J. Exp. Biol. (1974), 61, 455–461
With 1 figure
Printed in Great Britain

THERMOACCLIMATORY VARIATION IN THE HAEMOGLOBIN SYSTEMS OF GOLDFISH (*CARASSIUS AURATUS*) AND RAINBOW TROUT (*SALMO GAIRDNERI*)

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(*Received* 6 *May* 1974)

SUMMARY

Significant increases in total haemoglobin concentrations, and microhaematocrit values were associated with acclimation of rainbow trout and goldfish to increased temperature. Goldfish held at 2 °C were characterized by two haemoglobin components, whereas those acclimated to 20° and 35 °C exhibited three. Nine haemoglobin variants were observed in trout at 2°, 10 ° and 18 °C. The data provide evidence that both species selectively alter the concentrations of specific haemoglobin fractions during the thermoacclimatory process.

INTRODUCTION

The haemoglobin of teleost fishes normally includes a number of electrophoretically-distinguishable components (Riggs, 1970). These differ in subunit composition and, in some instances, exhibit markedly different physico-chemical and physiological properties (Riggs, 1970; Binotti *et al.* 1971; Ronald & Tsuyuki, 1971; Tsuyuki and Ronald, 1971; Iuchi, 1973). There is at least some possibility that systems of this kind may be adaptively responsive to specific environmental situations. In adjusting to temperature-induced variations in oxygen demand, for example, species characterized by haemoglobin polymorphism may selectively alter the concentrations of specific system components in response to particular temperature conditions. As an initial step in evaluation of this hypothesis an attempt has been made to separate and quantify the haemoglobins of thermally acclimated goldfish and rainbow trout: species selected for their noteworthy differences in thermal tolerance and respiratory dependence as well as in the complexity of their haemoglobin systems.

MATERIALS AND METHODS

Maintenance of experimental stocks

Animals were purchased from local commercial suppliers. Goldfish were maintained in static fibreglass aquaria at a density of approximately 20 litres per fish inch in filtered and aerated water with 35 % replacement per week. Trout were held at comparable densities in recirculating fibreglass troughs (Frigid Unit MT-700) which

* Supported in part by National Research Council of Canada Operating Grant A6972.

were equipped with supplementary dechlorinated water inflows providing complete replacement twice each day. Stocks were fed once daily in the morning, *ad lib.*, on a commercial pellet. Their feeding behaviour, general activity and the absence of obvious disease symptoms indicated that both species remained in healthy condition throughout the period of study.

Test groups were acclimated to temperatures which nominally spanned their respective thermal tolerances (goldfish: 2°, 20°, 35 °C; rainbow trout: 2°, 10°, 18 °C) for periods of not less than two weeks. In-tank heaters and refrigeration units provided temperature control in goldfish tanks to within ± 0·2 °C of set-point, and in trout tanks to within ± 1·0 °C. Oxygen and pH determinations were carried out for each tank on alternate days. Oxygen concentrations, as anticipated, varied inversely with temperature, but never fell below 70–75% of saturation. pH fluctuated irregularly between 7·4 and 7·6.

Sampling and analytical procedures

Sampling, blood haemoglobin and microhaematocrit determinations. Acclimated specimens were stunned and blood samples drawn by caudal puncture into heparinized syringes. MS-222 anaesthesia was rejected to avoid the significant alterations in several haematological parameters which accompany this procedure (Houston *et al.* 1971). Duplicate samples were centrifuged at 7000 rpm for 5·0 min to obtain microhaematocrit values. Haemoglobin concentrations were also performed in duplicate by the alkaline haematin method (Anthony, 1960), using Clinton Laboratories 'Hemotrol' (a stabilized human haemoglobin) as a reference standard.

