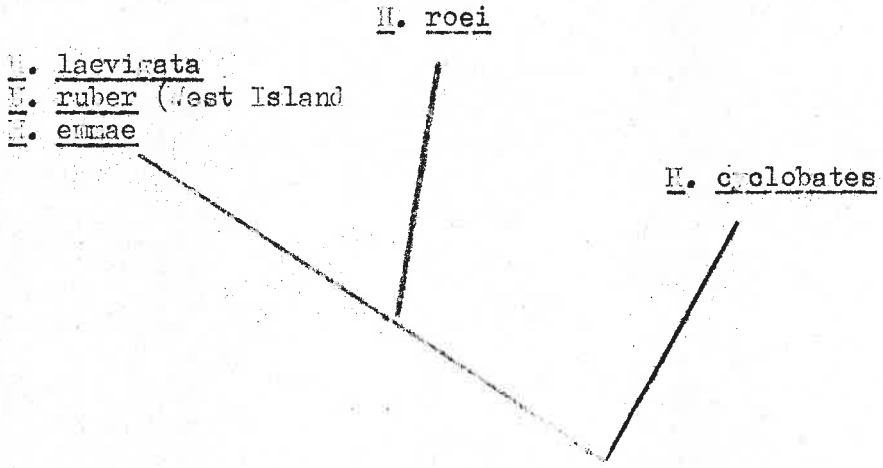


Fig. 6.2 (continued)

West Island species. H. cyclobates has a haemocyanin pattern which is quite distinct to that of any of the other four species. It has two haemocyanin bands, both of which are considerably faster than the single bands of H. ruber, H. laevigata and H. emmae, and the leading band of H. roei haemocyanin.

Figure 6.2 (i) is a composite picture comparing the results obtained for electrophoresis of the haemocyanins of the five South Australian abalone species at pH 8.0. H. laevigata, H. ruber (West Island), and H. emmae are indistinguishable; H. ruber from Cape Jervis has a slightly slower haemocyanin than the above three species, but still demonstrates only a single haemocyanin band. H. roei has two haemocyanin bands, one of which is identical to the single band of the above West Island species. H. cyclobates haemocyanin has two bands which are quite different to those of any of the other four species. Constructing a "tree" of relationship of the South Australian abalone simply on the basis of similarities/differences of the haemocyanin electrophoretic pattern (at pH 8.0) would give the following picture.



However this relationship must be recognised as only a first hypothesis, and too much emphasis cannot be placed on haemocyanin characters as a guide to evolutionary relationship without much more data; particularly in view of the fact that the mobility of H. ruber haemocyanin appears to differ in different localities. It should be noted however that in

Californian abalone, some characteristics of the haemocyanin, namely the antigenic characteristics, have been found to agree closely with predictions of relationship based on other parameters (morphology, distribution) Meyer, 1967).

On electrophoresis at pH 10.12 0.5M Tris-HCl, 0.5M NaCl H. ruber, H. laevigata and H. roei from West Island each have one major haemocyanin band with the same mobility (Fig. 6.2 (j)). In addition, in each species, there was evidence of dissociation of the haemocyanin, two to three narrow bands leading the major haemocyanin. The mobility of these leading bands was virtually indistinguishable between species, and the number visible appeared to depend on haemocyanin concentration rather than to reflect differences between species. In cases where the haemocyanin was most concentrated, judging by the density of the major haemocyanin band, the leading bands showed some activity with the dianisidine-H_2O_2 stain, indicating the presence of haemocyanin. Figure 6.2 (k) represents an interpretation of the haemocyanin pattern of the three experimental species after electrophoresis at pH 10.12 0.5M Tris-HCl, 0.5M NaCl.

6.4 Hybridisation of South Australian abalone

Introduction

Early on in this study several abalone specimens with an appearance intermediate between H. ruber and H. laevigata were reported to me by S. Shepherd (Department of S.A. Fisheries).

Owen, Mclean and Meyer (1971) offered evidence which strongly suggested that natural hybridization occurs between several species of Eastern Pacific abalones. Their evidence was based on (1) intermediate appearance of the soft parts of the animals, (2) intermediate appearance and dimensions of the shells and (3) immunological data which showed that the haemocyanins of suspected hybrids had antigenic properties of the haemocyanins of both parental species.

During collection for the physiological experiments of this project two live specimens demonstrating an appearance intermediate to H. ruber and H. laevigata were taken. These specimens were outside the "normal" limits of variation of the two large species at West Island. The morphology of soft parts, shell characteristics and immunological characteristics of the haemocyanins of the two specimens were examined.

6.4.1 Appearance and Shell Morphology

The sides of the foot were a dark olive green colour, intermediate between the characteristic lettuce green of H. laevigata and the brown-black of H. ruber. (Plate 4). The epipodia were intermediate between those of H. laevigata and H. ruber in appearance, being wider than the fine epipodium of H. laevigata but not as developed as those of H. ruber specimens of comparable size. When clamped to the substrate the cirri and narrow epipodium were just visible. The smaller specimen was immature and the sex indeterminate, but the larger specimen was a mature female with well developed eggs in the gonad. The shells both had a definite intermediate appearance, both in colour and form (Plate 4). One characteristic difference between the shells of H. laevigata and those of H. ruber is the relative size of the spires when viewed from the dorsal aspect. A series of measurement of spire length (IW) compared to total shell length (LT) were made for both H. ruber and H. laevigata specimens, and compared to these parameters on the intermediate shells (Table 6.1 Fig. 6.3). It can be seen that both shells with an intermediate appearance are also quantitatively intermediate to the shells of H. ruber and H. laevigata.

6.4.2 Immunological Evidence of Hybridization

Meyer (1967) compared the immunological characteristics of the haemocyanins of several species of California Haliotis, finding that their haemocyanins shared varying degrees of antigenic character but that each species was distinguishable from the others. Owen, Mclean and

Plate 4

'Hybrid' abalone specimen compared to parental species H. ruber and H. laevigata

Shell morphology

H. laevigata Hybrid H. ruber

Foot and epipodium

H. laevigata Hybrid H. ruber

Table 6.1

Shell measurements of West Island H. ruber, H. laevigata and "Hybrids"

	H. ruber				H. laevigata		
	LW (cm)	LT (cm)	LW/LT		LW (cm)	LT (cm)	LW/LT
(1)	5.17	12.19	2.35	(1)	4.06	12.9	3.2
(2)	3.74	8.50	2.27	(2)	2.27	8.44	3.7
(3)	4.55	9.85	2.16	(3)	1.98	6.97	3.5
(4)	3.85	8.85	2.29	(4)	2.7	8.58	3.2
(5)	4.79	10.37	2.16	(5)	2.75	8.7	3.2
(6)	4.17	9.07	2.17	(6)	3.3	10.42	3.2
(7)	2.52	6.01	2.38	(7)	3.95	12.32	3.1
(8)	3.35	8.05	2.4	(8)	3.25	9.85	3.04
(9)	3.74	8.20	2.19	(9)	1.88	6.26	3.32
(10)	3.71	8.35	2.25	(10)	4.17	12.9	3.1
(11)	3.45	8.2	2.37	(11)	2.7	10.1	3.74
(12)	3.6	8.4	2.35	(12)	2.2	9.1	4.1
(13)	4.4	10.35	2.35	(13)	2.3	8.45	3.67
(14)	5.0	11.3	2.26	(14)	1.9	7.9	4.16
(15)	3.9	9.4	2.4	(15)	2.2	7.8	3.5
(16)	3.5	8.2	2.34	(16)	3.2	10.2	3.2
(17)	2.9	8.1	2.79			mean	3.43
		mean	2.32				

"Hybrids"

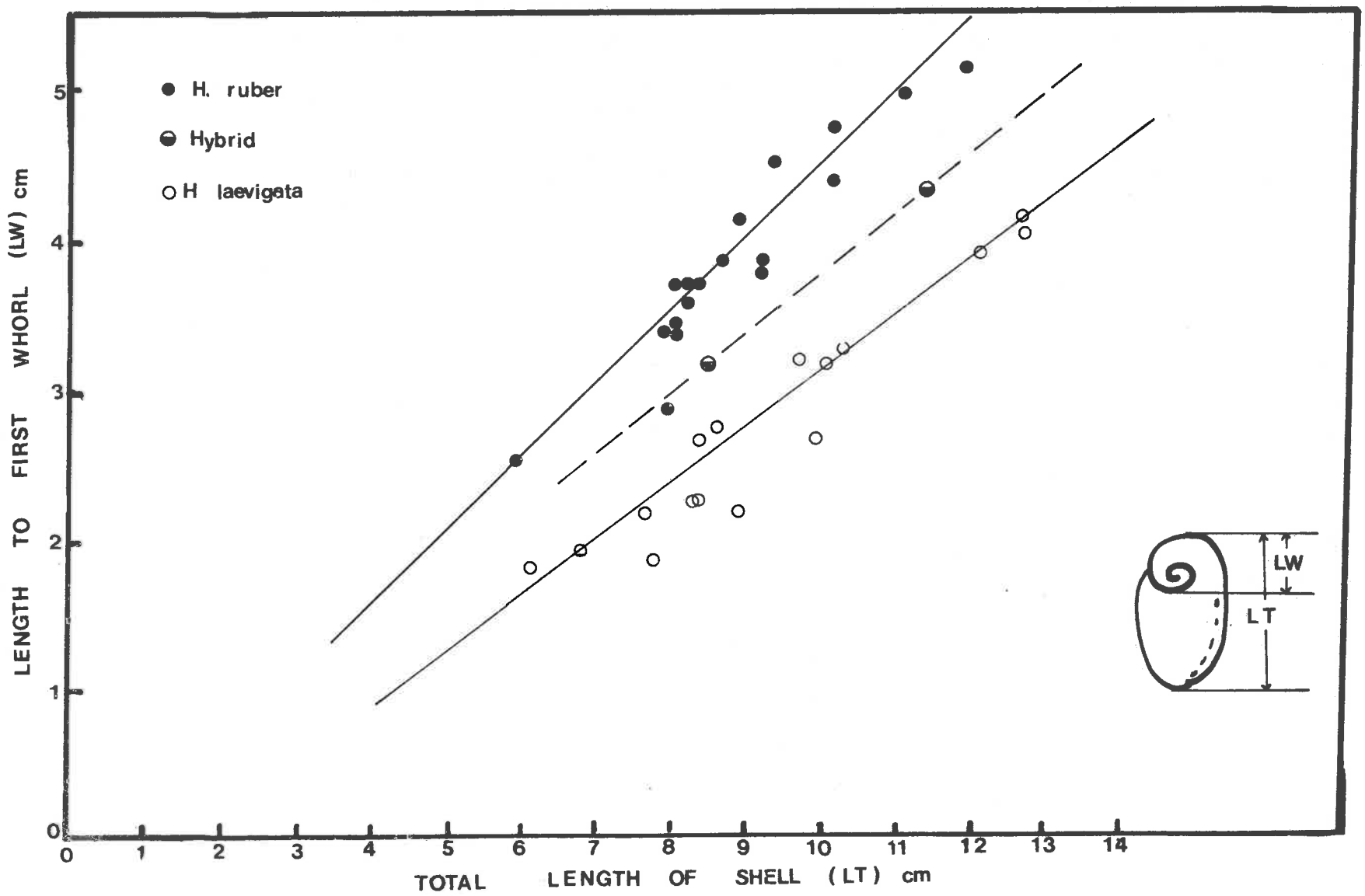
	LW (cm)	LT (cm)	LW/LT
(1)	3.2	8.56	2.5
(2)	4.34	11.8	2.7
		mean	2.6

Meyer (1971) used Meyer's (1967) techniques to demonstrate that the suspected hybrids of California Haliotis had immunological characters of both parents.

The haemocyanins of H. ruber and H. laevigata cannot be distinguished by electrophoresis. It was therefore decided to investigate the possibility that they could be distinguished immunologically, with the idea of comparing the antigenic characteristics of the haemocyanin of the two "intermediate" specimens with those of the haemocyanin of H. ruber and H. laevigata.

Figure 6.5

Shell measurements of H. ruber, H. laevigata, and hybrid abalone specimens; total shell length (LT), compared to length of the whorls (LW). Inset shows the measured parameters.


H. ruber
Hybrid
H laevigata
LENGTH TO FIRST WHORL (LW) cm
TOTAL LENGTH OF SHELL (LT) cm
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
LW
LT

6.4.3 Method

With slight variation, the techniques were the same as those described by Meyer (1967), and used by Owen et al. (1971). The haemocyanin was purified by gel filtration on Sephadex G200 in 3% NaCl, and concentrated by pressure dialysis. Meyer's regime of six 0.5 ml injections containing 10 mgm of protein in 3% NaCl were given intradermally to rabbits over a period of two weeks. The rabbits were bled one week after the final injection.

When running the Ouchterlony tests, undiluted rabbit serum containing the antibodies was used in all cases; the antigen (haemocyanin) concentration was adjusted to 10 mg/ml (see section 5.2.1 for methods used in determination of haemocyanin concentration).

The Ouchterlony tests were run in agar prepared as described by Meyer (1967), and cut with a template patterned on that used by him. Meyer had difficulty in some cases in defining his precipitin lines. In an attempt to overcome this, a thinner agar layer (1 mm thick) was used; after the precipitin lines had formed the gels were stained in Xylene Brilliant Cyanin G, dissolved in methanol:H_2O:glacial acetic acid 5:5:1. On destaining in the solvent the precipitin lines showed a bright blue.

6.4.4 Results and Discussion

When the antibody of H. ruber or H. laevigata was run directly against the haemocyanin antigen, a thick uninterpretable mass of precipitin lines was formed. This multiple nature of haemocyanin antibody-antigen precipitin lines has long been known (Hooker and Boyd, 1943; Bartel and Campbell, 1959; Tornabene and Bartel, 1962; Meyer, 1967). However, if for example H. ruber antibody was run against H. laevigata (or H. roei) haemocyanin a similar, slightly less dense mass of precipitin lines was formed. From this it can be deduced that H. ruber and H. laevigata haemocyanins share much but not all of their

antigenic characteristics. However, the precipitin lines were so complex and confused that no further information could be gained.

The absorption technique was therefore used in which antibodies of a given species were incubated with a blocking antigen, before being run against other antigens. In practice absorption was accomplished by filling the centre well (Fig. 2.4) with the chosen blocking antigen and allowing the well to go dry three times before setting up the plate with antibody in the central well, and antigens in the peripheral wells (Fig. 2.4). This technique, used by Meyer (1967), gave consistently good absorption results, and further absorption did not change the observed precipitin patterns.

In this case antibody to H. ruber haemocyanin was incubated in the central well with H. laevigata haemocyanin before being run against H. ruber and H. laevigata haemocyanin (cf. Meyer, 1967). As would be expected the shared characteristics of H. laevigata and H. ruber were blocked at the central well, and no precipitin line was formed about the H. laevigata peripheral wells. However anti-ruber haemocyanin antibodies absorbed on H. laevigata haemocyanin showed two precipitin lines when run against its own antigen. Similarly when antibodies to H. laevigata haemocyanin were incubated in the central well with H. ruber haemocyanin, no precipitin lines were formed at the peripheral wells containing H. ruber haemocyanin, but a single strong precipitin line was formed when reacted to its own antigen. Again this data points simply to the fact that H. ruber and H. laevigata haemocyanins share some, but not all of their antigenic character.

