

THE EFFECT OF GRADED METHAEMOGLOBIN LEVELS ON THE SWIMMING PERFORMANCE OF CHINOOK SALMON (*ONCORHYNCHUS TSHAWYTSCHA*)

C. J. BRAUNER, A. L. VAL* AND D. J. RANDALL

*Department of Zoology, University of British Columbia, Vancouver, British Columbia,
Canada, V6T 1Z4*

Accepted 3 August 1993

Summary

Nitrite oxidizes haemoglobin (Hb) to methaemoglobin (MetHb), which is unable to bind oxygen. Nitrite exposure can therefore be used as a tool to manipulate the oxygen-carrying capacity of the blood without changing haematocrit. The objective of this study is to examine the relationship between the critical swimming velocity (U_{crit}) and the functional haemoglobin concentration ([Hb]) of the blood in adult chinook salmon. Functional [Hb] was reduced by increasing MetHb levels through intraperitoneal administration of a mass-dependent volume of sodium nitrite. In resting fish, MetHb levels were found to stabilize at 25% of total [Hb] 3h after the injection of 30mgkg^{-1} sodium nitrite. Methaemoglobin levels increased in proportion to the amount of sodium nitrite injected and reached a maximum (following the injection of 90mgkg^{-1} sodium nitrite) of 51.8% in resting fish and 72% in fish forced to swim to U_{crit} . At 60 and 90mgkg^{-1} sodium nitrite, MetHb formation was greater in exercised than in resting fish. A second-order regression revealed that U_{crit} was virtually independent of functional [Hb] between 51 and 100% of control functional [Hb], but was positively correlated with functional [Hb] below 51% of total [Hb] (4.5g dl^{-1}). The insensitivity of U_{crit} to a functional [Hb] greater than 51% may be partly due to the exponential increase in aerobic metabolism required to provide the power to overcome hydrodynamic drag at higher water velocities. There were no significant changes in intraerythrocytic organic phosphate (adenylates and guanylates) concentrations standardized to [Hb] in swimming or resting fish over the range of MetHb levels induced in this study. Fish may encounter nitrite naturally; if MetHb levels become severely elevated as a result, swimming ability will be significantly impaired.

Introduction

Nitrite is toxic to fish and can be found in both natural aquatic systems and aquacultural facilities. Its presence in natural water systems occurs when nitrate, which is used as an

*Present address: Instituto Nacional de Pesquisas da Amazônia, Alameda Cosme Ferreira, 1756, 69083 Manaus, AM Brasil.

Key words: U_{crit} , methaemoglobin, functional haemoglobin, organic phosphates, chinook salmon, *Oncorhynchus tshawytscha*.

agricultural fertilizer in many countries, is reduced to nitrite by microbial anaerobic metabolism (Kiese, 1974). In fish-rearing facilities, inadequate water flow rates may lead to an accumulation of ammonia which can subsequently be oxidized by bacteria to nitrite (Collins *et al.* 1975).

Nitrite is taken up across the gills of fish and may accumulate to levels far in excess of that dissolved in the environmental water (Eddy *et al.* 1983; Jensen *et al.* 1987). Once within the circulatory system, nitrite crosses the membrane of the erythrocyte (leaving it intact), where it oxidizes the iron in haemoglobin (Hb) from its ferrous (II) to its ferric (III) state, creating methaemoglobin (MetHb), which does not bind oxygen. Thus, a primary toxic action of nitrite is the impairment in oxygen transport subsequent to MetHb formation. Recent work by Jensen *et al.* (1987) and Jensen (1990) has demonstrated that exposure of carp to aqueously dissolved nitrite is associated with ion-regulatory and acid-base disturbances, which are another mode of nitrite toxicity.

There have been relatively few studies designed to look specifically at the importance of the oxygen-carrying capacity of the blood to aerobic swimming. Attempts to increase the oxygen-carrying capacity of the blood by exposure to hyperoxia did not increase the critical swimming velocity (U_{crit}) in salmonids (Davis *et al.* 1963; Brett, 1964). Environmental hypoxia has been demonstrated to reduce U_{crit} in salmonids (Jones, 1971; Bushnell *et al.* 1984); however, the exact relationship between the oxygen content of the blood and U_{crit} remains unknown. Jones (1971) induced haemolytic anaemia in rainbow trout by intraperitoneal injections of phenylhydrazine hydrochloride and found a 34–40% reduction in maximal aerobic swimming velocity with 50–66% reductions in haematocrit (Hct); however, no strict relationship between U_{crit} and Hct could be determined from these data. Pearson and Stevens (1991) recently demonstrated a relationship between U_{crit} and Hct in splenectomized and sham-operated rainbow trout, but Gallagher *et al.* (1992) were unable to demonstrate such a relationship. The relationship between U_{crit} and Hct therefore remains both controversial and unresolved.

Changes in haematocrit affect blood viscosity, which may influence oxygen delivery and thus aerobic swimming performance. Nitrite oxidizes Hb to MetHb, which does not bind oxygen, so nitrite can be used as a tool to manipulate the oxygen-carrying capacity of the blood without altering haematocrit and thus blood viscosity. The main objective of this study is to examine the effect of graded reductions in the functional [Hb] of the blood, by inducing methaemoglobinaemia, on the U_{crit} of chinook salmon.

