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A TECHNIQUE FOR REPEATED SAMPLING OF THE BLOOD OF INDIVIDUAL RESTING FISH

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SUMMARY

A dorsal cannulation technique is described. It has been employed for repeated blood sampling in unanaesthetized rainbow trout (*Salmo gairdneri*) kept singly in special receptacles described in the paper. The level of the studied haematological parameters (Haematocrit, Hb, glucose, lactate, K^+, Na^+, Ca^{2+}) differed between fish kept in receptacles for 1 week and free-swimming fish, most probably owing to differences in the motility of the fish. The receptacle seems to minimize visual and handling disturbances, and permits both the standardization of experimental conditions and quick and easy sampling via the dorsal aorta cannula. The general variation in the blood parameter values was very small compared with the previously reported variation in such values for rainbow trout.

INTRODUCTION

There are considerable difficulties in obtaining a series of representative blood samples from an individual fish. A number of different techniques have been developed for taking blood samples from anaesthetized fish (discussed by Klontz & Smith, 1968), but the validity of the haematological information so obtained has recently been questioned, as the anaesthetic agents and the handling involved induce significant alterations in the physiological status of the fish (e.g. Houston, DeWilde & Madden, 1969; Houston *et al.* 1971*a*, *b*; Chavin & Young, 1970; Klontz, 1972; Wedemeyer, 1972). Various cannulation methods have been developed to permit repeated sampling from unanaesthetized, free-swimming specimens (Smith & Bell, 1964; Hickman, 1967; Garey, 1969; Houston, 1971; Soivio, Westman & Nyholm, 1972). With dorsal aorta cannulation, time can be allowed for the fish to recover from the stress of the operation (Houston *et al.* 1969, 1971*a*, *b*), and experiments can then be run for several weeks. Some recent observations indicate that attention should also be paid to the stressing effect of various other physical factors during experimentation (Soivio & Oikari, 1975).

We designed a special receptacle for the fish in order to minimize visual disturbances

and to standardize experimental conditions, while allowing quick, undisturbed sampling via the cannula. It has already been tested for pike, *Esox lucius* (Soivio & Oikari, 1975). The aim of the present study was to investigate the suitability of the receptacle for a typical salmonid, the rainbow trout (*Salmo gairdneri*), by following the changes in blood parameters of fish kept in the receptacle and sampled via dorsal aortic cannulae.

MATERIAL AND METHODS

The experiments were made at the Laukaa Fish Culture Research Station of the Finnish Game and Fisheries Research Institute between 24 February and 4 March 1973. The experimental fish were 4-year-old rainbow trout (*Salmo gairdneri* Richardson) from the stock of the Research Station. The fish were acclimatized for 4 weeks to the experimental temperature of $10 \pm 0{\cdot}5$ °C in 4 m^2 fibre-glass tanks with a water volume of 2 m^3, housed in a rearing hall. Natural daylight conditions prevailed in the hall during the day, and artificial light was used at night. Water was supplied to the tanks (at about 40–50 l/min) from the nearby Lake Peurunkajärvi (water quality: pH 6·5 and 6·6, specific conductivity 45 and 45 μS/cm, $KMnO_4$ 17·3 and 18·0 mg/l, total hardness 0·99 and 1·12 °dH (German degrees of hardness), as measured on 5 February and 6 March). The water was 80 % saturated with oxygen.

The fish were fed pelleted salmon food (Ewos) four times a day: 07.00, 09.00, 13.00 and 17.00.

CANNULATION OF THE DORSAL AORTA

The cannulae were prepared from polyethylene tubing (Clay Adams Intramedic PE 50) with a bubble about 40 mm from the tapering free tip of the catheter, as recommended by Soivio *et al.* (1972). A steel wire (ϕ 0·5 mm) with the tip filed to a sharp cone was inserted into the cannula.

Before the operation the cannula was filled with heparin solution containing Na-heparinate (Trade mark pularin, Evans Medical Ltd.), 1 mg/ml in isotonic (1 %) NaCl solution.