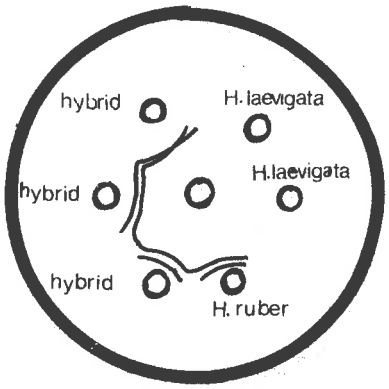
Now, when the H. laevigata antibody was absorbed on H. ruber haemocyanin, and run against H. ruber, H. laevigata and suspected hybrid antigen, the H. ruber well showed no precipitin line (as would be expected), but both the haemocyanins of the intermediate form and of H. laevigata formed a single strong precipitin line (Fig. 6.4 (b).

Figure 6.4 (a)

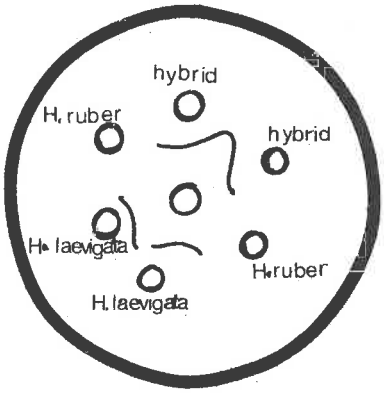
Precipitin pattern formed when Haliotis ruber, Haliotis laevigata and 'hybrid' haemocyanins are run against H. ruber antibody absorbed on H. laevigata antigen.

Figure 6.4 (b)

Precipitin pattern formed when Haliotis ruber, Haliotis laevigata and 'hybrid' haemocyanins are run against H. laevigata antibody absorbed on H. ruber antigen.



(a) H. ruber antibody absorped on H.laevigata antigen



(b) H. laevigata antibody absorped on H. ruber antigen

Conversely when the H. ruber antibody was incubated with H. laevigata antigen, the H. laevigata wells showed no precipitin line (again, as would be expected), but both the H. ruber and the intermediate forms showed two clear precipitin lines (Fig. 6.4 (a)). The result was the same for both intermediate forms. Another set of experiments were then conducted in which anti-ruber and anti-laevigata were incubated with the "intermediate" haemocyanin before being run against H. ruber, H. laevigata and intermediate haemocyanin: no precipitin lines formed in any case. It is apparent then, that as with the proposed hybrids described by Owen et al. (1971) in California abalone, the intermediate forms found at West Island have haemocyanins with the antigenic properties which are the same as a mixture of H. ruber and H. laevigata haemocyanin.

On the basis of animal morphology, shell morphology, and antigenic character of the haemocyanins it would appear that the two "intermediate" forms collected at West Island are naturally occurring hybrids, between H. ruber, and H. laevigata. A total of over 500 animals of these two species were collected for physiological studies during the course of this week. No other suspected hybrids were found. The occurrence of hybrids between H. ruber and H. laevigata at West Island is therefore extremely low, less than .5%.

No hybrids between H. roei and either of the other two species were found.

6.5 Summary

1. Haemocyanin is the major, if not the only, protein occurring in the haemolymph of the three abalone species. Unlike the haemocyanin of the large land gastropod Achatina fulica (Abderin and Kareem, 1971) the haemocyanin of the three abalone species apparently occurs in a single molecular form at physiological pH. Like other gastropod haemocyanins it is an extremely large molecule, having a molecular weight somewhat less than 5,000,000 daltons at physiological pH. Again like other gastropod haemocyanins, highly alkaline conditions

cause the abalone haemocyanin to dissociate into lower molecular weight subunits.

2. On the basis of electrophoretic comparisons of the haemocyanins of the three abalone species it is suggested that although all the haemocyanins have shared electrophoretic characteristics those of West Island H. ruber and H. laevigata are more similar to each other than either is to that of H. roei. It is suggested that in the case of H. ruber different populations have haemocyanins which demonstrate different mobilities of their haemocyanins.

3. Evidence has been presented that naturally occurring hybrids exist between H. laevigata and H. ruber at West Island. These hybrids have haemocyanins which possess the antigenic characters of both parents. The immunological evidence supports the electrophoretic data in regard to the "closeness" of the haemocyanins of these two species.

General Summary

1. The oxygen equilibrium characteristics of the haemocyanins of the three species of abalone are described, considering a range of temperature conditions and a range of CO_2 concentrations. The effect on the oxygen equilibrium curves of directly varying pH using a buffer (Redmond, 1962) is also investigated.

At any given temperature, in the absence of CO_2 the P_{50} of H. ruber haemocyanin is greater than that of H. laevigata haemocyanin which is in turn greater than that of H. roei haemocyanin. The P_{50} values of all three species change markedly with temperature. Over the investigated temperature range (7-25°C) there is little difference in the overall sensitivities of the three species' haemocyanins to temperature change; however H. roei haemocyanin is slightly less temperature sensitive than those of the other two species over the range from 7-20°C. H. roei haemocyanin is also more resistant to heat denaturation (38°C) than those of the other two species.

The haemocyanins of all three species demonstrate a marked reverse Bohr effect in the presence of increasing concentrations of CO_2. At all temperatures this reverse Bohr effect is slightly greater in H. ruber than in H. laevigata which is in turn greater than that of H. roei. Therefore, in the presence of increasing concentrations of CO_2 the observed differences in the P_{50} values of the three species rapidly diminish. In fact, at less temperatures little difference can be seen in the P_{50} values of the three species at a P_{CO_2} over about 4 mm Hg (Chapter 2, Fig. 2.4). Nevertheless, at CO_2 tensions like those in the arteries of the abalone the P_{50} of H. ruber is greater than that of H. laevigata, which in turn is greater than that of H. roei, reflecting the pattern found in the complete absence of CO_2.

Directly varying the pH of abalone haemocyanin by means of buffers also gives rise to a reverse Bohr effect in all species. Again this effect is greatest in H. ruber haemocyanin, with H. laevigata haemocyanin showing a slightly more pronounced effect than that of H. roei. However although the "buffer"experiments indicated the same general trend, comparison of results with those obtained by directly varying P_{CO_2} indicate that the buffers used to vary pH (Redmond, 1962b) cause abnormalities in the position of abalone oxygen equilibrium curves (e.g. even at physiological pH).

2. Oxygen capacities of the haemocyanins of all three species have been measured, and related to haemocyanin concentration in the haemolymphs. With increased haemocyanin concentration the oxygen capacity of the haemolymph also increased. However, unexpectedly over a wide range of haemocyanin concentrations, 1.1 m/ml - 12.9 m/ml (all species), this relationship was not strictly linear. At higher concentrations the haemocyanin was found to be binding proportionally less O_2 than lower concentrations. This fact (not without precedent - Johansen, 1965) is discussed in detail in Chapter 2 (section 2.3.2).

At the same haemocyanin concentrations, the oxygen capacities of the haemolymphs of the three species are the same. However measurements indicate that haemolymphs of H. roei specimens consistently have higher haemocyanin concentrations (hence O_2 capacities) than those of the other two species.

3. Measurements have been made of internal P_{O_2} and P_{CO_2} at arterial and venous sites, in all three species of abalone.

This data has then been related to the oxygen equilibrium characteristics of the haemocyanins, the O_2 capacities of the haemocyanins, and the dissolved O_2 content of the haemolymph to describe the O_2 delivery of the blood of the abalone both at an acclimation temperature of 20°C and also after an abrupt temperature increase from 20°C to 25°C. A detailed comparison of the in vivo function of the haemocyanins of the

three species at 20°C is given in section 2.3.42 and summarised in Table 2.16. Generally the form of O_2 exchange is very similar in all three species. In an "average" specimen of any species of abalone the haemocyanin delivers most of the O_2 to the tissues (88% in H. ruber, 90% in H. laevigata, and 91% in H. roei), with only a relatively small amount being delivered in solution. Even at very low concentrations (too low to be detected by eye) the haemocyanin delivers much more O_2 than that carried in solution.

Despite the general similarity between species, simply by virtue of its higher haemocyanin concentration - hence higher O_2 capacity, - the haemolymph of H. roei delivers considerably more oxygen to the tissues for respiratory cycle than that of the other two species at 20°C.

Because of the considerable arterial-venous gradient in P_{CO_2} in all species, the reverse Bohr effect plays a marked role in reducing the amount of O_2 unloaded to the tissues of the abalone under normal circumstances. Possible physiological advantages of this effect are discussed in section 2.3.43 in the light of knowledge of the habits and ecology of South Australian abalone.

Although after an abrupt temperature change from 20° to 25°C the oxygen equilibrium curves of all species are displaced markedly to the right, the *in vivo* P_{O_2} (and P_{CO_2}) does not immediately adjust (cf. Falkowski, 1973). The result of this is that the higher temperature, when O_2 demands are greater (see 4.) the haemocyanin in fact delivers *less*, not more, O_2 to the tissues per respiratory cycle - although still delivering over 80% of the total O_2 in each case.

This reduction in "efficiency" is greatest in H. roei where the mean arterial P_{O_2} after the abrupt temperature transfer was found to be slightly lower than the mean arterial P_{O_2} of 20°C animals. Nevertheless although after the abrupt temperature increase to 25°C the haemocyanin of H. roei only delivers 64% of the oxygen which it

delivery at 20°C, because of the greater O_2 capacity of the haemocyanin of this species the actual amount of O_2 delivered by the haemolymph is still greater than that of the other two species.

Possible reasons for the absence of an immediate adjustment of *in vivo* T_{O_2} in response to an abrupt temperature increase are discussed in section 5.3.46.

4. Oxygen consumption, and heartrate of the three abalone species have been measured under controlled conditions. The inter-relationships between these factors, and the *in vivo* function of haemocyanins has been examined, and values for cardiac output, and stroke volume have been calculated both for animals at the acclimation temperature 20°C, and for animals which have been subjected to an abrupt temperature increase to 25°C.

The active species H. roei demonstrates a higher oxygen consumption than either of the other two species at the acclimation temperature 20°C. However due to the greater delivery of oxygen to the tissues per respiratory cycle by the haemocyanin in this species, the cardiac output of H. roei is actually considerably lower than that of the other two species at acclimation temperature. The higher haemocyanin concentration in the blood of this species therefore appears to enable it to expend relatively less "circulatory energy" to maintain its greater oxygen consumption, than the other two species.

After an abrupt temperature change to 25°C, although all species demonstrate increased oxygen consumption, the increase (relative to the 20°C value) is greatest in H. roei. The A-V difference in vol % of oxygen of this species has however been shown to undergo the greatest relative reduction in response to an abrupt temperature change. Hence the relative increase in cardiac output of H. roei from 20-25°C is greater than that of either of the other two species. Nevertheless, even after the abrupt temperature change, in absolute terms the A-V difference vol %

of oxygen of H. roei is still greater than that of the other two species, and is in fact large enough to ensure that at 25°C its cardiac output is still quite comparable to those of the other species, even in the face of a considerably higher oxygen consumption.

The increase in heartrate and stroke volume necessary to maintain the increased cardiac outputs of all three species are discussed in section 3.3.16. As would be expected the greatest increases in these parameters relative to the 20°C values occur in H. roei. Nevertheless, the 25°C values for these parameters for H. roei do not differ greatly to those for the other two species, the heartrate being slightly greater, and the stroke volume being slightly smaller than those of H. laevigata and H. ruber (Table 3.2.2)

A summary of all the essential parameters measured in this study of the O_2 exchange systems of the three species of abalone is given in section 3.4. In the light of knowledge of the ecology of the species, the possible significance of the higher O_2 capacity haemolymph of H. roei is discussed.

5. The possibility of anerobic respiration supplementing (or even replacing) aerobic respiration in abalone during times of oxygen stress has been investigated.

It has been found that in completely anaerobic conditions all abalone apparently make a "last ditch stand", using up "high energy" phosphate stores to maintain metabolic processes for a limited period. It is apparent that beyond this mechanism abalone have no ability to survive prolonged periods of anoxic conditions.

An abrupt 10°C temperature increase apparently has a similar though not quite so drastic affect as complete anoxia on the high energy phosphate store. It is proposed that as a result of such a temperature rise, in the face of increased oxygen consumption, but decreased in vivo O_2 delivery by the haemocyanin, the circulatory mechanisms might not be

able to maintain the oxygen supply at a high enough level to meet the animal's metabolic demands. In such cases the dephosphorylation of high energy phosphate bonds should tide the animal over the period of adjustment to the new temperature - until *in vivo* gas tensions adjust so that the haemocyanin contributes more oxygen to the tissues per respiratory cycle.

It is proposed that this mechanism might from time to time enable *H. roei* in particular to survive large temperature increases in its shallow water habitat during calm, summer weather.

6. Regular field sampling has shown that all species of abalone have a wide range of haemocyanin concentrations at any given time. Further, samples taken at different times had different amounts of variability, and the mean concentrations of the haemocyanins of the samples varied significantly during the period of study.

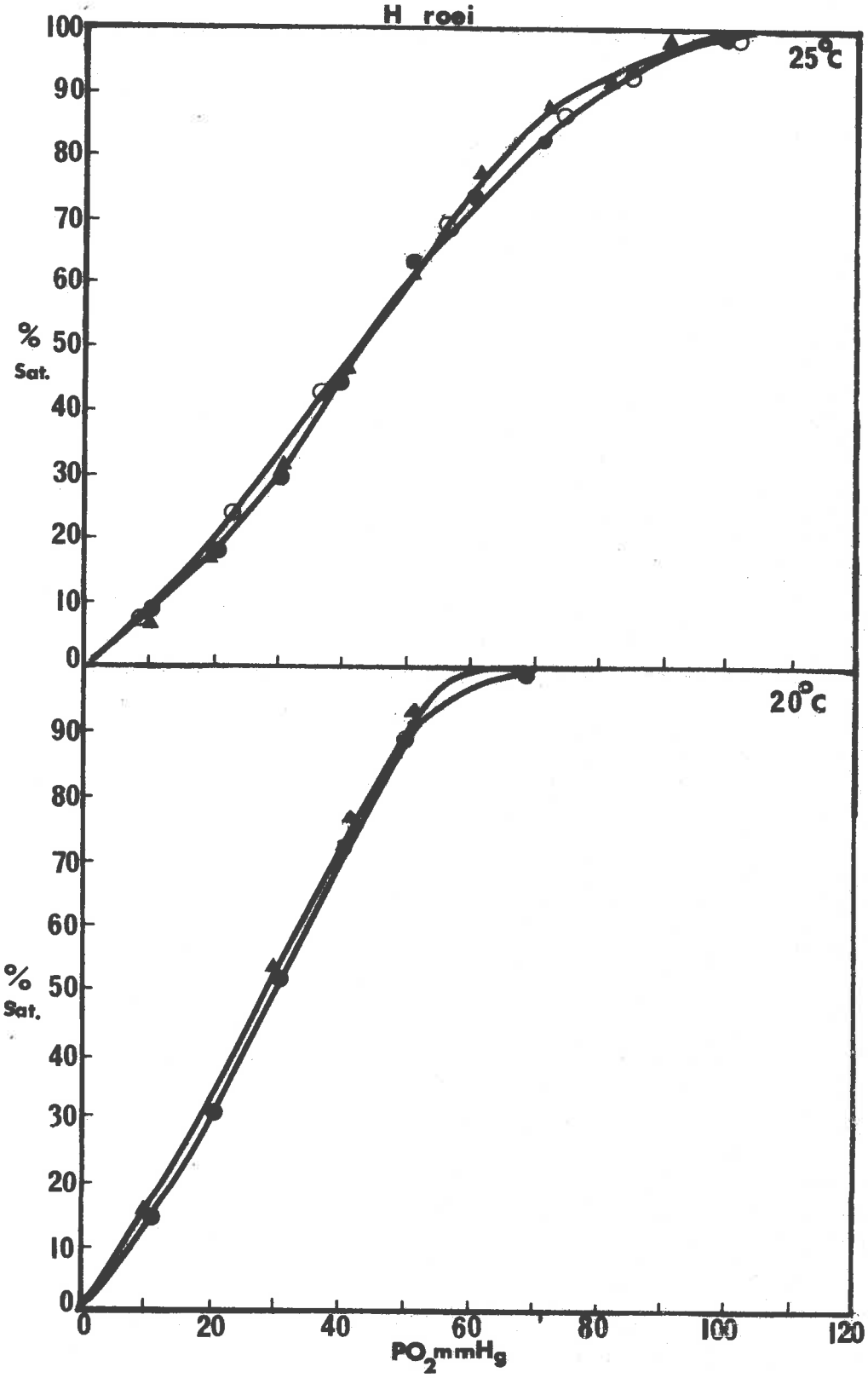
One consistent fact emerged; in the field studies as in laboratory studies *H. roei* specimens demonstrated higher haemocyanin concentrations than either of the other two species. However, considering all specimens of all species examined in the field study (350 specimens) it was found that the lowest haemocyanin concentration recorded was such that the respiratory pigment would still contribute considerably more O_2 to the tissues than that carried in solution.

