J. Exp. Biol. (1969), 50, 307–317
With 3 text-figures
Printed in Great Britain

RESPIRATORY RESPONSE TO EXPERIMENTALLY INDUCED ANAEMIA IN THE PINFISH (*LAGODON RHOMBOIDES*)

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(*Received* 16 *July* 1968)

INTRODUCTION

Although much literature describes interspecific differences in blood characteristics, relatively little deals with intraspecific differences and the significance of fluctuations in these values. Some experimental work with temperature has yielded conflicting results (cf. Spoor, 1951; Anthony, 1961*a*; Black, Kirkpatrick & Tucker, 1966; DeWilde & Houston, 1967), and a few data exist on the effects of oxygen acclimation, which shows differences in blood haemoglobin levels (Prosser *et al.* 1957).

As DeWilde & Houston (1967) point out, most of the parameters involved in gas exchange can be expected to show little adjustment with respect to altered metabolic demands arising from varying temperature or oxygen content of the water. This is certainly true of the total blood volume and gill surface area, though only a portion of the latter may be utilized at any given time (Steen & Kruysse, 1964). The commonly observed changes in ventilation volume ($\dot{V}_G$) and cardiac volume ($\dot{Q}$) are the most obvious short-term compensation mechanisms, but estimates of the energy requirements for increased ventilation seem rather prohibitive (Schumann & Piiper, 1966), and the work of Rahn (1966) indicates that increases in one of these without corresponding increases in the other would be largely wasted energy. Therefore, increases in blood oxygen capacity (haemoglobin) represent the least expensive long-term method of compensating for changes in respiratory demand.

The purpose of this study was to measure respiratory responses to reduced haemoglobin in order to estimate the ability of the pinfish to compensate for lowered oxygen capacity, either through lowered respiratory demand, or increases in other parameters of the gas-exchange relationship. It was also addressed to the general question of the role of haemoglobin in the lower vertebrates.

METHODS AND MATERIALS

Collecting and treatment

Fish were collected in the vicinity of Port Aransas, Texas, with a seine, 4 m. otter-trawl, or by hook-and-line, and held in aquaria for at least 2 days in advance of treatment with the drug. The holding period allowed some assessment of capture mortality, and in the case of the seine-caught fish this was very small. Mortality was somewhat heavier from trawling and hook-and-line capture; injury led to a higher proportion of infections, and subsequent death in later series of experiments.

Prior to injection the fish were anaesthetized in MS 222, weighed, and measured

for later identification and calculation of doses. Control groups were injected with either distilled water or varying mixtures of ethanol and water (usually 10% EtOH) in the amount of 0·01 ml./g. of wet weight. Experimental groups were injected similarly with solutions of phenylhydrazine hydrochloride, either in distilled water or ethanol: water. The concentrations of these solutions were adjusted so that the correct dose was also 0·01 ml./g. Injections were made intraperitoneally, just above the origin of the left pectoral fin, in all experiments but one. Subcutaneous injections were tried in one group, but results were similar, although loss of the drug from the puncture was greater.

Haematological methods

Blood samples from the larger fish (> 100 mm. standard length) were collected directly from the heart with a syringe whose dead space had first been filled with a solution of heparin sodium (usually 200 i.u./0·5 ml. of blood). These samples were later corrected for the resulting dilution. For smaller fish (< 100 mm.), blood was obtained by blotting the fish dry and quickly severing the caudal peduncle. A good flow of blood was obtained in this way, and could be collected into heparinized capillaries or pipettes.

Microhaematocrits were determined following the recommendations of Larsen & Snieszko (1961), using capillaries treated with 10% heparin (commercially prepared capillaries were unsuitable).