The fish were anaesthetized for about 10 min in well-aerated water containing 100 mg/l unbuffered MS 222 (tricaine methanesulphonate, Sandoz).

The cannulation procedure was a modification of that presented by Soivio *et al.* (1972), which is a modification of that presented by Smith & Bell (1964). The tip of the steel wire and the cannula were inserted into the vessel at the point where the dorsal aorta begins (Fig. 1). The steel wire was then pulled out while the cannula was held in place. The cannula was pushed into the aorta to its final position, and blood moving up the cannula under its own pressure indicated that the tip had entered the vessel. The cannula was filled with heparinized saline (1 mg/ml Na-heparinate) and closed with a spine of a hedgehog. The fish were marked with Carlin tags and rapidly transferred to the stock tanks, where they were kept under free-swimming conditions, without any further handling, until they were used for the experiments. During this time the fish were fed twice daily with pelletted salmon food (Ewos).

During the operations, which lasted about 5 min, no respiratory water was supplied to the gills.

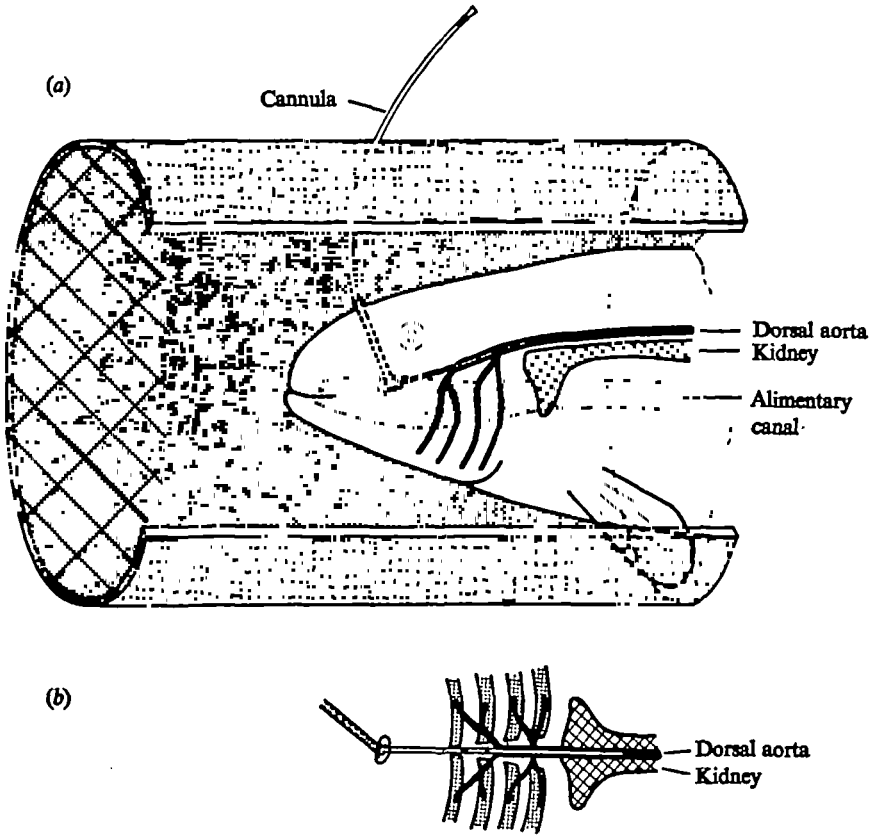

Fig. 1. A fish in the special receptacle. The position of the cannula in the dorsal aorta is viewed from two directions.

EXPERIMENTATION

After 3 weeks' recovery from the operation the fish were allowed or persuaded to swim into the handling receptacle placed in the water 1 day before the experiments. A check was made to ensure that the cannula was functioning, and the haematocrit value was determined.