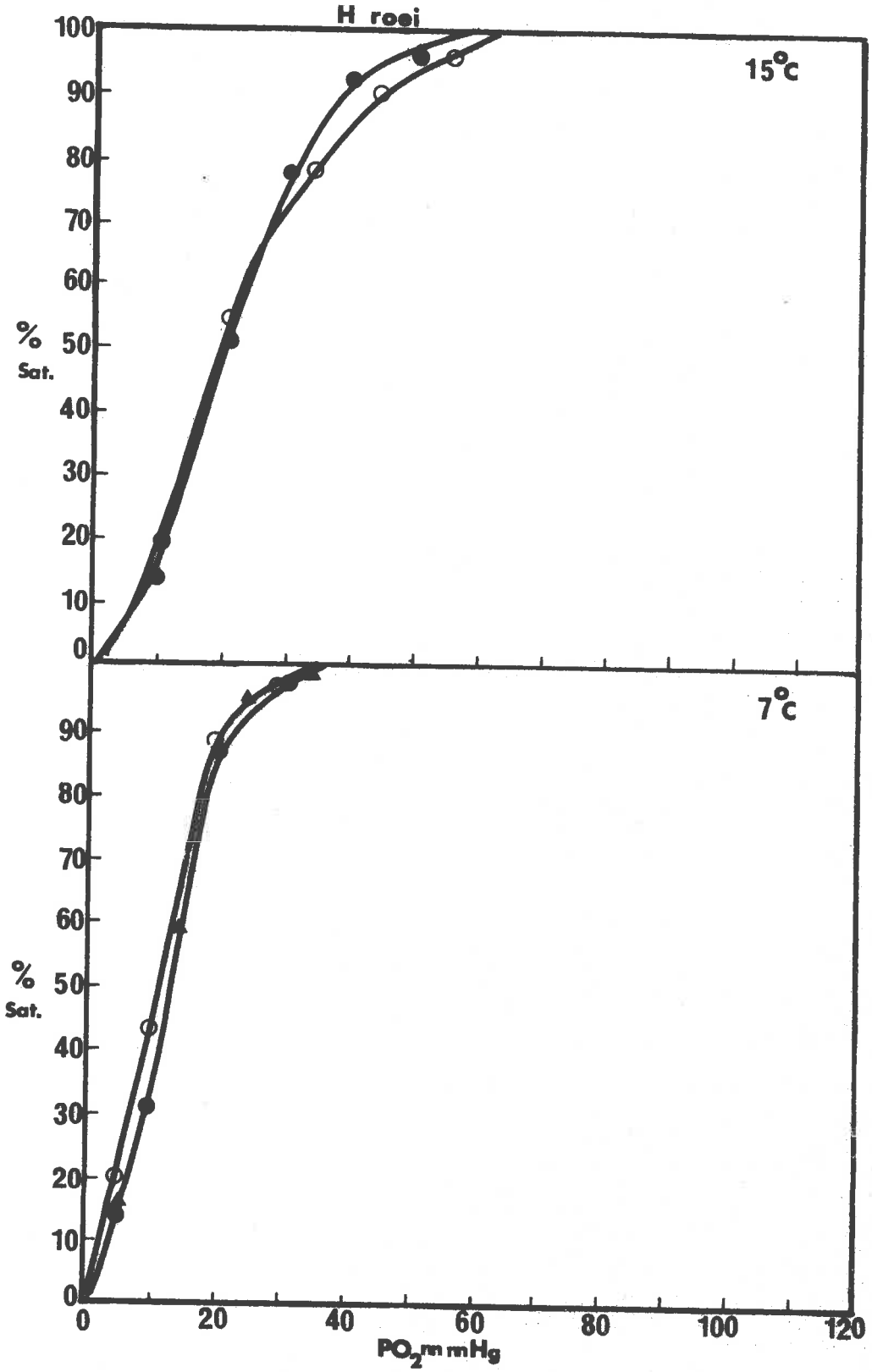
The concentration of haemocyanins in the sera of some of the animals sampled over an extended period of time showed fluctuations, while those of other specimens remained relatively steady for considerable periods. Generally the variation, and fluctuation of haemocyanin concentrations in the population samples, and the fluctuations in the haemocyanin concentrations of individual abalone over a period of time could not be correlated with any other physiological, or environmental patterns of fluctuation already described for abalone.

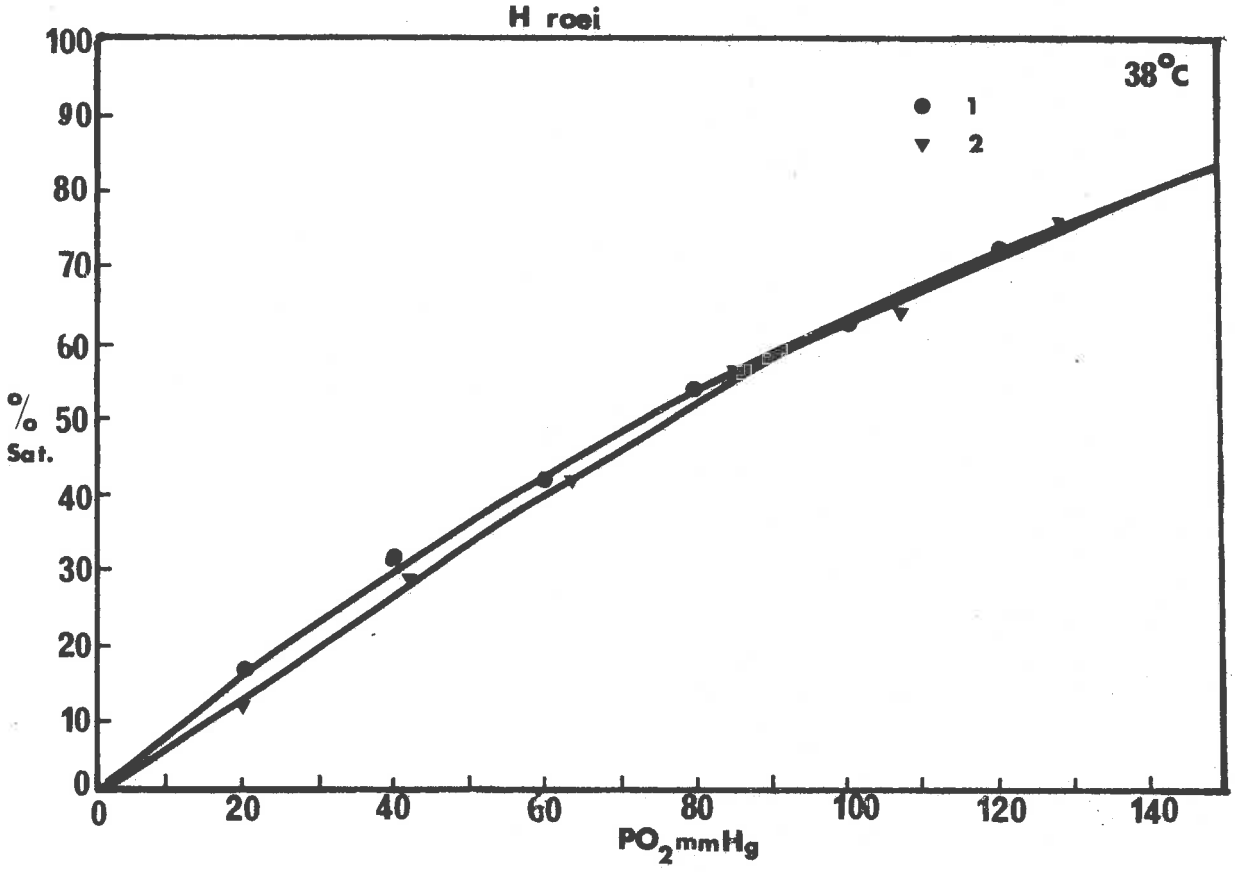
7. A brief comparison has been made of electrophoretic, and immunological characteristics of the haemocyanins of the three South Australian abalone species H. roei, H. ruber and H. laevigata. It is suggested on the basis of these comparisons that although all three species have similar haemocyanins, those of H. ruber, and H. laevigata are more similar to each other than either is to that of H. roei.

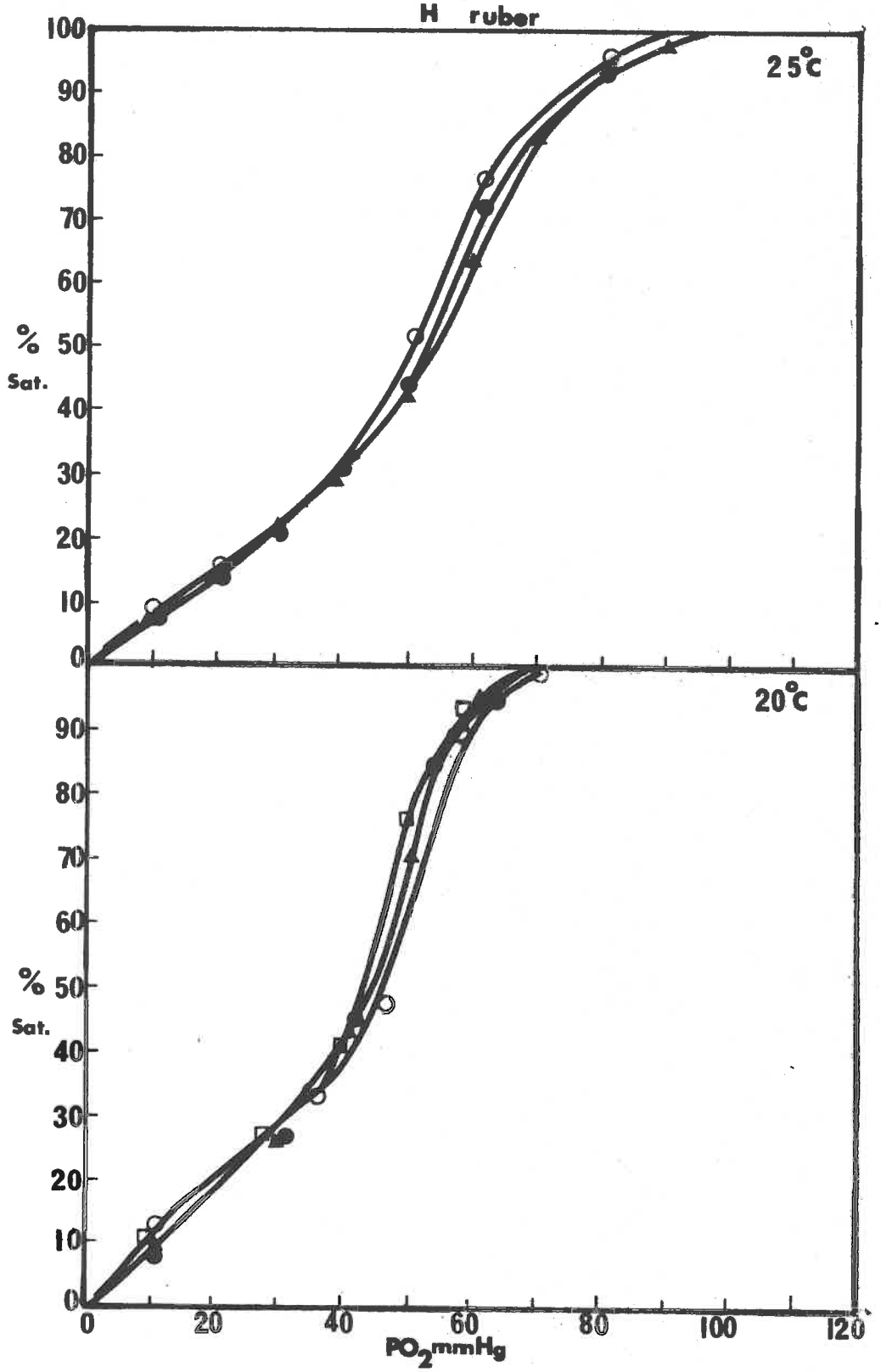
Appendix 1 (a)

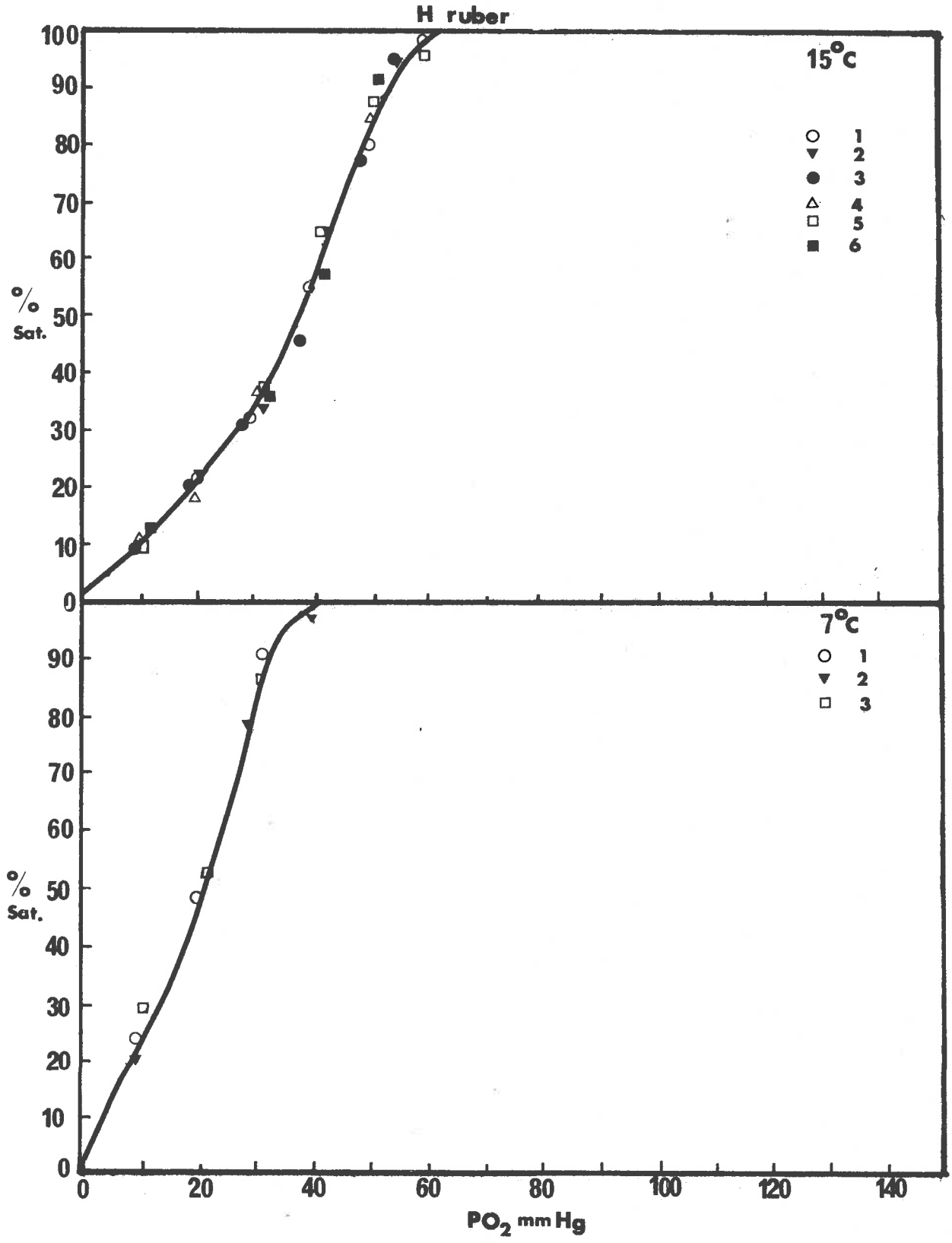
Effect of temperature on oxygen equilibrium curves of the haemocyanins of *H. roei*, *H. ruber* and *H. laevigata*.