Haemoglobin determinations were carried out either by the oxyhaemoglobin method or by the cyanmethaemoglobin method, the latter using Uni-Tech reagents and standards. The cyanmethaemoglobin method was reported to be unsatisfactory by Anthony (1961*a*), but was considered accurate by McKnight (1966) and by Sindermann & Mairs (1961). Comparison of the two methods carried out in this laboratory show a consistent difference of about 1% between the two methods, with a correlation factor of 0·96. Additionally, the iron content of the standard and of several samples was analysed by a modification of the method of Diehl & Smith (1960), and by atomic absorption spectrophotometry. All methods agreed within 3%. Occasional samples developed some turbidity, and these were centrifuged at 9000 rev./min. for 3 min. before reading in a spectrophotometer (at 542 mμ for HbO_2 and 540 mμ for FeHbCN).

Metabolism measurements

Measurements of resting metabolism were carried out in 2900 ml. Fernbach flasks fitted with tubes for sampling at intervals. Fish were placed in the flasks at least 1 hr. prior to the beginning of the measurement period, and showed little or no movement after the first few minutes. All measurements were carried out in darkness to avoid undue stimulation. After the initial acclimation period in the flask, successive Winkler oxygen determinations were made to follow the rate of disappearance of oxygen in the flasks. After applying the appropriate corrections for sampling volume replacement and blank runs, the slope of the oxygen line was calculated by the least squares method (Steel & Torrie, 1960) and expressed as a rate per hour.

Oxygen tolerance tests

A continuous supply of deoxygenated water was provided by a column similar to that described by Fry (1951). The content could be controlled to $\pm$0·1 mg./l. at flow

rates of 0–2500 ml./min. Concentrations from 0·30 mg./l. to full saturation could be produced.

Fish were placed in flasks with running aerated water for at least half an hour before beginning the flow of deoxygenated water. This acclimation period is sufficient, according to Shepard (1955). Mixing times were usually short in relation to the duration of the trials, but at very low concentrations starting time was considered to be the time at which mixing was 90% complete. Cessation of respiratory movements, usually preceded by a period of spasmodic quivering, was taken to be the point of death (Shepard 1955).

Cardiac rate measurements

Fine silver-wire electrodes were inserted above and below the heart on the same side of the body to lessen the interference from the pectoral musculature. The signal was fed through a standard cardiac pre-amplifier to a dynograph-type recorder with a time-and-event marker. In many instances it was possible to count the opercular and cardiac frequencies from the same trace.

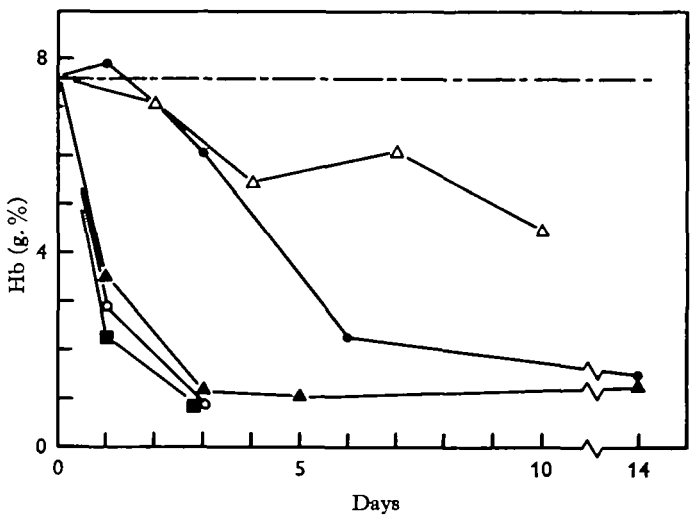

Fig. 1. Haemoglobin in grams per 100 ml. versus time in days from injection. Open triangles, 3 μg./g.; solid circles, 5 μg./g.; solid triangles, 15 μg./g.; open circles, 25 μg./g.; and solid squares, 40 μg./g.