The handling receptacle (Fig. 1) is similar to that described by Soivio & Oikari (1975). It is a cylinder made of black polyethylene tubing with a diameter of 15 cm, and has galvanized net with a 12 mm mesh at each end. The length of the receptacle is at least 25 cm greater than that of the fish. The cannula can be reached through a slit 1 cm wide, which extends along the length of the cylinder. The receptacles were placed, slit upwards, in two 4 m^2 tanks, suspended from the rim of the tanks with the slit level with the water surface. The tanks were kept in continuous artificial light. The circulating water streamed slowly (about 2 cm/sec) through the receptacles, with the fish facing 'upstream'. During the experiments the fish were not fed.

The effect of repeated blood sampling and immobilization was examined by dividing the fish in the receptacles into two groups:

Group I (controls): 8 ♀♀ (weight 864 ± 55 g (mean ± S.E.M.) and length 41·3 ± 0·6 cm)

from which blood was collected at the beginning (zero-time sample) and end (7-day sample) of a 1-week experimental period.

Group II: 8 ♀♀ (weight 816 ± 21 g and length 40·8 ± 0·3 cm), from which blood was collected at 0, 5, 20 and 60 min, 4, 12 and 24 h, and 2, 4 and 7 days after the beginning of the experiment (Fig. 2).

After completion of the first part of the experiment a possible 'receptacle effect' was investigated by letting the fish of both groups swim freely in the experimental tanks for 1 week, enclosing all of them again in the receptacles, as described above, and taking samples after 1 day. The possibility that the normal daily activities in the rearing hall produce variations in the haematological data was examined with Group III, which was formed from a number of trout earlier used in Groups I and II (12 ♀♀, weight 816 ± 27 g and length 40·9 ± 0·4 cm). After they had been kept in the receptacles for 1 day, samples were taken during the night, when there were no disturbances connected with the rearing work, according to the schedule given in Table 2.

BLOOD SAMPLING AND ANALYSES

All the blood samples were taken via the cannula. The tip of the cannula was dried on the outside thoroughly, the hedgehog spine was removed and blood was allowed to rise into the tubing; 0·5 ml samples were collected in 1 ml tuberculin syringes fitted into the cannula with a hypodermic needle. No heparin was added to the sample. After each sampling, the cannula was filled with isotonic saline (1 % NaCl) containing 1 mg/ml of Na-heparinate.

Haematocrit (Hct) was determined on each sample in Clay-Adams heparinized microhaematocrit tubes (No. 1025) within 3 min of the start of sampling (cf. Soivio, Nyholm & Westman, 1973; Soivio, Westman & Nyholm, 1974). The haemoglobin (Hb) concentration was determined by the cyanmethaemoglobin method using a Hitachi 101 spectrophotometer. Because of the colourless jelly-like formation often seen in the haemoglobin samples, they were centrifuged before they were measured. Blood lactate and glucose were determined according to the Biochemica-Test-Combination of Biochemica Boehringer GmBH (L-Lactat, GOD-Perid).

The remaining blood was transferred to polypropylene microtest-tubes and centrifuged for 1 min in a Beckman Spinco Microfuge 152 within 5 min of sampling. The inorganic electrolytes of the plasma were determined with a Perkin-Elmer atomic absorption spectrophotometer (Mod. 305), as described elsewhere (Kristoffersson, Broberg & Oikari, 1972), the diluent containing only 0·1 % lanthanum.

RESULTS

The test fish were all in good condition and all the cannulae were patent at the end of the experimental period, which lasted for more than 2 weeks (i.e. 5–6 weeks after cannulation). The Hct values (0·241 ± 0·021 in Group I and 0·235 ± 0·014 in Group II) recorded just after the fish were enclosed in the receptacles were slightly higher than the zero-time values in both groups recorded after 1 day's rest in the receptacle (Table 1).