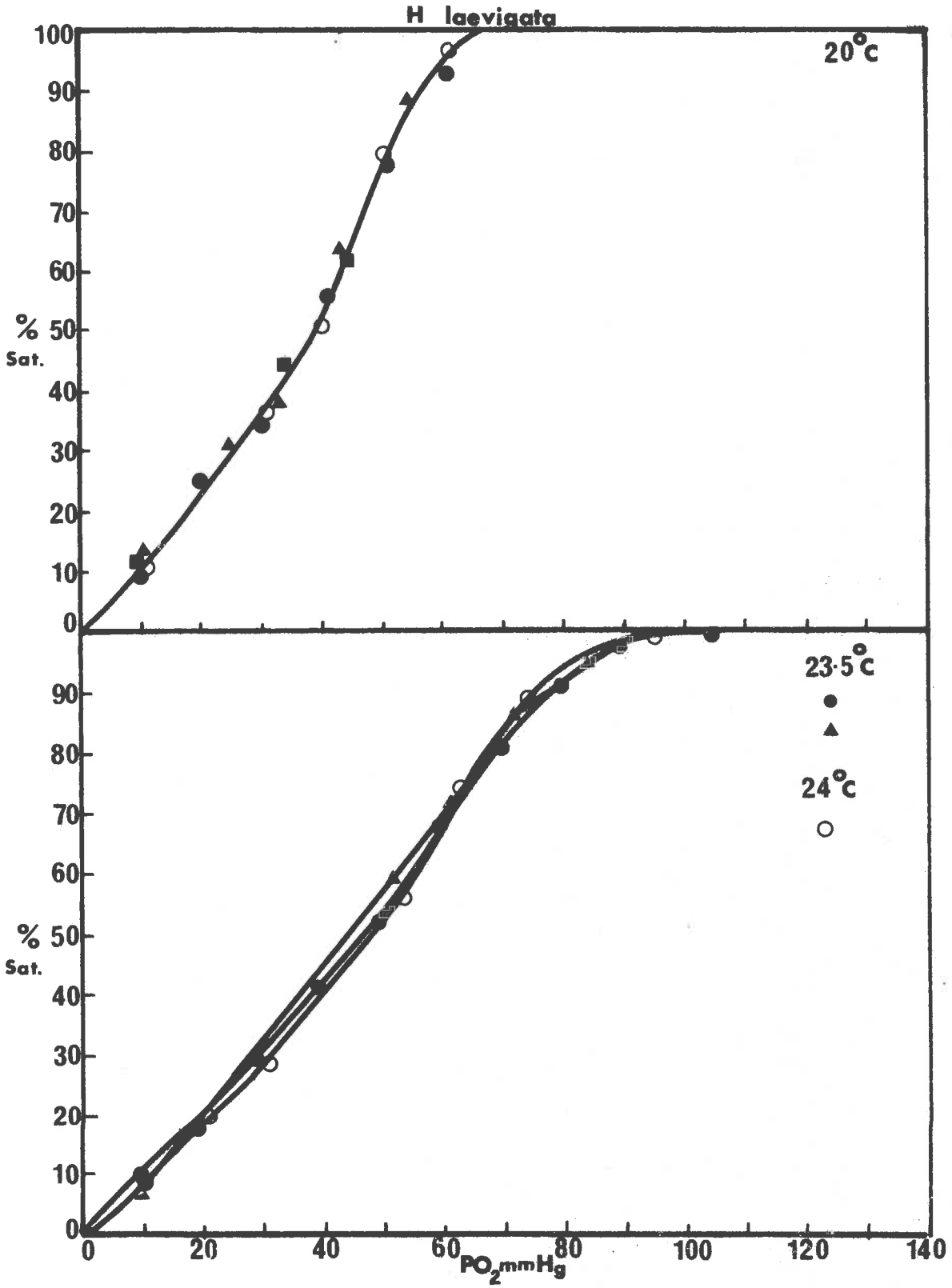

H roei
25°C
20°C
% Sat.
% Sat.
0
10
20
30
40
50
60
70
80
90
100
0
20
40
60
80
100
120
PO_2 mmHg

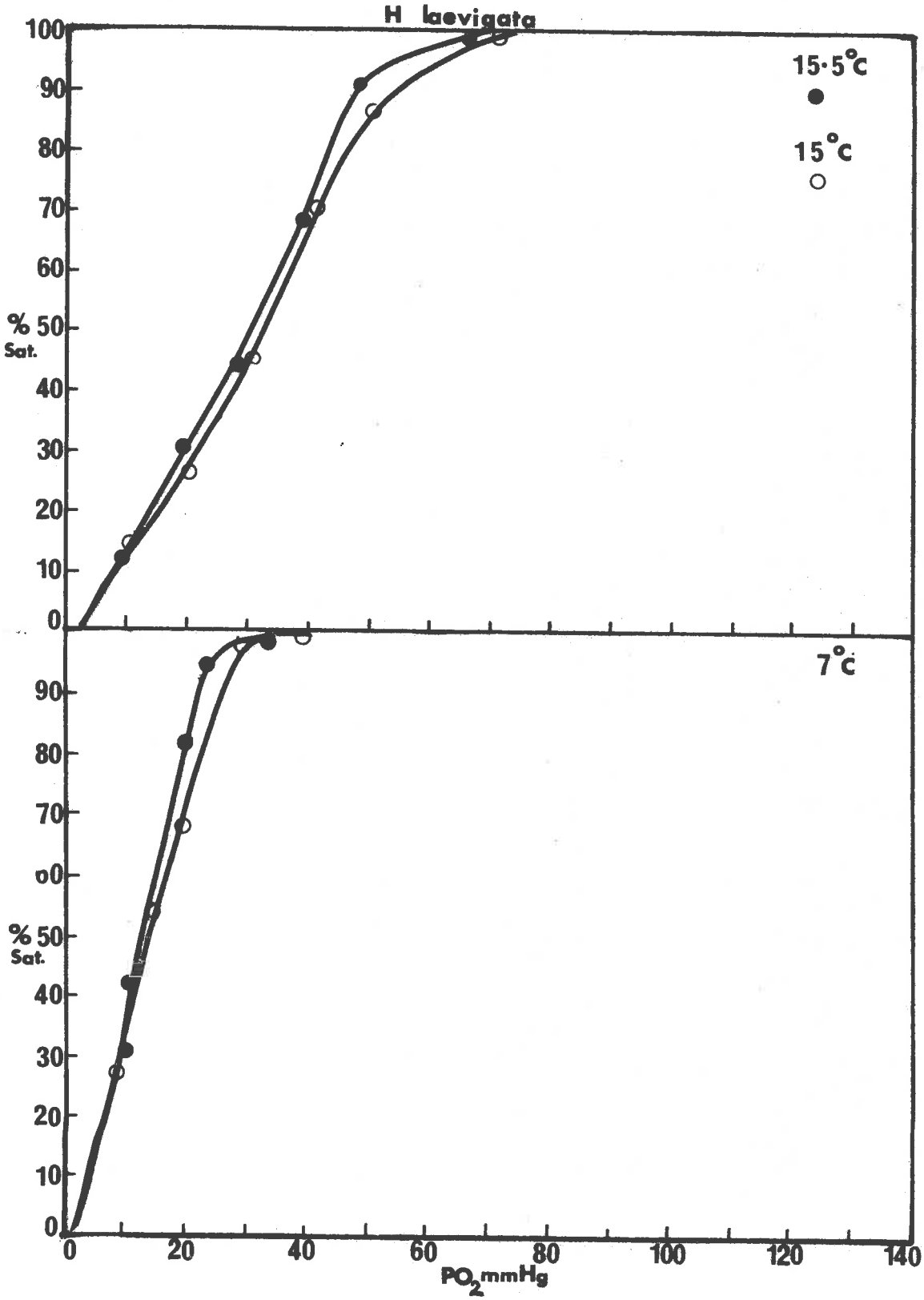

H rosi
15°c
7°c
% Sat.
% Sat.
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
120
PO2 mm mHg


H roei
38°C
1
2
% Sat.
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
120
140
PO2 mmHg


H ruber
25°C
20°C
% Sat.
% Sat.
0
10
20
30
40
50
60
70
80
90
100
0
20
40
60
80
100
120
PO2 mmHg

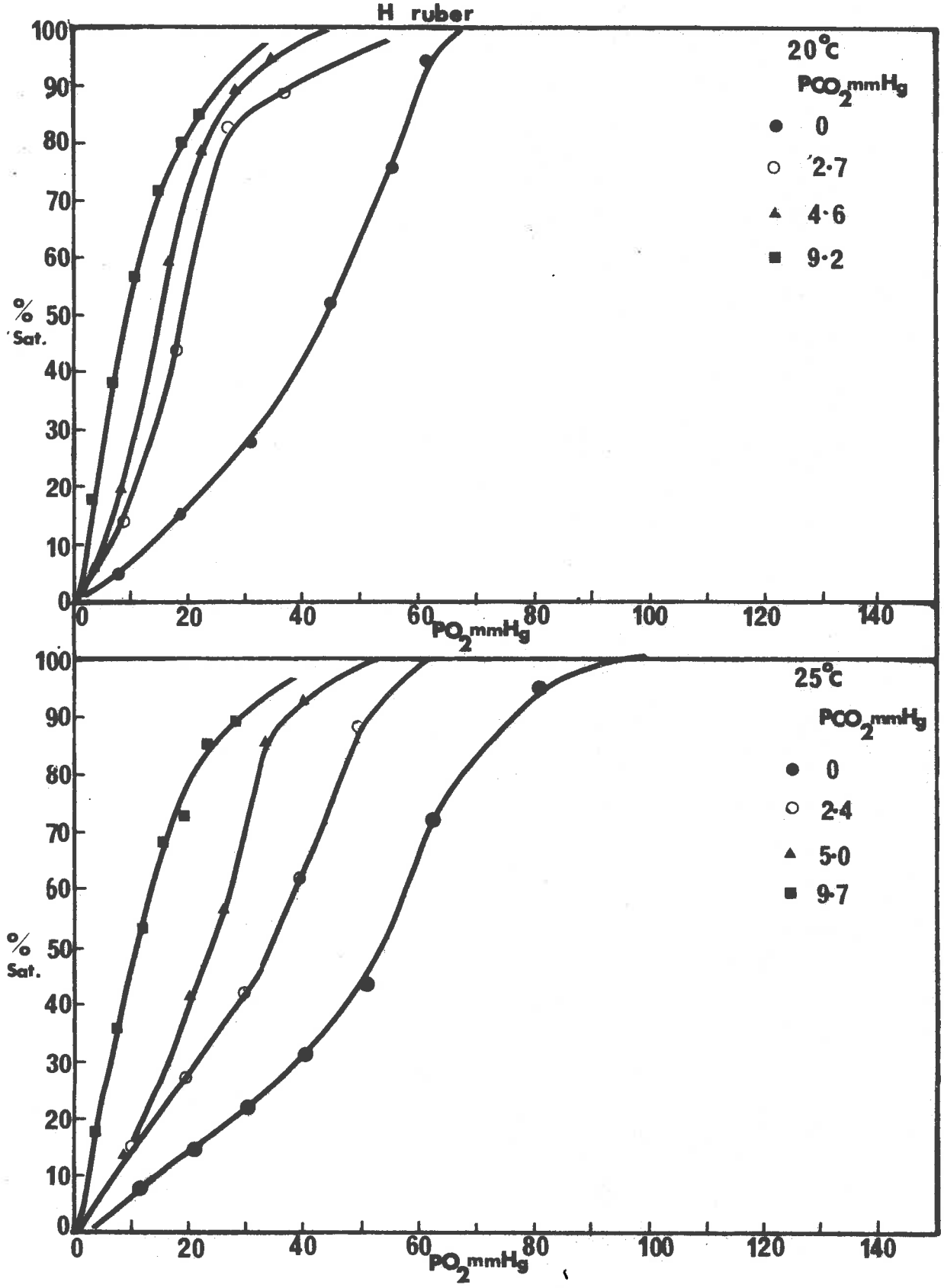

H ruber
15°C
1
2
3
4
5
6
% Sat.
7°C
1
2
3
% Sat.
0
10
20
30
40
50
60
70
80
90
100
0
20
40
60
80
100
120
140
PO2 mm Hg