RESULTS

Phenylhydrazine action

As shown in Fig. 1, haemoglobin of the pinfish dropped rapidly in the first few days after administration of phenylhydrazine, especially in the higher dosage groups. Haematocrit values were also taken, and are closely correlated with haemoglobin, as shown by Fig. 2. A steady level was reached after about 3–6 days, and most deaths also occurred in this period. Dosages on the order of 3–15 μg./g. were used in subsequent experiments, since this dosage seemed to produce maximal depression with the least mortality.

Blood sampling up to 2 weeks later showed little or no replacement of haemoglobin

in the 5 μg./g. group, but no long-term experiments to measure recovery rate have been undertaken.

Although haemoglobin levels of most of the fish seemed to level out at about 1 g.% vaues as low as 0·26 g.% have been recorded; these fish were apparently healthy at the time of sampling.

Metabolism measurements

Resting metabolism measurements showed no significant difference between control groups and either of the two treatment groups (Student's t test). The data are shown in Table 1, with the standard deviations. Values for oxygen consumption are in

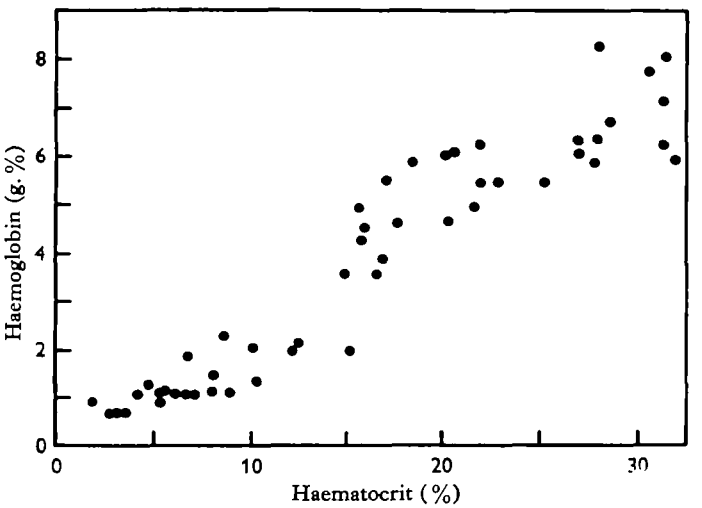

Fig. 2. Scatter diagram of haematocrit (Hc%) versus haemoglobin (Hb) in g./100 ml.

mg..hr.$^{-1}$.kg.$^{-1}$ (Y), converted to the average weight of all the fish (15·9 g.) and to the average temperature (14° C.) according to the equation $Y = -1{\cdot}865 + 1{\cdot}132\chi_w + 0{\cdot}0606\chi_t$, where Y is $\log_{10}$ mg.,kg.$^{-1}$.hr.$^{-1}$ consumed, χ_w is $\log_{10}$ wet weight, and χ_t is degrees C. The equation was calculated from a series of metabolism measurements carried out on pinfish in late winter over a size and temperature range using multiple regression analysis (J. N. Cameron, unpublished). The data are grouped according to haemoglobin values, the high group of 6·50 g.% or more, the intermediate group of 5·00 to 6·50 g.%, and the low group, less than 5·00 g.%. Packed cell volumes (haematocrits) are also given. None of the differences in metabolic rates are significant at the 5% level, although the treated groups were somewhat higher.

Oxygen tolerance trials

Three experiments were run at approximately 20°C., using 20 fish each, 10 phenylhydrazine-treated and 10 controls. Percentage death versus time was plotted for each set on a log-probit basis (Bliss, 1952), and the geometric mean tolerance times were compared. At 0·4, 0·8 and 0·9 mg./l., the treated fish had tolerance times of 22, 142 and 151 min.; the controls had times of 14, 184, and 135 min. Haemoglobin counts for the controls averaged 7·60 mg./100 ml., and ranged from 2·0 to 4·5 for the treated fish.

In addition, preliminary trials were run with several fish at different oxygen concentrations to determine the approximate lethal levels, and the tolerance times for the treated fish were always slightly higher. The incipient lethal level, from inspection of the data, appears to be about 1·3 mg./l. at 20° C.