Electrophoresis. Haemoglobins were separated by acrylamide gel electrophoresis using procedures comparable to those described by Davis (1964) and Dietz, Lubrano & Rubinstein (1971). Haemolysates were prepared immediately following haemoglobin determinations, the erythrocytes being washed and re-suspended in isotonic saline four times prior to haemolysis in distilled water. Several authors (e.g., Yamanaka, Yamaguchi & Matsuura 1965*a*; Tsuyuki & Ronald, 1971; Iuchi, 1973) have stressed the instability of fish haemoglobins (particularly their tendency to form spurious fractions during storage) while Ronald & Tsuyuki (1971) have noted the nearly identical mobilities of oxy-, carboxy- and cyanmethaemoglobin derivatives. Freshly prepared, oxygenated haemolysates were used throughout this study. These were diluted as required with saline to achieve loads of 100 μg protein/200 μl gel (goldfish) or 120 μg protein/200 μl gel (trout). Purified cytochrome *c* (40 μg) was also added to each sample as a marker protein for subsequent estimations of electrophoretic mobility. Duplicate electrophoretic runs were carried out at a current of 5 mA/tube for 70–75 min. Haemoglobin bands were identified by colour, and confirmed by benzidine staining of one member of each pair of gels (Dietz *et al.* 1971). The remaining gel was stained overnight with Coomassie Blue and subsequently destained in aqueous 10% acetic-2·5% perchloric acid solution containing strips of silk cloth. Stained gels were stored in 7% aqueous acetic acid.

R_x values were calculated by reference to the cytochrome marker, measurements being made under × 2 magnification with a vernier micrometer. A mirror placed under the gel carrier helped to reduce errors of parallax. The mean standard error of R_x estimations made in this fashion on trial samples replicated six times was ± 0·005.

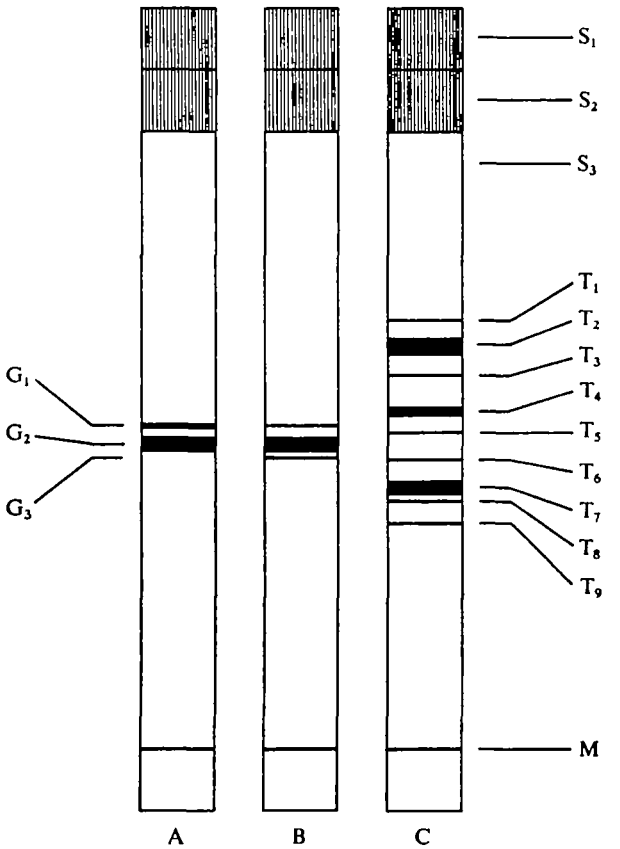

Fig. 1. Diagrammatic representation of the haemoglobin patterns of goldfish (A, 2 °C; B, 20 °C and 35 °C) and rainbow trout (C, 2 °C, 10 °C and 18 °C). S_1, sample gel; S_2, spacer gel; S_3, separation gel; G_1 to G_3, haemoglobin polymorphs of goldfish; T_1 to T_9, haemoglobin polymorphs of rainbow trout; M, cytochrome marker.

Gels were scanned at 600 μm using a Gilford model 2400 recording spectrophotometer, the area under each peak being estimated planimetrically. As the error of estimate for any given peak was roughly $\pm 5\%$ on replicate samples, subsequent calculations of polymorph concentrations (based upon total haemoglobin concentration and peak proportion of total area) should be regarded as approximations.