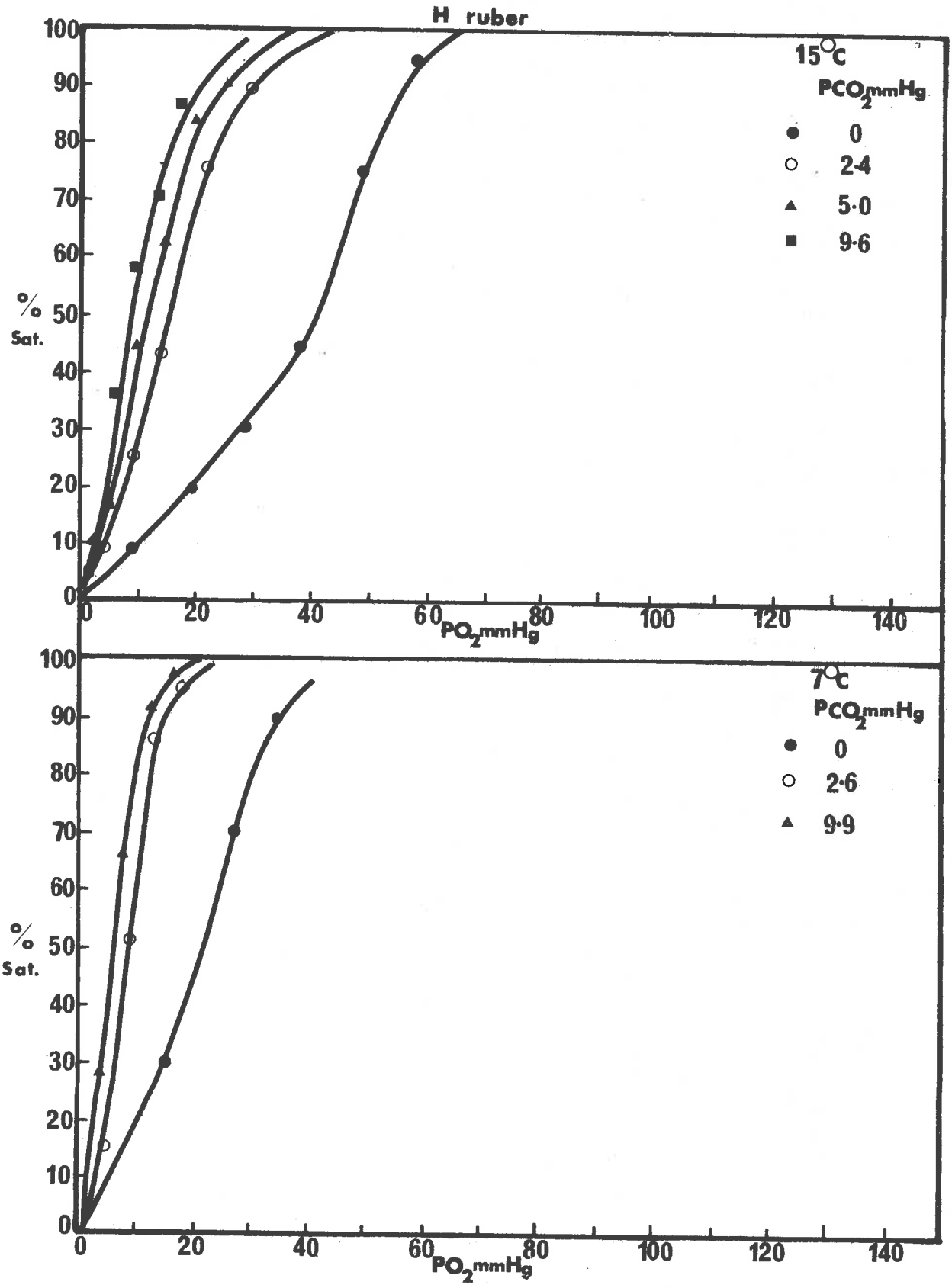

H laevigata
20°C
23·5°C
24°C
% Sat.
% Sat.
0
10
20
30
40
50
60
70
80
90
100
0
20
40
60
80
100
120
140
PO2 mmHg

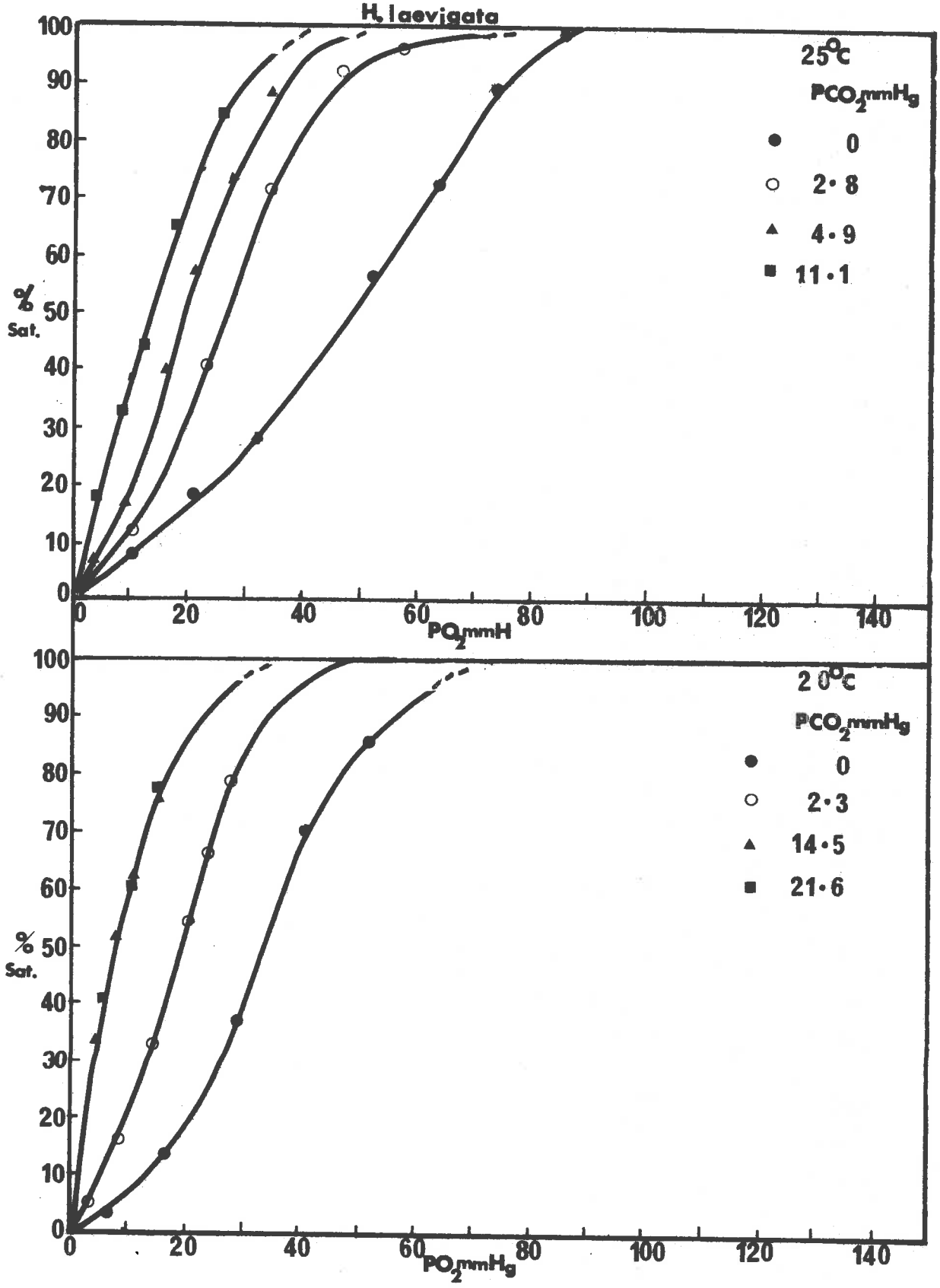

H laevigata
15·5°C
15°C
7°C
% Sat.
% Sat.
0
10
20
30
40
50
60
70
80
90
100
0
20
40
60
80
100
120
140
PO2 mmHg

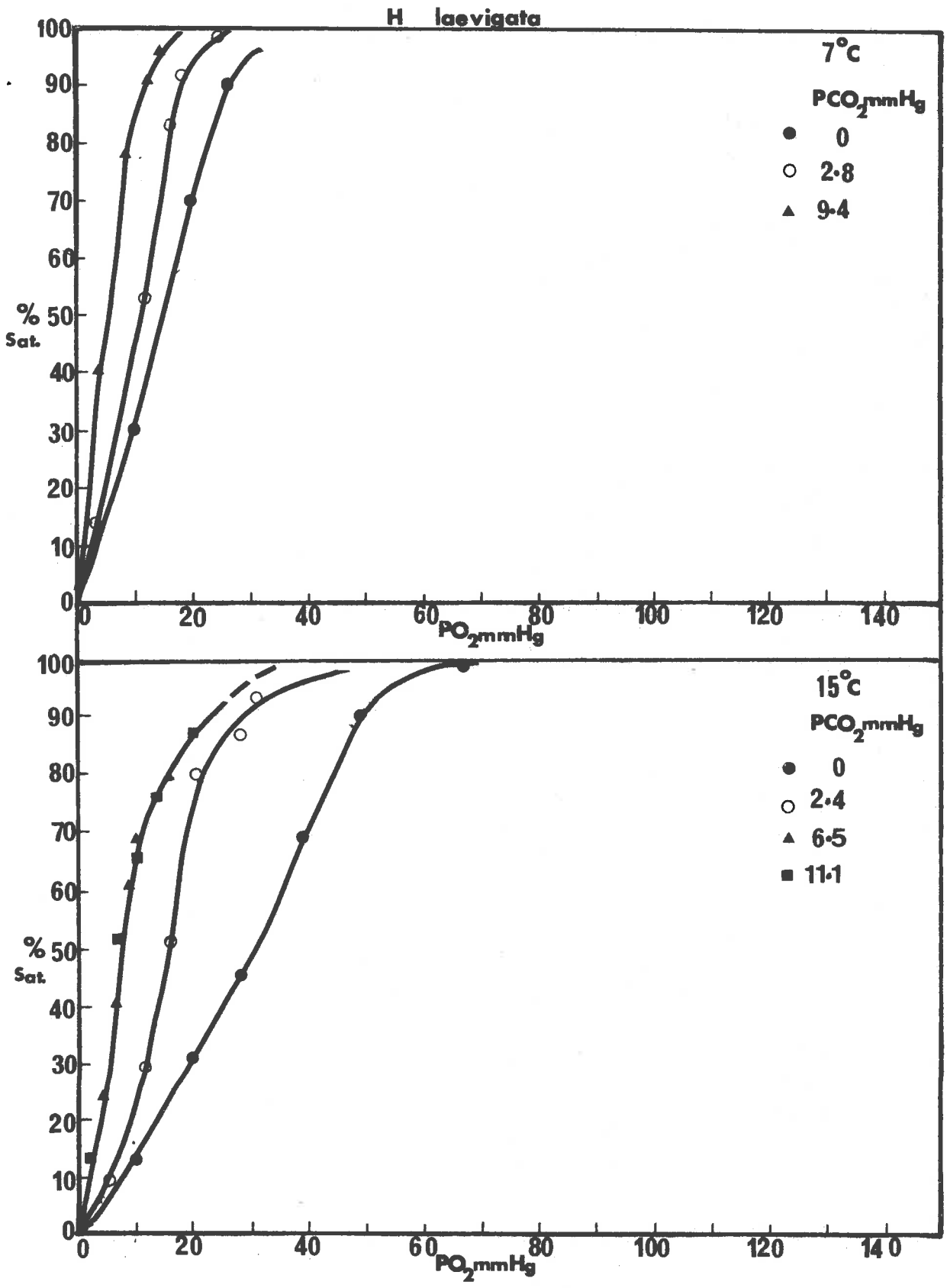
Appendix 1 (b)

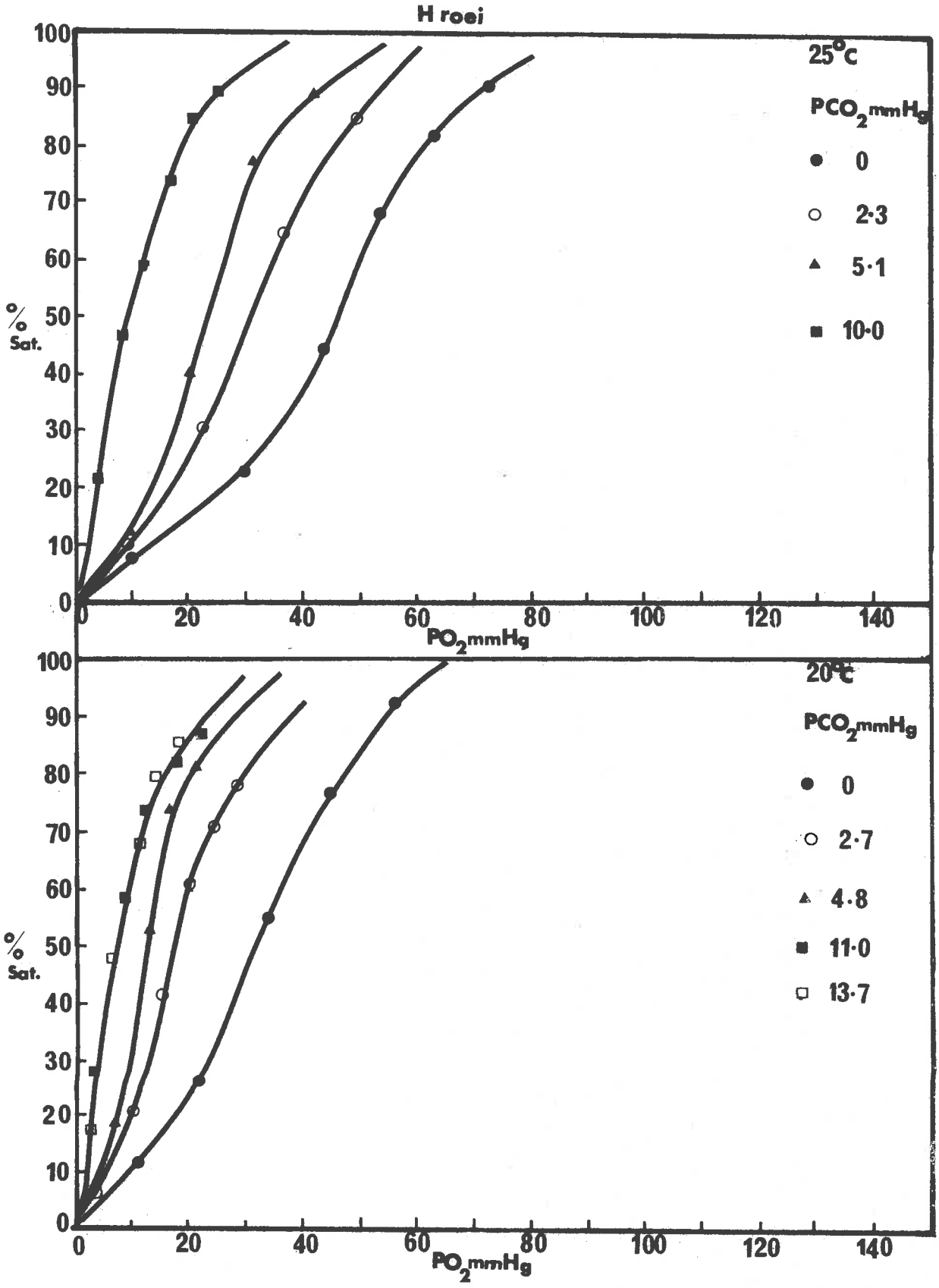
Effect of P_{CO_2} on the oxygen equilibrium curves of abalone haemocyanin at various temperatures.