Table 1. *Oxygen consumption at various haemoglobin levels*

(Data are grouped according to the haemoglobin values in grams per 100 ml. Haematocrit values (Hc) are in percentages, and oxygen consumption values (Y) in mg..kg.$^{-1}$. hr.$^{-1}$, as calculated for comparison (see text). Differences in Y values are not significant, but the differences in haematocrit between high and low groups, and between intermediate and low groups are significant, as are all the haemoglobin differences.)

	High			Intermediate			Low		
	Y	Hb	Hc	Y	Hb	Hc	Y	Hb	Hc
	145	6·76	28·6	111	6·31	38·9	221	4·06	31·9
	111	7·17	31·4	133	5·83	27·8	237	4·62	20·3
	138	6·89	34·0	208	5·44	25·3	215	1·94	15·2
	192	8·19	34·8	354	6·05	27·0	225	3·59	15·0
	165	7·75	30·6	140	6·39	28·0	191	3·89	17·0
	160	6·52	23·7	220	6·24	30·9	167	3·59	16·6
	146	8·04	31·5	168	5·92	32·0	104	4·23	15·9
	349	6·52	23·7	128	5·44	22·9			
	145	8·29	28·0	200	6·31	27·0			
	144	6·65	29·3	240	5·83	28·3			
	139	7·50	26·8	175	5·57	19·7			
	127	6·87	23·9						
Means	163	7·26	28·9	189	5·94	28·0	194	3·70	18·8
S.E.	±18	—	—	±21	—	—	±17	—	—

Cardiac rate measurements

Since cardiac rate decreases exponentially with size (Prosser & Brown, 1961), the following equation was used:

$$\log (\text{rate}) = a + b \log (\text{weight}) + c \log (Hc^{-1}). \quad (1)$$

The term for haematocrit was derived from the equation

$$\dot{Q} = \dot{V}_{O_2}/(Pa_{O_2} - Pv_{O_2}).\alpha B_{O_2} \quad (2)$$

where $\dot{Q}$ = cardiac output in ml./min.; $\dot{V}_{O_2}$ = oxygen consumption in ml. O_2/min. STPD; Pa_{O_2} = arterial oxygen tension in mm. Hg; Pv_{O_2} = venous oxygen tension in mm. Hg; αB_{O_2} = solubility coefficient for oxygen in blood in ml. O_2/(mm. Hg) (ml. blood); and from the assumption that αB_{O_2} is directly proportional to haemoglobin and to haematocrit (Holeton & Randall, 1967). Taking $\dot{V}_{O_2}$ to be constant, as previous results show, and ignoring the variability in arteriovenous oxygen difference, the calculated equation is:

$$\log (\text{rate}) = 2{\cdot}168 - 0{\cdot}273 \log (\text{weight}) + 0{\cdot}381 \log (Hc^{-1}). \quad (3)$$

The over-all correlation coefficient is $R_{y \cdot 12} = 0{\cdot}73$, which is significant at the 5% level. Calculated rates are plotted out in Fig. 3 for three different weights over a range of haematocrit values. As an example, a 50 g. fish would have a heart rate of 72/min. with a haematocrit of 40% and a rate of 122/min. with a haematocrit of 10%. From equation (2) it is apparent that the 75% decrease in αB_{O_2} would require a fourfold

increase in the product of $\dot{Q}$ and $(Pa_{O_2} - Pv_{O_2})$. Half this compensation is occurring in the cardiac rate, and presumably changes in stroke volume and arteriovenous oxygen difference account for the rest.