Data analysis was carried out by one-way anova. Proportional data (R_x values, relative polymorph concentrations, microhaematocrit values) were subjected to arcsin transformation before analysis; other values (total haemoglobin concentrations, haemoglobin polymorph concentrations) to base-10 logarithmic transformation. Significance was attached to differences below the 0·05 level.

RESULTS AND DISCUSSION

Total haemoglobin and microhaematocrit

Tables 1 and 2 summarize data obtained for total haemoglobin and microhaematocrit values in goldfish and rainbow trout respectively. Analysis revealed significant

temperature-correlated differences in both parameters in both species. According these findings support the conclusion reached by several authors (Spoor, 1951; Bondar, 1957; DeWilde & Houston, 1967; Houston & DeWilde, 1968, 1969; Cameron, 1970) that acclimation to increased environmental temperatures is associated with haematological alterations which tend to enhance the oxygen carrying capacity of the blood. They contrast, however, with studies in which insignificant variations, or changes which are inconsistent with this interpretation have been encountered (Anthony, 1960; Falkner and Houston, 1966; Grigg, 1969; Eddy, 1973).

Haemoglobin polymorphism

Typical electrophoretic patterns for goldfish and trout are diagrammatically represented in Fig. 1. Tables 1 and 2 include R_x values and estimated proportional and absolute concentrations for the haemoglobin variants found in each species.

Goldfish. Three haemoglobin fractions (designated G_1, G_2 and G_3 in order of increasing mobility) were observed in goldfish; G_1 and G_2 occurring in every specimen. G_3 was restricted to groups acclimated to 20° and 30 °C. No significant differences associated with temperature were found in comparisons of the R_x values for any given band. Thus, it would appear that the same haemoglobin types are formed, but that G_3 is not synthesized at low temperatures.

The presence of three haemoglobin components in goldfish has been previously reported by Yamanaka *et al.* (1965*b*) and Falkner & Houston (1966). A comparable situation was observed in the closely related carp by Yamanaka *et al.* (1965*b*) and Gillen & Riggs (1972), although Noble, Parkhurst & Gibson (1970) found evidence of two major and two minor variants in this species. Falkner & Houston (1966) have, in addition, reported that two haemoglobin variants are found in goldfish at 5 °C, whereas three normally occur in specimens acclimated to 12°, 20° and 30 °C. There is, then, substantial evidence that this teleost forms specific molecular variants under particular temperature conditions. It is, of course, unclear at present whether the process is a regulated one or whether higher temperatures simply permit random assembly of an increased range of stable subunit structures. In any event these findings are consistent with what would, in the terminology of Hochachka and Somero (1973), be categorized as a 'qualitative adaptive strategy'.

Estimates of fractional concentrations suggest, however, that alterations in the abundance of particular haemoglobin components (what Hochachka and Somero would term a 'quantitative adaptive strategy') may be of greater importance in the thermo-acclimatory process. Indeed, these data indicate that the novel haemoglobin, G_3, is unlikely to be of any great functional importance. Concentrations of the least mobile fraction (G_1) were apparently not influenced by acclimation, remaining essentially constant at $1{\cdot}3 \pm 0{\cdot}05$ to $1{\cdot}4 \pm 0{\cdot}07$ g%. The contribution of this component to total haemoglobin, however, declined from roughly 30% at 2 °C to 15–16% at 20° and 30 °C. By contrast, the major variant (G_2) increased from $3{\cdot}2 \pm 0{\cdot}11$ g% (2 °C) to $6{\cdot}7 \pm 0{\cdot}09$ g% (35 °C), and its relative abundance from about 70 to 80% of the total haemoglobin present. The overall increase of approximately 80% in haemoglobin concentration between 2 °C ($4{\cdot}6 \pm 0{\cdot}17$ g%) and 35 °C ($8{\cdot}4 \pm 0{\cdot}04$ g%) can, therefore, be largely accounted for in terms of a selective increase in G_2; the remainder being attributable to the G_3 polymorph. However, the maximum G

Table 1. *Sample number (N), mean weight (g), total haemoglobin (g %), haematocrit (%), relative electrophoretic mobility (R_x), relative polymorph concentration (% total haemoglobin) and polymorph concentration (g %) in thermally-acclimated goldfish*