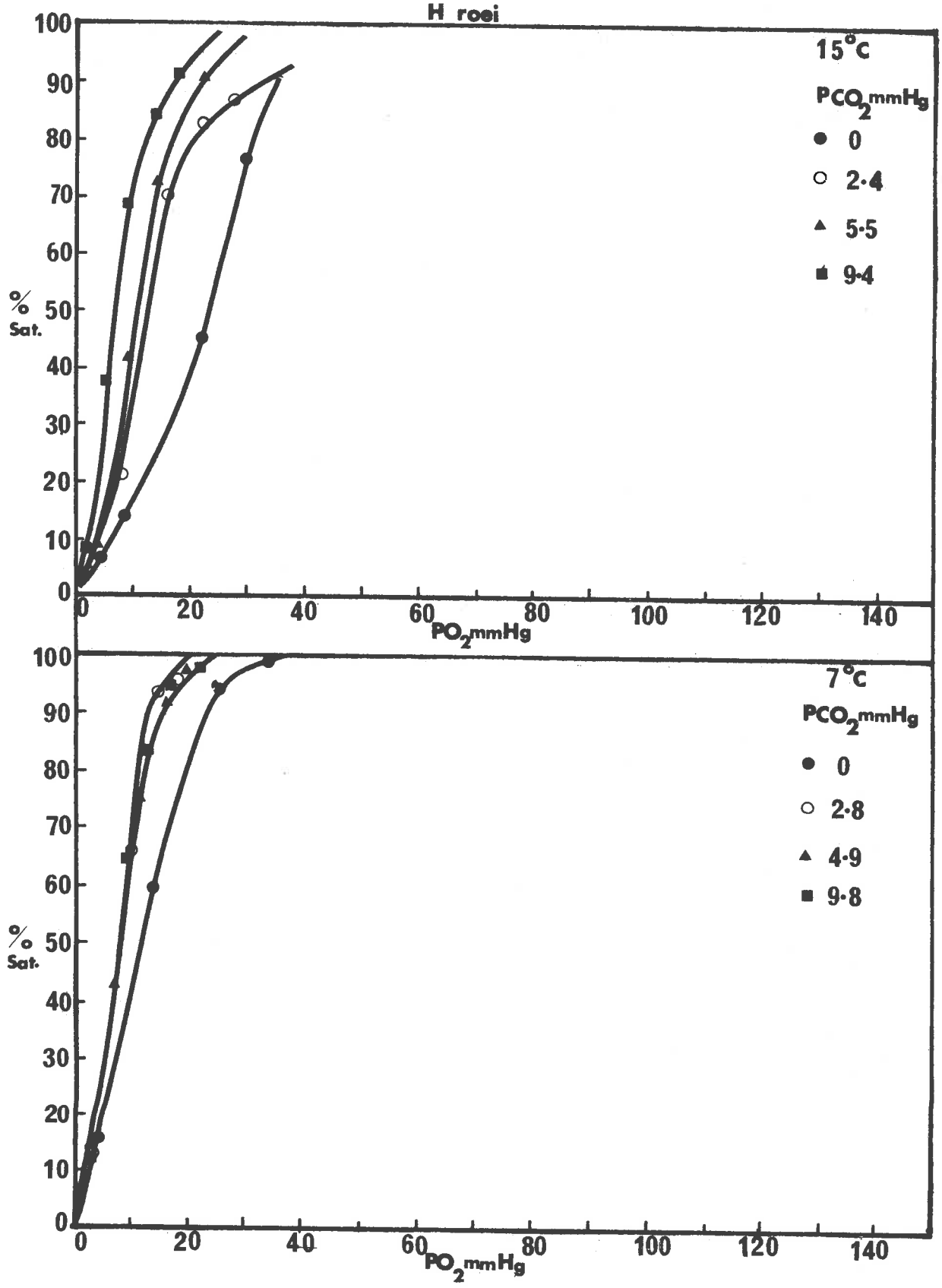

H ruber
20°C
PCO2 mmHg
0
2·7
4·6
9·2
% Sat.
PO2 mmHg
25°C
PCO2 mmHg
0
2·4
5·0
9·7
% Sat.
PO2 mmHg


H ruber
15°C
PCO2mmHg
0
2·4
5·0
9·6
% Sat.
PO2mmHg
7°C
PCO2mmHg
0
2·6
9·9
% Sat.
PO2mmHg


H. laevigata
25°C
PCO2 mmHg
0
2·8
4·9
11·1
% Sat.
PO2 mmHg
20°C
PCO2 mmHg
0
2·3
14·5
21·6
% Sat.
PO2 mmHg


H laevigata
7°C
PCO2mmHg
0
2·8
9·4
% Sat.
PO2mmHg
15°C
PCO2mmHg
0
2·4
6·5
11·1
% Sat.
PO2mmHg

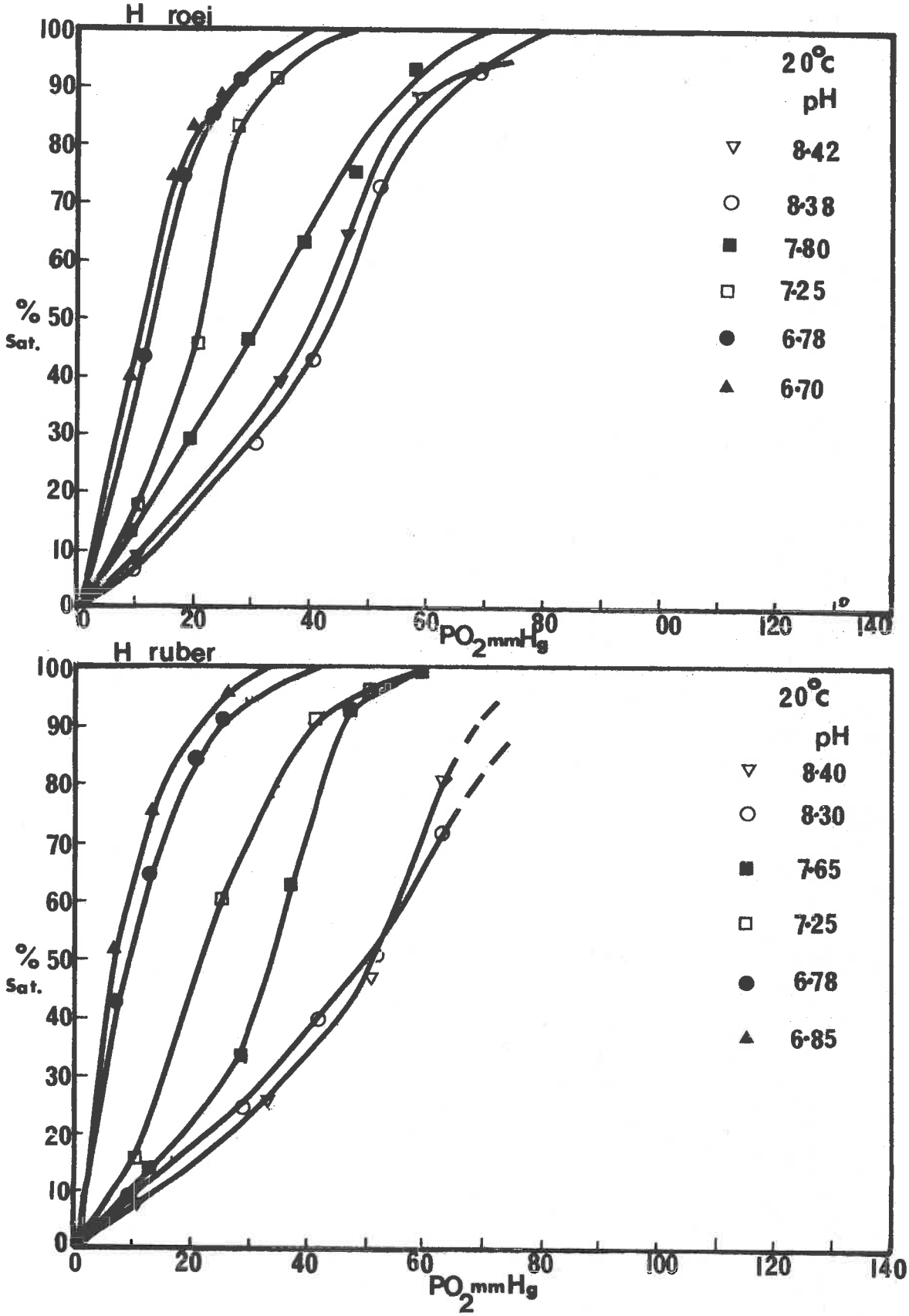

H roei
25°C
PCO2 mmHg
0
2·3
5·1
10·0
% Sat.
PO2 mmHg
20°C
PCO2 mmHg
0
2·7
4·8
11·0
13·7
% Sat.
PO2 mmHg

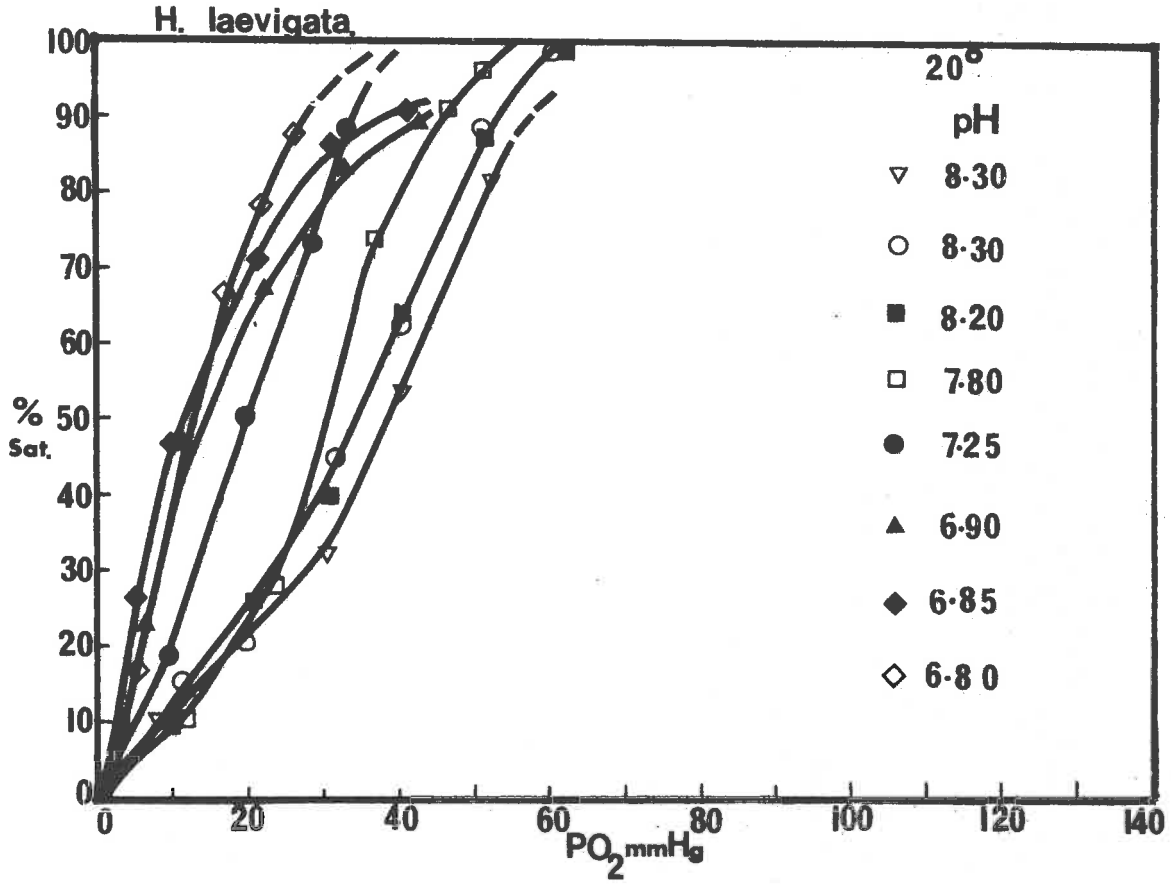

H roei
15°C
PCO2mmHg
0
2·4
5·5
9·4
% Sat.
PO2mmHg
7°C
PCO2mmHg
0
2·8
4·9
9·8
% Sat.
PO2mmHg

Appendix 1 (c)

Effect of pH on the oxygen equilibrium curves of abalone haemocyanin at 20°C

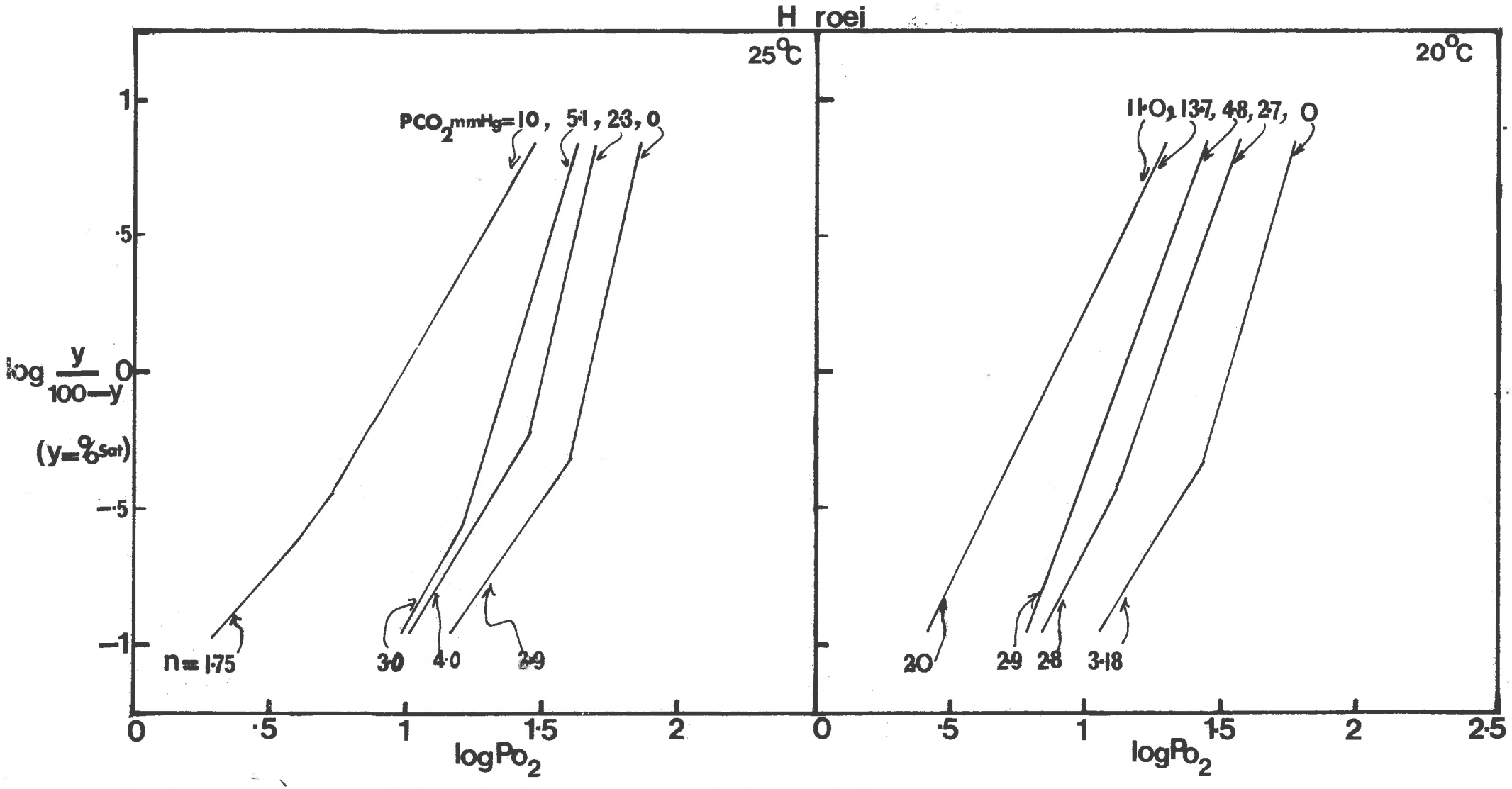
pH adjusted using Tris-HCl-seawater buffer recommended by Redmond 1962.