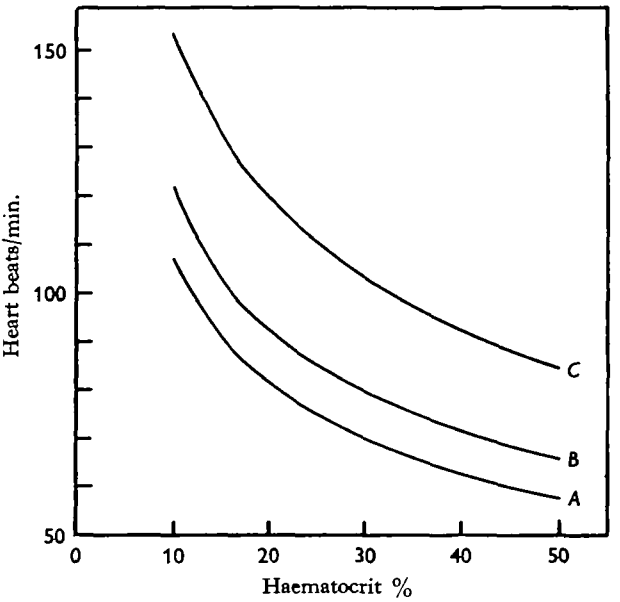

Fig. 3. Heart beats per minute versus haematocrit for various sizes calculated from the equation given in the text. *A* is the curve for an 80 g. fish, *B* for a 50 g. fish, and *C* for a 20 g. fish.

DISCUSSION

Reports of fishes without haemoglobin go back at least as far as the 1920's when Schlicher (1926) reported an erythrocyte-less carp. Ruud (1954) confirmed earlier rumours of Antarctic fishes with no haemoglobin or erythrocytes. Experimentally, Nicloux (1923) and later Anthony (1961*b*) used carbon monoxide to reduce the oxygen-carrying capacity of the blood, and studied survival under various conditions. There are also several reports of anurans without haemoglobin (de Graaf, 1957; Ewer, 1959; Flores & Frieden, 1968) but only the last paper dealt with live specimens, and respiration was not studied in any detail.

The conventional explanation of the Antarctic 'ice-fish' is that they live in cold, highly oxygenated waters and are of sluggish habits, thus obviating the need for a respiratory pigment. Also, in Nicloux's experiments, and in some of Anthony's, the fish were held at low temperatures and very low activity levels. Additionally, the use of goldfish in the latter experiments can be criticized on the grounds that they are known to become unusually sluggish in the laboratory and are of limited use for comparisons with fish in natural situations.

The use of carbon monoxide to inactivate the haemoglobin involved some uncertainties which we believe to be lacking in the methods described. Direct effects of carbon monoxide are well known for certain enzyme systems (Conn & Stumpf, 1963), and could well have lowered the metabolic rate in these experiments. Also, the calculation of haemoglobin blockage depended on a single value for the equilibrium constant of

the CO–Hb reaction. The use of phenylhydrazine obviates both of these objections, since elimination rather than blockage is occurring, and the remaining haemoglobin can easily be measured by any of the standard techniques. Side effects of the drug are unknown, but because several weeks can elapse before further experimentation, the chances for residual effects are thus reduced.

The action of phenylhydrazine seems to be specific for the erythrocyte, interfering with methaemoglobin reduction and TPNH formation (Kellermeyer *et al.* 1962; Carson, 1956). This may be accomplished by interfering with the first step in pentose phosphate metabolism via glucose-6-phosphate dehydrogenase (G6PD) (Marks, 1964).

Measurements of resting metabolism which showed little or no difference at various levels of haemoglobin may reflect the large amount of variability in the data, and the rather small number of fish used, but the degree of variability is comparable with other work on this species (Wohlschlag & Cameron, 1967; Wohschlag *et al.* 1968) and on fish in general. It may be interesting that the treated groups were higher than the others, even though there was no statistically significant difference. This small increase should be due to the increased cardiac output observed in the anaemic individuals.