Acclimation temperature	N	Weight	Haemoglobin	Haematocrit	G_1 R_x	G_1 Hb (%Hb)	G_1 Hb (g %)	G_2 R_x	G_2 Hb (%Hb)	G_2 Hb (g %)	G_3 R_x	G_3 Hb (%Hb)	G_3 Hb (g %)
2 °C	12	15·6 ± 0·61*	4·6 ± 0·17	30·7 ± 1·04	0·475 ± 0·0012	29·5 ± 0·56	1·4 ± 0·07	0·506 ± 0·0014	70·5 ± 0·56	3·2 ± 0·114	—	—	—
20 °C	12	13·1 ± 0·43	7·6 ± 0·21	35·3 ± 0·49	0·474 ± 0·0012	16·5 ± 0·32	1·3 ± 0·05	0·503 ± 0·0012	72·4 ± 0·49	5·5 ± 0·150	0·525 ± 0·0017	11·5 ± 0·27	0·9 ± 0·03
35 °C	12	13·4 ± 1·88	8·4 ± 0·04	44·7 ± 0·43	0·475 ± 0·0012	15·8 ± 0·67	1·3 ± 0·05	0·503 ± 0·0012	79·5 ± 0·81	6·7 ± 0·090	0·529 ± 0·0012	4·8 ± 0·22	0·4 ± 0·02
Significance			$P < 0·05$	$P < 0·05$	NS	$P < 0·05$	NS	NS	$P < 0·05$	$P < 0·05$	NS	$P < 0·05$	$P < 0·05$

* Mean ± 1 S.E.M.

Table 2. *Sample number (N), mean weight (g), total haemoglobin (g %), haematocrit (%), relative electrophoretic mobility (R_x), relative polymorph concentration (% total haemoglobin) and polymorph concentration (g %) in thermally-acclimated rainbow trout*

Acclimation temperature	N	Weight	Haemoglobin	Haematocrit	T_1 R_x	T_1 Hb (%Hb)	T_1 Hb (g %)	T_2 R_x	T_2 Hb (%Hb)	T_2 Hb (g %)	T_3 R_x	T_3 Hb (%Hb)	T_3 Hb (g %)
2 °C	12	31·2 ± 3·15*	6·3 ± 0·20	32·7 ± 0·69	0·308 ± 0·0014	4·9 ± 0··03	0·3 ± 0·02	0·348 ± 0·0012	22·0 ± 0·89	1·4 ± 0·06	0·400 ± 0·0020	5 7 ± 0·38	0·4 ± 0·03
10 °C	12	26·3 ± 1·99	7·3 ± 0·13	42·9 ± 0·46	0·307 ± 0·0014	4·9 ± 0·35	0·4 ± 0·01	0·343 ± 0·0021	27·7 ± 0·69	2·0 ± 0·06	0·397 ± 0·0032	4·3 ± 0·28	0·3 ± 0·02
18 °C	12	54·1 ± 4·16	8·3 ± 0·02	46·6 ± 0·66	0·306 ± 0·0009	3·5 ± 0·33	0·3 ± 0·01	0 343 ± 0·0014	28·0 ± 0·80	2·3 ± 0·07	0·396 ± 0·0020	4·3 ± 0·33	0·4 ± 0·01
Significance			$P < 0·05$	$P < 0·05$	NS	$P < 0·05$	NS	NS	$P < 0·05$	$P < 0·05$	NS	$P < 0·05$	NS