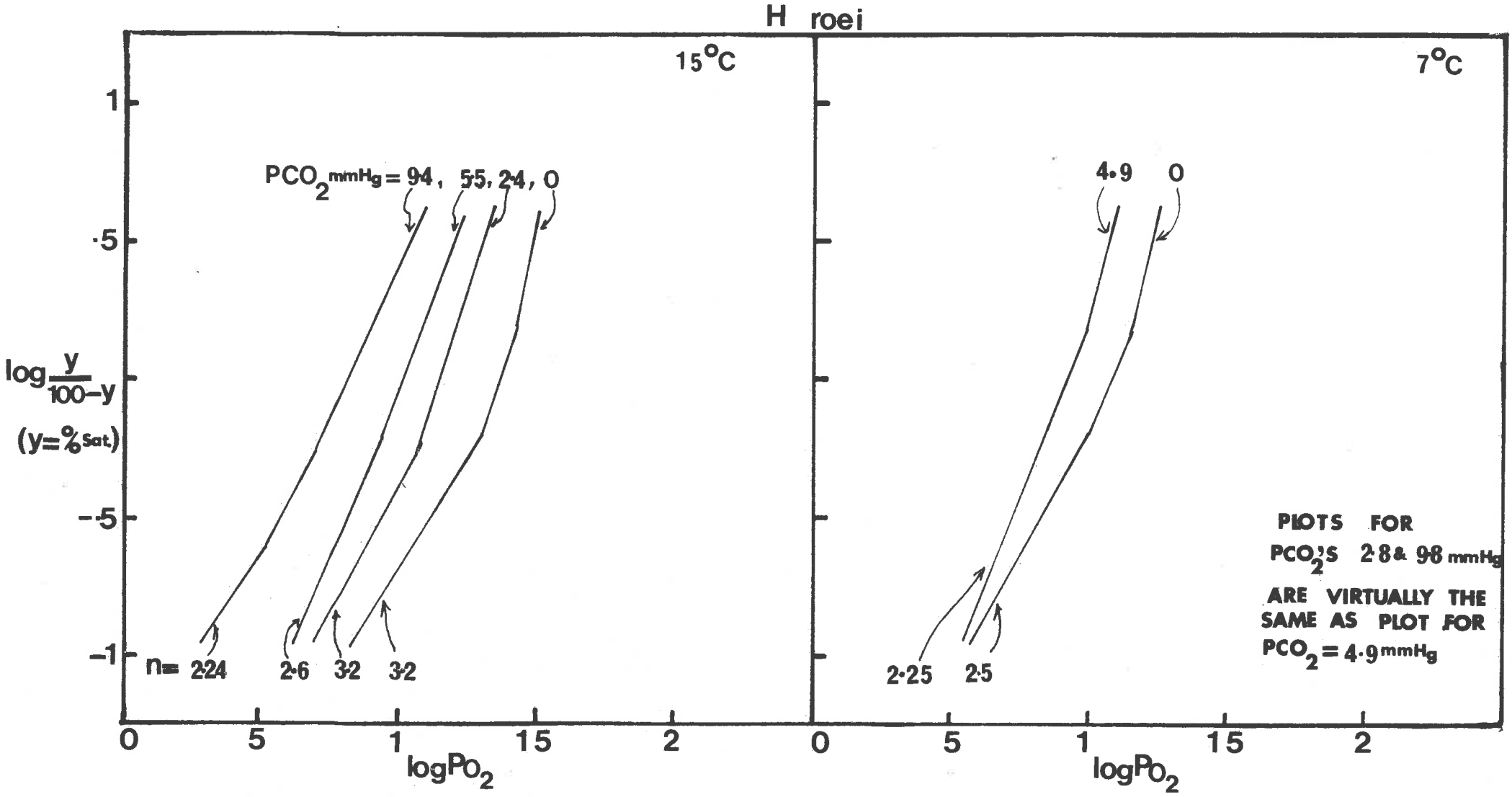

H roei
100
90
80
70
60
% 50
Sat.
40
30
20
10
0
0
20
40
60
80
00
120
140
PO2mmHg
20°C
pH
8·42
8·38
7·80
7·25
6·78
6·70
H ruber
100
90
80
70
60
% 50
Sat.
40
30
20
10
0
0
20
40
60
80
100
120
140
PO2mmHg
20°C
pH
8·40
8·30
7·65
7·25
6·78
6·85

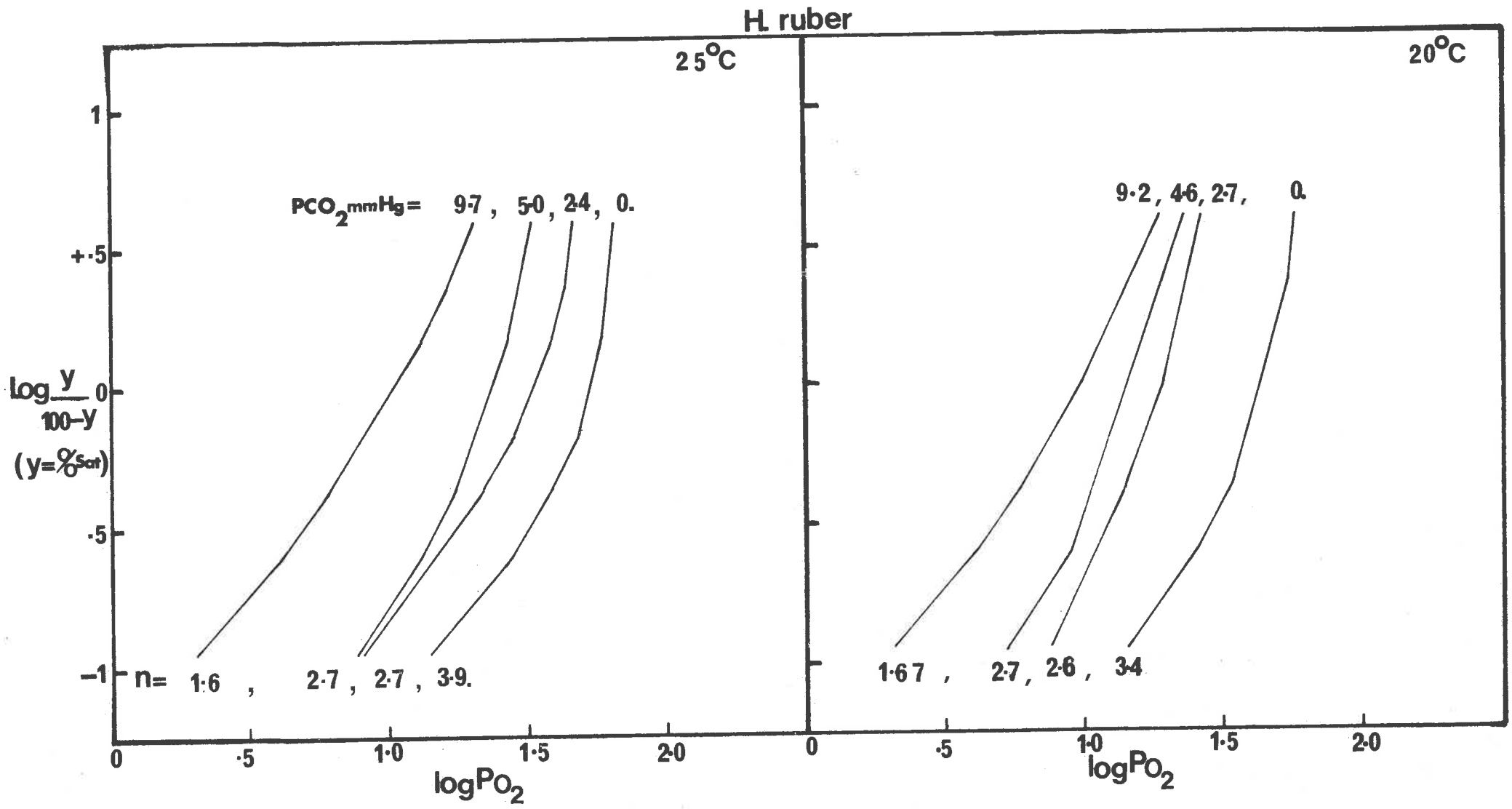

H. laevigata.
20°
pH
8·30
8·30
8·20
7·80
7·25
6·90
6·85
6·80
% Sat.
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
120
140
PO2 mmHg

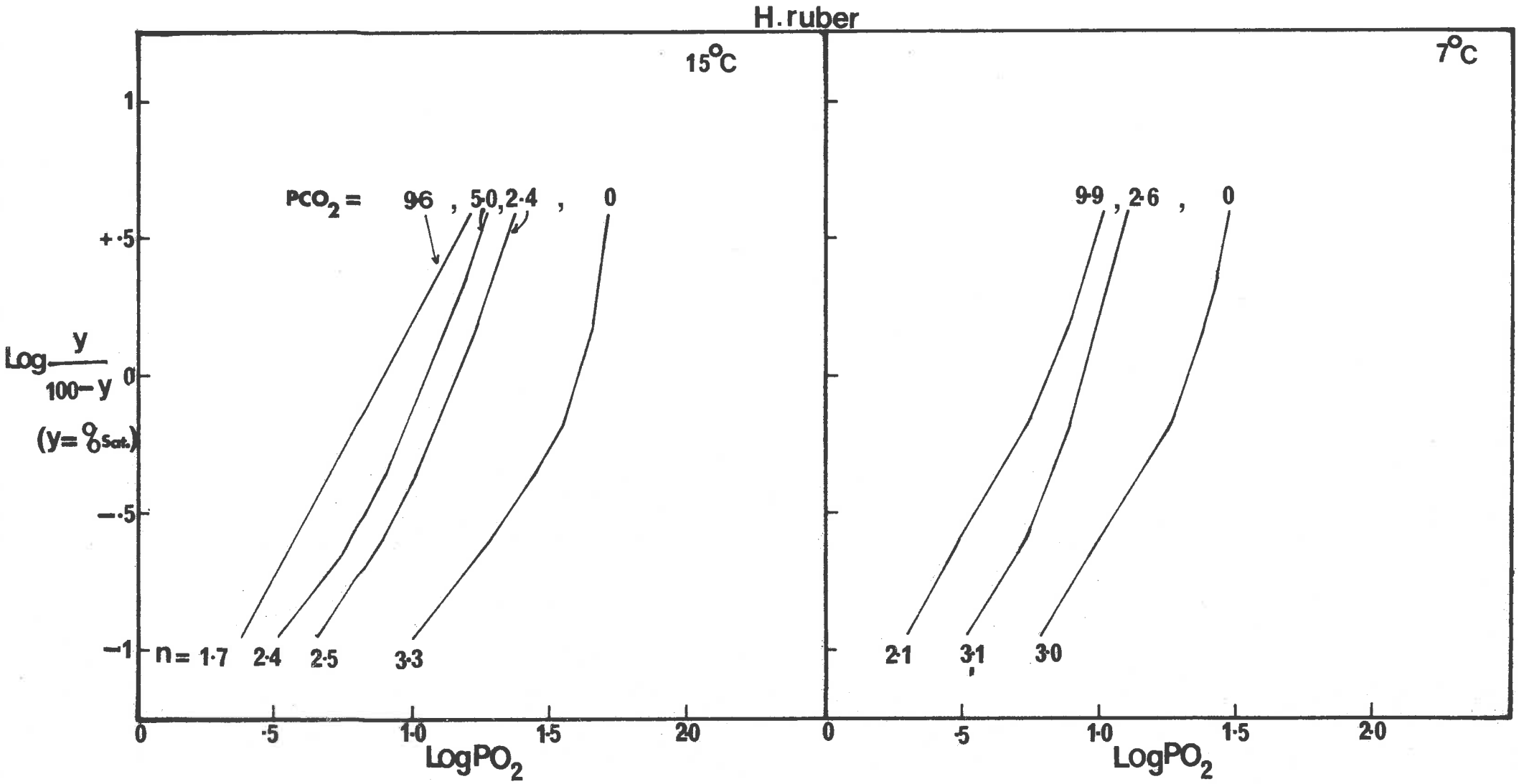
Appendix 1 (d)

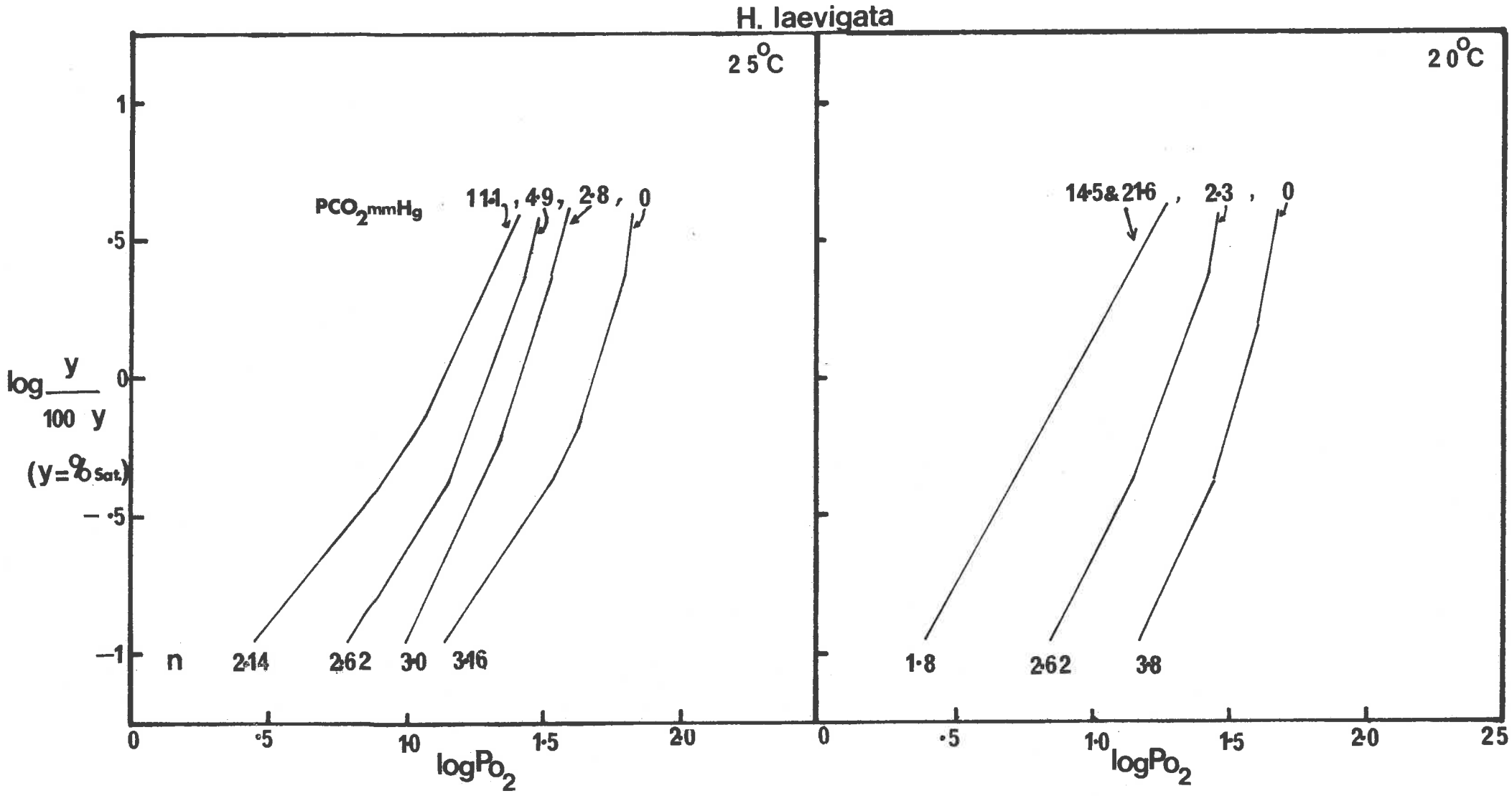
Hill plots of oxygen equilibrium curve data of *H. roei*, *H. ruber* and *H. laevigata* haemocyanins at 25, 20, 15 and 7°C, and various P_{CO_2}'s.