The methods used in measuring cardiac rates (restrained fish and implanted electrodes) leave some doubt as to whether these are realistic values for free-swimming fish, but it is the difference between groups that was of interest, not the absolute values. Furthermore, since cardiac output is the significant parameter, measurement of one component of it (rate) might not accurately indicate changes in output (Holeton & Randall, 1967). Stroke–volume measurements were precluded by the small size of the fish and limitations of available equipment. Increases in rate would be further discouraged by the advantages of synchrony between the gill movements and blood flow (Randall & Smith, 1967), and by the capacity for increase in stroke volume, which may be more than fivefold (Holeton & Randall, 1967). Therefore, we think it significant that there *was* an increase in heart rate with decreasing haemoglobin, and that it apparently accounted for about 50% of the predicted increase. Presumably changes in stroke volume and arteriovenous oxygen concentrations account for the rest of the difference.

The observed increases in heart rate may account for the slight increase in metabolic rate, but no figures on the energy required for cardiac pumping are available. This energy expenditure further supports the view that haemoglobin levels are adjusted to allow operation of the heart pump at some optimum level. Other considerations of metabolite removal, nutrient supply, hormonal communication, etc., may contribute to the lower threshhold of cardiac pumping, while physical limitations on the muscle itself must set some upper limit of operation.

This leads us to the consideration of cardiac response to activity demands. Normally increases of four to sixfold in the cardiac output accompany moderate bursts of activity (Stevens & Randall, 1967), so if an anaemic fish is already operating closer to the physical limit of his cardiac capacity, his ability to meet these extra activity demands will be greatly impaired. While this hypothesis has not been systematically tested, it was observed several times that in the course of chasing the treated fish around the aquarium, one of them would suddenly go into a spasmodic state of quivering, such as described in the final stage of asphyxiation (Shepard, 1955), and die. This could have resulted either from rapid lactic acid build-up, or from abrupt

cardiac failure. Simply measuring maximum sustainable activity levels should provide an adequate test of the hypothesis.

The last experiments, which we believe are incidental to the whole question of blood oxygen transport, are the ones on oxygen tolerance times. That haemoglobin may act as an oxygen store in these tests is made doubtful by the following calculation: if a pinfish of 50 g. has a blood volume of 3·0 ml., and an oxygen capacity of 10 ml./100 ml., the total storage will be at most 0·30 ml. O_2, which will be exhausted in about 2·2 min. with the consumption rate given in Table 1. Storage of this magnitude can hardly be considered significant.

Anthony (1961*b*) concluded correctly that haemoglobin level was unrelated to the asphyxial oxygen tension, but thought this demonstrated the adequacy of the plasma oxygen capacity under stress conditions. Our hypothesis is that as soon as the oxygen supply falls below the demand, oxygen reserves in the fish will be exhausted at a rate dependent on the metabolic rate only, and unrelated to the haemoglobin content of the blood. The rate-limiting steps in oxygen supply are the opercular ventilation and the diffusion rate across the gill membranes. This diffusion rate may already be slow under normal conditions (Rahn, 1966), and declines with concentration gradient. At very low oxygen concentrations any solution may be adequate to carry away the oxygen diffusing, and haemoglobin may indeed be superfluous. Therefore, in so far as their metabolic rates are the same, normal and anaemic fish can be expected to have the same tolerance times. The small but non-significant increase observed in anaemic fish understandably did not cause a significant difference in tolerance times. A more realistic measurement might be the minimum value tolerated indefinitely, but the difference between normal and anaemic fish will probably be small, and numerous operational difficulties are involved in an experiment of this kind.

The observed results of longer tolerance times in the anaemic fish could be due to two causes: a reduction in arteriovenous oxygen tensions may be producing an effect akin to the low-oxygen acclimation observed by Shepard (1955), or the decreased reserve in cardiac output may lead to curtailment of the activity increase normally associated with sudden introduction of water low in oxygen (Jones, 1952; Höglund, 1961).