There were no significant differences in the zero-time values of Hct and Hb between Groups I and II. After 1 week in the receptacle the Hct value of Group I

Table 1. *The zero-time and final values (7 days' sample) for the blood constituents studied in Groups I (controls) and II (sampled repeatedly for 1 week) compared with the values obtained after 1 week's free-swimming period*

(The mean ± S.E.M. and (number of analyses) are given)

Parameter and unit	Group I (controls)			Group II (sampled repeatedly)		
	Zero-time	7 days'	After 1 week's free-swimming period	Zero-time	7 days'	After 1 week's free-swimming period
Hct	0·216 ± 0·011 (8)	0·184 ± 0·009 (8)	0·206 ± 0·007 (7)	0·230 ± 0·009 (8)	0·151 ± 0·007 (8)	0·171 ± 0·006 (7)
Hb (g/l)	63·4 ± 3·2 (8)	53·3 ± 2·3 (8)	55·0 ± 2·1 (7)	63·2 ± 2·3 (8)	44·4 ± 2·2 (8)	48·6 ± 4·0 (5)
Glucose (g/l)	0·606 ± 0·065 (7)	0·550 ± 0·041 (7)	0·526 ± 0·038 (6)	0·479 ± 0·032 (7)	0·449 ± 0·030 (7)	0·586 ± 0·056 (5)
Lactate (g/l)	0·120 ± 0·016 (8)	0·074 ± 0·010 (8)	0·087 ± 0·006 (7)	0·122 ± 0·017 (8)	0·063 ± 0·007 (8)	0·078 ± 0·006 (5)
K^+ (m-equiv/l)	2·22 ± 0·07 (8)	2·41 ± 0·05 (7)	2·52 ± 0·09 (7)	2·30 ± 0·05 (8)	2·45 ± 0·02 (8)	2·34 ± 0·03 (5)
Na^+ (m-equiv/l)	149·6 ± 2·0 (8)	153·0 ± 1·1 (8)	152·7 ± 1·0 (7)	151·0 ± 1·1 (8)	155·6 ± 1·6 (8)	150·1 ± 1·7 (5)
Ca^{2+} (m-equiv/l)	4·19 ± 0·09 (5)	4·41 ± 0·03 (4)	4·37 ± 0·10 (7)	4·33 ± 0·10 (8)	4·40 ± 0·07 (8)	4·40 ± 0·04 (5)

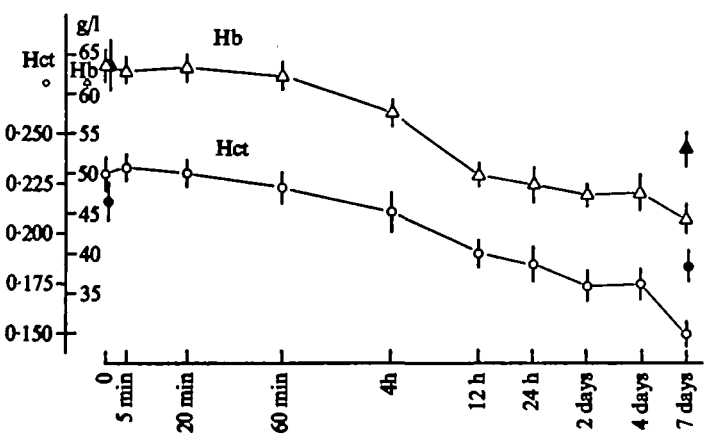

Fig. 2. Effects of repeated sampling on blood Hct and Hb values during experimental period of 1 week. Closed symbols for Group I; open symbols for Group II. The vertical bars indicate ± S.E.M.

fish decreased by 12·5% and the Hb value decreased by 15·8%. In Group II, where the fish were sampled repeatedly, the Hct and Hb values decreased steeply during the first 12 h period, and the decrease continued until after 1 week the Hct value had decreased by 34% and the Hb value by 30% below the zero-time value (Fig. 2). The final values for Hct and Hb were significantly smaller in this group than in Group I ($P < 0·01$ and $P < 0·02$, respectively).