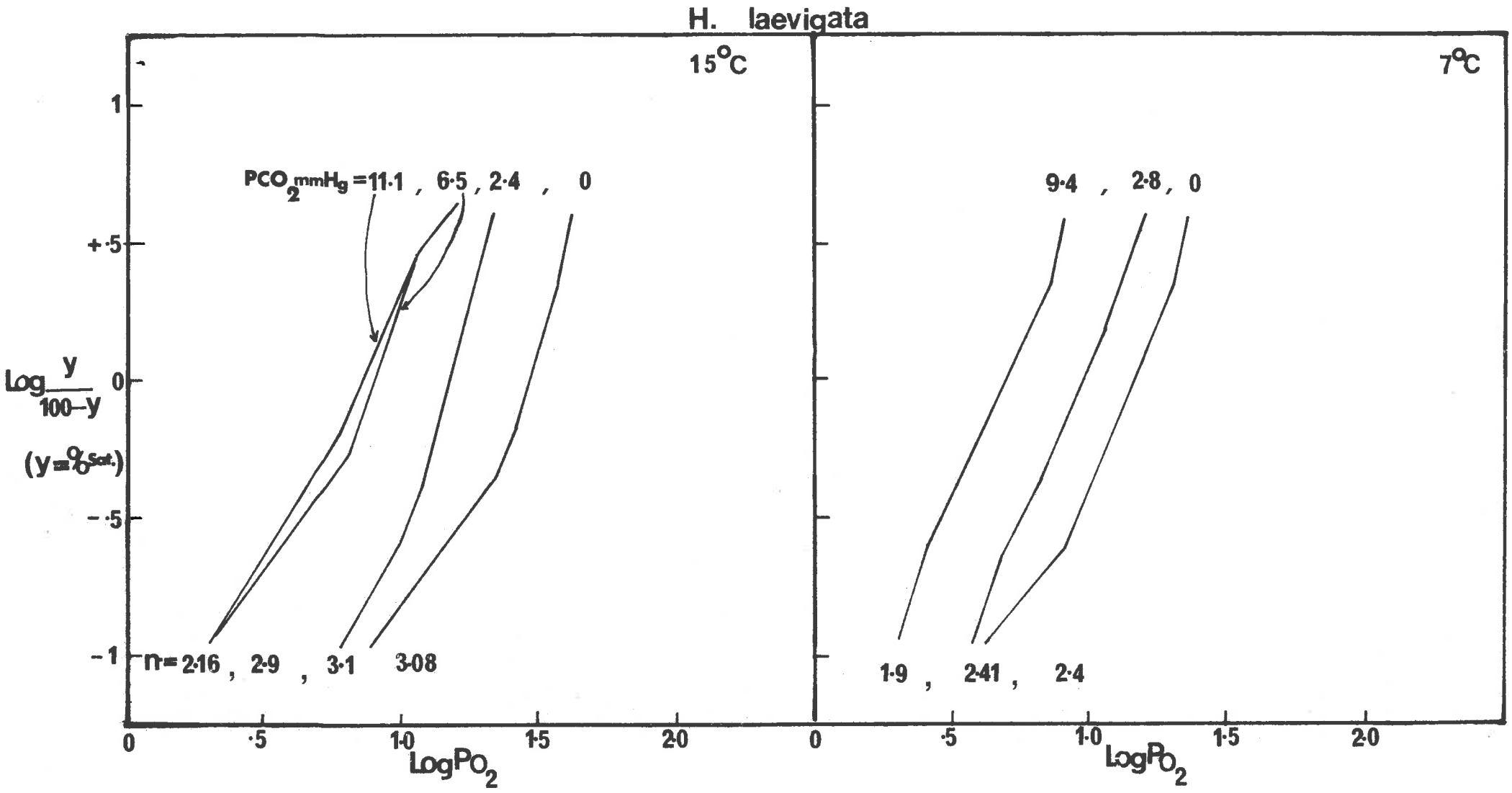

H roei
25°C
20°C
PCO2 mmHg = 10, 5·1, 2·3, 0
n = 1·75
3·0
4·0
2·9
1·O2 13·7, 4·8, 2·7, 0
2·0
2·9
2·8
3·18
log y/(100−y)
(y = %Sat)
log Po2
log Po2
1
·5
0
−·5
−1
0
·5
1
1·5
2
2·5


H roei
15°C
7°C
1
·5
-·5
-1
$\log \frac{y}{100-y}$
(y=%Sat.)
PCO_2 mmHg = 9·4, 5·5, 2·4, 0
4·9 0
n= 2·24 2·6 3·2 3·2
2·25 2·5
PLOTS FOR
PCO_2'S 2·8 & 9·8 mmHg
ARE VIRTUALLY THE
SAME AS PLOT FOR
PCO_2 = 4·9 mmHg
0 5 1 15 2
logP_{O_2}
0 5 1 15 2
logP_{O_2}


H. ruber
25°C
20°C
PCO2 mmHg = 9·7 , 5·0 , 2·4 , 0.
9·2 , 4·6, 2·7 , 0.
n= 1·6 , 2·7 , 2·7 , 3·9.
1·67 , 2·7 , 2·6 , 3·4
log y/(100−y)
(y=%Sat)
1
+·5
0
·5
−1
0
·5
1·0
1·5
2·0
logPO2

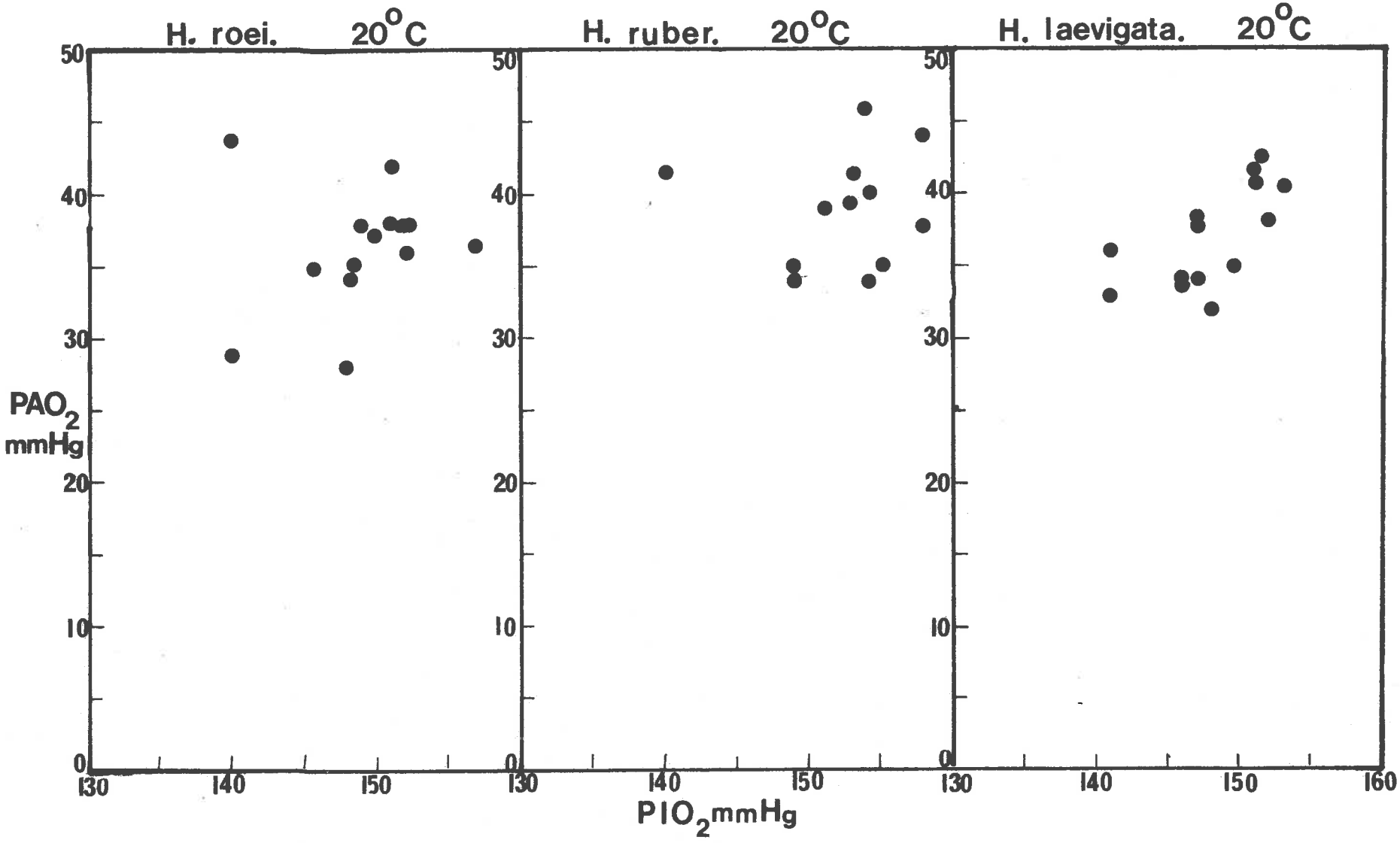

H. ruber
15°C
7°C
PCO2 = 9·6 , 5·0, 2·4 , 0
9·9 , 2·6 , 0
n = 1·7 2·4 2·5 3·3
2·1 3·1 3·0
Log y / 100−y
(y = % Sat.)
1
+·5
0
−·5
−1
0 ·5 1·0 1·5 2·0
Log PO2


H. laevigata
25°C
20°C
PCO2mmHg
11·1, 4·9, 2·8, 0
14·5& 2·16, 2·3, 0
log y/100 y
(y=% Sat.)
n
2·14
2·62
3·0
3·16
1·8
2·62
3·8
logPO2
logPO2


H. laevigata
15°C
7°C
1
+·5
0
−·5
−1
Log y/100−y
(y=%Sat.)
PCO2 mmHg = 11·1 , 6·5 , 2·4 , 0
9·4 , 2·8 , 0
n = 2·16 , 2·9 , 3·1 3·08
1·9 , 2·41 , 2·4
0 ·5 1·0 1·5 2·0
Log PO2
0 ·5 1·0 1·5 2·0
Log PO2

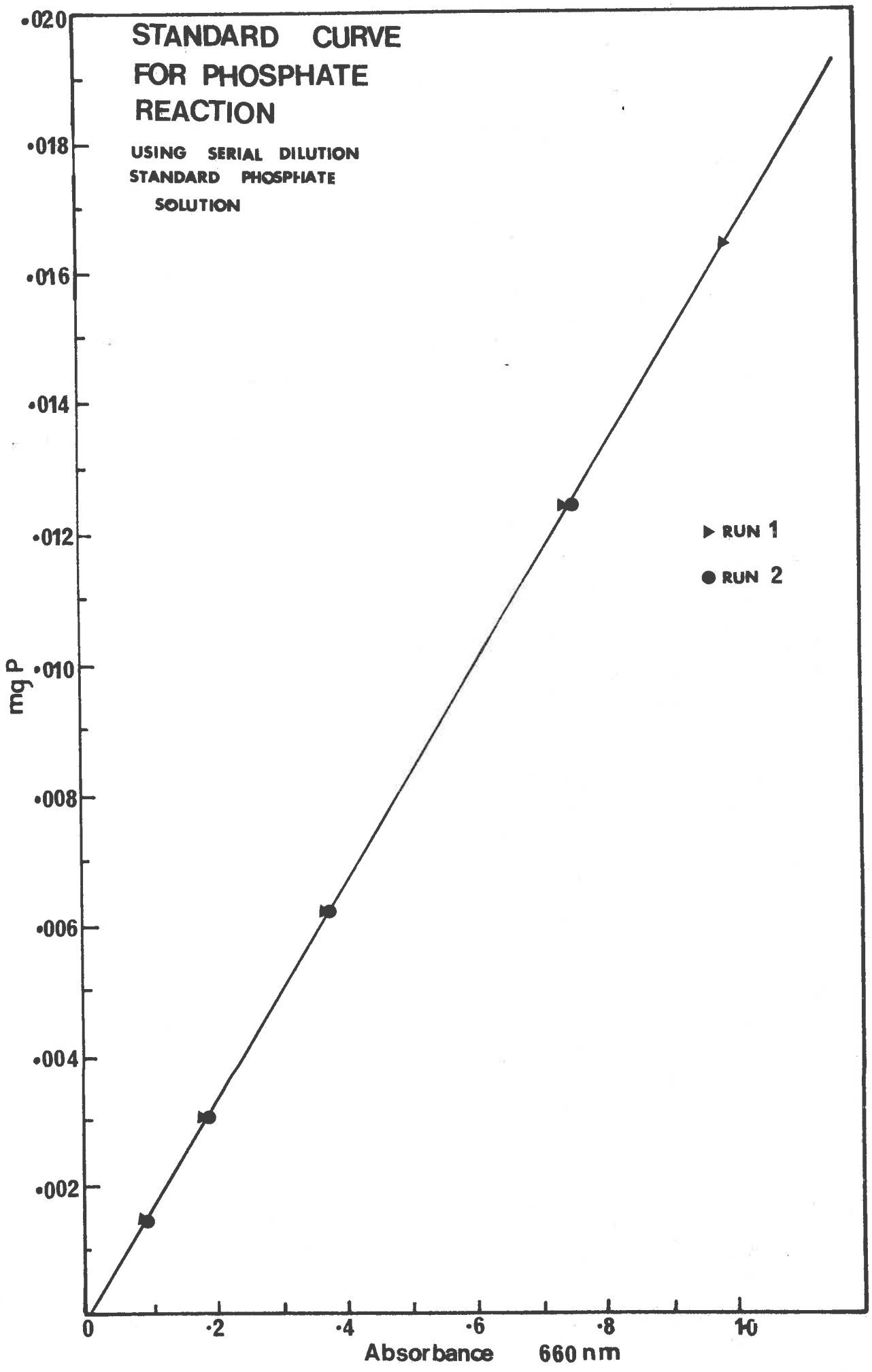
Appendix 2

Plots of P_{O_2} of inhaled water (PI_{O_2}) against arterial P_{O_2} (PA_{O_2}) for the three abalone species at 20°C.


H. roei. 20°C
H. ruber. 20°C
H. laevigata. 20°C
PAO2 mmHg
PIO2 mmHg
0
10
20
30
40
50
130
140
150
160

Appendix 5

Standard curve for phosphate reaction, using serial dilutions of standard phosphate solution 100 mg/100mls (King and Wooton, 1956).


STANDARD CURVE
FOR PHOSPHATE
REACTION
USING SERIAL DILUTION
STANDARD PHOSPHATE
SOLUTION
•020
•018
•016
•014
•012
•010
•008
•006
•004
•002
mg P
RUN 1
RUN 2
0
·2
·4
·6
·8
1·0
Absorbance 660 nm

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