Acclimation temperature	T_4 R_x	T_4 Hb (%)Hb	T_4 Hb (g %)	T_5 R_x	T_5 Hb (%Hb)	T_5 Hb (g %)	T_6 R_x	T_6 Hb (%Hb)	T_6 Hb (g %)	T_7 R_x	T_7 Hb (%Hb)	T_7 Hb (g %)
2 °C	0·452 ± 0·0014	14·4 ± 0·49	0·9 ± 0·05	0·484 ± 0·0009	5·4 ± 0·22	0·3 ± 0·02	0·527 ± 0·0012	6·5 ± 0·24	0·4 ± 0·01	0·570 ± 0·0014	23·0 ± 0·81	1·5 ± 0·08
10 °C	0·449 ± 0·0012	13·4 ± 0·27	1·0 ± 0·02	0 488 ± 0·0017	3·9 ± 0·31	0·3 ± 0·02	0·532 ± 0·0021	3·0 ± 0·23	0·2 ± 0·02	0 574 ± 0·0014	26·3 ± 1·04	1·9 ± 0·09
18 °C	0·452 ± 0·0020	17·6 ± 0·64	1·5 ± 0·05	0·483 ± 0·0014	6·5 ± 0·37	0·5 ± 0·01	0·530 ± 0·0014	6·7 ± 0·42	0·5 ± 0·04	0·571 ± 0·0023	22·4 ± 0·52	2·0 ± 0·02
Significance	NS	$P < 0·05$	$P < 0·05$	NS	$P < 0·05$	$P < 0·05$	NS	$P < 0·05$	$P < 0·05$	NS	$P < 0·05$	$P < 0·05$

Acclimation temperature	T_8 R_x	T_8 Hb (%Hb)	T_8 Hb (g %)	T_9 R_x	T_9 Hb (%Hb)	T_9 Hb (g %)
2 °C	0·595 ± 0·0009	12·6 ± 0·43	0·8 ± 0·04	0·634 ± 0·0014	6·3 ± 0·29	0·4 ± 0·02
10 °C	0·598 ± 0·0014	10·8 ± 0·24	0·8 ± 0·02	0·631 ± 0·0023	7·2 ± 0·28	0·5 ± 0·02
18 °C	0·596 ± 0·0014	6·7 ± 0·28	0·6 ± 0·01	0·629 ± 0·0017	4·5 ± 0·20	0·4 ± 0·02
Significance	NS	$P < 0·05$	$P < 0·05$	NS	$P < 0·05$	$P < 0·05$

* Mean ± 1 S.E.M.

concentration observed did not exceed 12·5% of the total haemoglobin in any individual fish.

Rainbow trout. The situation in rainbow trout was more complex, with nine fractions (T_1 to T_9) being observed in all specimens regardless of acclimation temperature. Earlier reports indicate that there is some uncertainty as regards the degree of haemoglobin polymorphism in this tetraploid species. A variety of studies employing different electrophoretic and chromatographic procedures have provided evidence of from three to sixteen haemoglobin fractions in trout (Buhler, 1963; Tsuyuki & Gadd, 1963; Burke, 1965; Yamanaka *et al.* 1965*b*; Binotti *et al.* 1971; Ronald & Tsuyuki, 1971; Tsuyuki & Ronald, 1971; Iuchi, 1973).

The present data provide no indication of a situation similar to that seen in goldfish. The individual fractions of the haemoglobin complex were comparable in electrophoretic mobility at each acclimation temperature, and all were observed in every specimen. There is, on the other hand, evidence of temperature-related variation in the abundancies of specific components. Significant increases in the concentrations of T_2, T_4, T_5, and T_7 occurred at higher temperatures, while one polymorph (T_8) declined in concentration under these conditions. The remaining fractions exhibited maxima or minima at 10 °C by comparison with 2° and 18 °C. This situation contrasts markedly with the relatively minor variations in relative abundance reported by Griggs (1969) for the complex haemoglobin system of brown bullhead acclimated to 9° and 24 °C.

While the present study suggests that both species may effect substantial alterations in their haemoglobin systems during the thermoacclimatory process any conclusions regarding the adaptive significance of these findings would be premature. Although evidence of functional heterogeneity has been reported for some species (Riggs, 1970; Binotti *et al.* 1971; Iuchi, 1973) the kinetic and equilibrium studies of Noble *et al.* (1970) on isolated haemoglobins failed to confirm this for the carp. In order to resolve this question it will be necessary to define the transport characteristics of the variants under physiologically realistic conditions of temperature, pH and organophosphate and ionic concentrations. Investigations of this character are now under way and will be the substance of a later report.

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