In Group I the blood glucose level at the beginning and at the end (7-day sample)

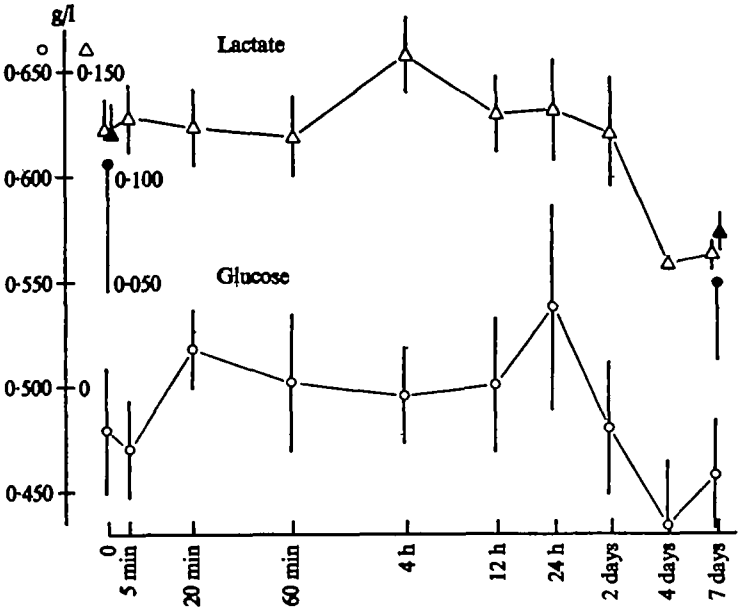

Fig. 3. Effects of repeated sampling on blood glucose and lactate concentrations. For further explanations see Fig. 2.

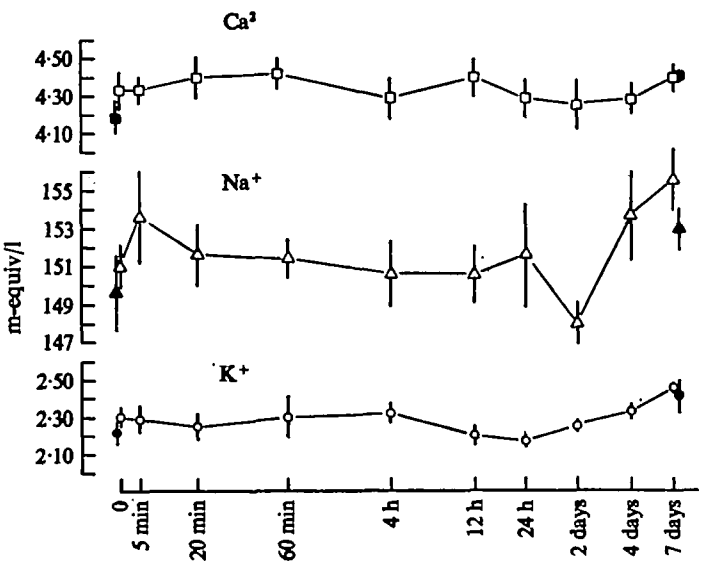

Fig. 4. Effects of repeated sampling on Na^+, K^+ and Ca^{2+} concentrations. For further explanations see Fig. 2.

of the first part of the experiment was on a higher level than in the repeatedly sampled animals of Group II, but the difference was not significant (Table 1). In Group II the glucose level was slightly elevated 20 min after the beginning of the experiment, remaining high until the 24 h sample, and then decreasing to a final value that was lower than the zero-time value (Fig. 3).

Table 2. *The values for blood constituents obtained in repeated sampling from Group III during the night and early morning*

(The mean ± S.E.M. and (number of analyses) are given.)

	Sampling time			
Parameter and unit	21.10–22.15	00.30–01.20	06.30–07.15	09.40–10.30
Hct	0·191 ± 0·008 (12)	0·187 ± 0·009 (12)	0·177 ± 0·008 (12)	0·165 ± 0·008 (12)
Hb (g/l)	53·0 ± 2·2 (12)	50·3 ± 2·4 (12)	47·8 ± 2·4 (11)	43·5 ± 2·2 (11)
Glucose (g/l)	0·539 ± 0·032 (10)	0·558 ± 0·034 (10)	0·598 ± 0·032 (10)	0·676 ± 0·031 (10)
Lactate (g/l)	0·083 ± 0·004 (12)	0·069 ± 0·006 (12)	0·075 ± 0·007 (12)	0·072 ± 0·004 (12)
K^+ (m-equiv/l)	2·44 ± 0·06 (12)	2·44 ± 0·05 (12)	2·41 ± 0·05 (12)	2·35 ± 0·04 (12)
Na^+ (m-equiv/l)	151·6 ± 1·0 (12)	151·0 ± 0·6 (12)	148·9 ± 1·0 (12)	148·5 ± 0·7 (12)
Ca^{2+} (m-equiv/l)	4·38 ± 0·06 (12)	4·41 ± 0·06 (12)	4·36 ± 0·08 (12)	4·41 ± 0·07 (12)
Mg^{2+} (m-equiv/l)	1·35 ± 0·04 (12)	1·32 ± 0·03 (12)	1·33 ± 0·03 (12)	1·32 ± 0·03 (12)