Contrary to Anthony's (1961*b*) statement that 'it (is) doubtful that fish as a class make much use of this respiratory pigment', it seems to us that haemoglobin allows the circulatory system under ordinary conditions to operate at a much lower level of energy expenditure than it could without it, thus providing a considerable reserve for extra demands made by activity or other stress. Removal or inactivation of haemoglobin merely pushes the level of circulatory operation up closer to the limits, so that the reserve is considerably reduced. On this basis it seems reasonable to examine the changes brought about in haemoglobin levels with environmental conditions, especially the relationship between the level found in the field and the *maximum* respiratory demand likely to occur in that season, rather than the resting or *basal* demand at that time. DeWilde & Houston (1967) proceed from the assumption that haemoglobin levels will reflect the increase in resting metabolism at different temperatures, when in fact many species show a maximum active metabolism at some intermediate temperature (cf. Fry, 1957; Brett, 1967). Anadromous salmonids might be expected to show haemoglobin increases before and during migration, e.g. to reduce the cardiac energy

required over prolonged periods of activity. Very active fish such as the mackerels may have high haemoglobin levels (Klawe, Barrett & Klawe, 1963) for the same reason. Haemoglobin, then, appears to be an effective homoeostatic mechanism for long-term balancing of the circulatory system.

Lowering haemoglobin levels in natural populations would have the immediate effect of increasing the maintenance metabolism requirements. In order to meet this stress either the feeding rate would have to be increased or the growth rate slowed correspondingly. In some situations increased food may not be available, or increased competition for existing food supplies may cause a reduction in population size. On the other hand maintaining the same rate of feeding, leading to a lower growth rate, has the effect of lengthening time to maturity in many fishes. Slower growing fish are therefore subjected to the same causes of mortality (predation, for example) for a longer period of time, which lowers the reproductive potential of the population.

The second effect of lowered haemoglobin levels is a restriction of activity levels. While this may not be so severe as to restrict normal feeding movements, escape from predators may require a maximum expenditure of energy which is no longer available. Also, the pinfish inhabits a shallow environment of an unstable and extreme nature, where rapid drops of temperature or oxygen may require migratory movements to deeper, more stable areas. Apparently the normal haemoglobin level of this species provides them with the large reserve capacity needed to meet these occasional demands, and a lower level may not be adequate. Investigation of the reserve in species inhabiting nearby deeper habitats, or inhabiting the same areas for more restricted periods of time, may show a smaller reserve capacity which may in part account for their different distribution in time and space.

Since a fairly large group of chemicals are known to have haemolytic effects, especially many phenol derivatives (Kellermeyer *et al.* 1962; Carson *et al.* 1956; and others), haemolysis may be a consequence of some kinds of industrial pollution. Another possible cause of haemoglobin deficiency is a short supply of iron. Again, no studies have been directed to the availability of iron in natural populations, but introduction of chelating substances or competing metal ions may produce the same effects as haemolysis. In both cases, increase of energy requirements of cardiac pumping and lowering of reserve capacity will have profound effects on fish populations.

SUMMARY

1. Injection of phenylhydrazine hydrochloride produced surviving pinfish with as low as 0·26 g.% haemoglobin (2·9% of the normal value). Haematocrits dropped as low as 1–2%, against a normal value of about 30%.

2. Measurements of resting metabolism showed no significant difference related to haemoglobin level, but anaemic fish were slightly higher.

3. Tolerance times at low oxygen levels were not clearly related to haemoglobin levels.

4. Cardiac rates show a compensatory increase in response to haemoglobin reduction which accounts for about 50% of the predicted increase in cardiac output.

5. Haemoglobin is thought to provide a reserve capacity in circulatory ability for oxygen transport through its influence on cardiac output.

6. The reserve capacity is important in meeting unusual demands of migration or escape, and may prove useful in comparing different species.

The authors wish to thank Dr C. Van Baalen for his many helpful comments, and Dr D. J. Randall and Dr H. Rahn for critical reading of the manuscript, although any remaining errors are ours.

The work was supported in part by grants NSF-GU 1963 and NSF-GB 6889. One of the authors (J. C.) holds an NSF Graduate Fellowship.

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