The blood lactate concentration in both groups decreased markedly during the week in the receptacles (by 32% in Group I and 18·5% in Group II, Table 1). In Group II the lactate concentration remained at approximately the zero-time level for the first 2 days and then fell markedly before the two final samples (Fig. 3).

A small increase in the concentrations of Ca^{2+}, Na^+ and K^+ was observed in both groups during the experiment (Table 1, Fig. 4). The differences between the two groups were not significant.

The Hct and Hb values both showed a decreasing trend during the night-time sampling of Group III (Table 2). The glucose level of the blood increased during the sampling period. The most marked increase took place between the two last sampling periods in the morning. No variations were evident in the lactate concentrations. The plasma Na^+ concentration had a marked decreasing trend. The plasma K^+ concentration also declined, but the difference between the first and last sample was not very marked. The divalent ions studied showed no differences during the night-time sampling period.

The blood values obtained after one week's free-swimming period subsequent to the period in the receptacles have been presented in Table 1. The Hct, Hb and lactate values showed an increasing trend and Na^+ a decreasing trend in both Groups. The other values showed different trends in Group I from those in Group II.

DISCUSSION

A number of recent haematological investigations refer to the fact that fish seem to be much more sensitive to the effects of physical and chemical changes than was earlier supposed. Anaesthetization and the associated handling of the experimental

fish have been demonstrated to produce considerable qualitative and quantitative variations in haematological data (e.g. Houston *et al.* 1969, 1971*a*, *b*; Chavin & Young, 1970; Klontz, 1972; Wedemeyer, 1972). These reports throw doubt on the validity of a considerable amount of the haematological information at present available, as has also been noted by Houston *et al.* (1969) and Klontz (1972).

In the light of these observations, more attention has recently been paid to the acclimatization of fish to standard experimental conditions (e.g. temperature, diet, photoperiod, aquaria, etc.) as well as to the procedures in handling and sampling fish. The cannulation of fish is perhaps the most promising method for repeatedly obtaining representative and reproducible blood samples from minimally stressed fish (e.g. Garey, 1969; Houston, 1971; Soivio *et al.* 1972). With the dorsal aorta cannulation technique it is possible to postpone experimental sampling until the haematological parameters indicate that the fish have completely recovered from the cannulation procedure together with the associated anaesthetization and other manipulation. According to present knowledge, the recovery of salmonids from the cannulation procedure takes place in several phases and 2–3 weeks seem to be needed before the most strongly affected parameters stabilize (e.g. Houston *et al.* 1971*b*; Soivio *et al.* 1972; Wedemeyer, 1972).

The zero-time values of Hct and Hb obtained in the present study are smaller than those earlier presented in the literature for rainbow trout (e.g. Black *et al.* 1959; Schiffman & Fromm, 1959; Snieszko, 1961; Normandeau, 1962; Hunn, Schoettger & Whealdon, 1968; Barnhart, 1969). The main reasons for this may be that in the present study the fish were not anaesthetized, removed from the water or handled prior to sampling; also an improved technique was employed for determining Hct (Soivio *et al.* 1973, 1974). In addition, the motility of the experimental trout was reduced by the receptacles; activity has been shown to elevate Hct and Hb values (discussed by Soivio & Oikari, 1975).

The differences between the values recorded for the blood parameters of the trout after the 1 week in the receptacle and those obtained after 1 week under free-swimming conditions may be the result of differences in the motility of the fish. This seems to indicate that the level of these blood parameters in fish kept in receptacles and in free-swimming fish is different.

The differences in the Hct and Hb values between Groups I and II (Table 1) may mainly be attributed to differences in haemodilution between the two groups. These are mostly due to the greater amount of erythrocytes withdrawn from the repeatedly sampled group; *ca.* 1 ml of erythrocytes (i.e. about 4·5 ml of blood) was taken, representing about 20% of the volume of the circulating red cells in the experimental fish (Conte, Wagner & Harris, 1963; Nyholm, unpublished data). In Group III, which was formed to find out whether environmental disturbance occurred in the daytime (cf. Table 2), repeated sampling also resulted in haemodilution. Immobilization for 8 days seems to decrease the Hct value by about 10% when compared to the free-swimming fish. Soivio *et al.* (1974) have shown that the haemoconcentration induced by hypoxic stress is evidently due to the shift of plasma from circulation to the tissues. Thus a 'hypo-stressed', or totally undisturbed, state may induce a reverse shift of plasma leading to the haemodilution observed. The Hct and Hb values in Group II did not decrease markedly before the 4 h sample. This indicates

that the time needed for a fish to respond to the withdrawal of a sample was between 4 and 12 h. An alternative explanation is that blood sampling and handling (e.g. introduction of the fish to the receptacle) results in a haemoconcentration. Then several days are required for these blood parameters to return to their normal lower values.

The blood glucose level seems to be a very sensitive indicator of disturbance and usually rises after short-term stress or exercise in many teleost fish (see Chavin & Young, 1970; Wedemeyer, 1972; Soivio & Oikari, 1975, for references), the rise being less after minor stress or in trained fish (Hammond & Hickman, 1966). In the light of these observations, the increased blood glucose values indicate some kind of disturbance from the 20 min sample on to the 24 h sample. After one more day in the receptacle (2-day sample), the zero-time level was regained and the blood glucose values subsequently stayed below this level. The increase in the glucose level between the morning samples in Group III indicates that the normal rearing routine in the hall (several people walking on the concerete floor, the feeding of the fish and possible sound effects) is sufficient to function as some kind of 'Zeitgeber' for a possible circadian rhythm, even in continuous light.

No signs of stress were indicated by the blood lactate values. The marked decrease in the lactate level may indicate that the fish do not become accustomed to the receptacle until after 3–5 days' acclimatization, differing from values given by Heath (1972) for respective measurements. The low lactate levels for the same fish after 1 weeks' free-swimming may indicate that fish become used to handling.

The values for the sodium ion concentration may possibly be somewhat too high, because the cannula was filled with 1% NaCl solution between samplings. The likelihood of this error was decreased by discarding several drops of blood before sampling and in any case it may be expected to be identical at every sampling time.

The changes seen in the potassium and sodium ion concentrations when the environmental effects were studied (Group III) indicate that the fish may still have been slightly disturbed after only 1 day in the receptacle. Another possibility is that fish are not able to maintain the ionic balance when the withdrawal of blood samples exceeds a certain rate. The increase in the levels of these ions during the first week of the experiment may be connected with the plasma shift discussed above (cf. Houston *et al.* 1969, 1971*b*; Soivio & Oikari, 1975).

In the present investigation the rainbow trout remained peaceful in the receptacles throughout the experiments. Blood sampling was very easy and quick, as the time-consuming netting of the fish prior to sampling was not needed. The sampling did not cause any visible disturbances in the fish, which often occur when samples are taken from free-swimming cannulated fish (Heath, 1972). The elevated glucose values indicate that the handling of the cannula and the sampling procedure itself disturb the fish to some extent, when sampling is frequent, but the stress caused was evidently slight. This is also indicated by the fact that the general variation in blood parameter values recorded in the present study is very small compared with that in the values presented in the literature for rainbow trout. The difference in the levels of the blood values observed between fish kept in receptacles and under free-swimming conditions, and the changes induced by the normal rearing routine

in the hall underline the need to standardize experimental conditions and the sampling schedule.

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