Nitrite accumulates within fish during direct exposure to ambient dissolved nitrite (Smith and Williams, 1974; Smith and Russo, 1975) or following intraperitoneal administration of sodium nitrite dissolved in saline (Bartlett *et al.* 1987). Although the mechanism of uptake of nitrite from the environment is unclear, nitrite entry is associated with a reduction in chloride uptake, leading to an electrolyte disturbance (Williams and Eddy, 1986). Intraperitoneal injections of sodium nitrite markedly elevate the rate of MetHb formation and circumvent part of the ionic imbalance associated with nitrite exposure (Jensen *et al.* 1987). For these reasons, we chose to elevate internal nitrite concentrations by intraperitoneal injection. The dynamics of MetHb formation following intraperitoneal injections of sodium nitrite has not previously been examined in chinook salmon, so temporal and dose-dependent changes in MetHb concentration following

sodium nitrite administration were examined prior to the swimming component of this study.

Materials and methods

Fish acquisition and holding facilities

Chinook salmon, *Oncorhynchus tshawytscha* (mass 799 ± 36.1 g; fork length 41.6 ± 3.4 cm), were obtained from the Pacific Biological Station, Nanaimo, Department of Fisheries and Oceans, Canada, or purchased from Barkley Marine Farms Barkley Sound, BC (mass 376 ± 11.6 g; fork length 32.0 ± 0.32 cm). They were maintained in sea water (14.3 ± 0.13 °C) in flow-through tanks. Fish were held for 2 weeks at the Bamfield Marine Station, where all experiments were conducted, and were starved for 2 days prior to experimentation. Over the duration of the study, the PO_2 of incoming water was 20.3 ± 0.21 kPa (152 ± 1.6 mmHg) and water pH was 7.6 ± 0.20 .

Experimental procedure

Series I: temporal effects of 30mgkg^{-1} sodium nitrite

Chinook salmon from the larger group were individually netted and anaesthetized in a 1:10000 solution of tricaine methanesulphonate (MS-222) in sea water. Each fish was placed on a surgery table similar to the one described by Smith and Bell (1967), the gills were irrigated with a solution of 1:30000 MS-222 and the dorsal aorta was cannulated with polyethylene tubing (Clay Adams PE-60) according to the method of Soivio *et al.* (1975). Mass and length were recorded and the fish was left to recover in sea water in a black Perspex box for 24h. After 24h, the fish was lightly anaesthetized in MS-222 and intraperitoneally injected with a mass-dependent volume (1mlkg^{-1}) of 30mgml^{-1} sodium nitrite dissolved in Cortland saline to achieve a body concentration of 30mgkg^{-1} sodium nitrite. Blood samples of $400\mu\text{l}$ were withdrawn from the cannula prior to anaesthesia and sodium nitrite injection, and every 30min after sodium nitrite injection for 3h. An identical volume of heparinized saline replaced the blood removed at each sampling time to minimize changes in blood volume. Blood was analyzed for pH, haematocrit (Hct), total haemoglobin [Hb] and percentage methaemoglobin (% MetHb) as described below.

Series II: dose-dependent effects of sodium nitrite

Chinook salmon from the smaller size group were lightly anaesthetized with MS-222 (1:30000), weighed and injected intraperitoneally with a mass-dependent volume (1mlkg^{-1}) of 0, 30, 60, 75 or 90mgml^{-1} sodium nitrite in Cortland saline to achieve body concentrations of 0, 30, 60, 75 or 90mgkg^{-1} , respectively. Following the injection, fish were left to recover in sea water in black Perspex boxes for 3h, after which they were again lightly anaesthetized and $700\mu\text{l}$ of blood was removed from the caudal vein by puncturing the vessel just caudal to the anal fin. Blood was analyzed for total [Hb], % MetHb and organic phosphates as described below.

Series III: effect of methaemoglobinaemia on U_{crit}

The respirometer used in this study was a 550l modified Brett-type respirometer, with a swim chamber diameter of 15.3cm. It is an open system, so oxygen consumption rates could not be measured. A continual overflow of sea water maintained P_{O_2} near air-saturation levels (P_{O_2} of incoming water was 20.3 ± 0.21 kPa, 152 ± 1.6 mmHg) and the respirometer was regulated at 14.3°C for the duration of the study. Fish from the smaller size group were lightly anaesthetized and their mass, length, width and height were recorded. They were intraperitoneally injected with a mass-dependent volume of 0, 30, 60, 75 or 90mg l^{-1} sodium nitrite in Cortland saline to achieve body concentrations of 0, 30, 60, 75 and 90mg kg^{-1} , respectively, and then placed in the respirometer for a 2h introductory period. Fish quickly regained equilibrium following handling, and water velocity was increased to 20cm s^{-1} for the first hour and then to 40cm s^{-1} for the second hour of the introductory period.

The critical swimming velocity (U_{crit}) test is a standardized method used to quantify maximal swimming velocity in fish (Beamish, 1978). The U_{crit} test was initiated following the 2h introductory period, and entailed increasing the water velocity by 20cm s^{-1} every 30min until the fish fatigued. Fatigue was defined as the velocity at which the fish could no longer maintain its position in the swim tube; at which time it adopted a pronounced 'burst and glide' swimming strategy or collapsed completely onto the downstream electrified (5V) grid. The U_{crit} was determined by calculating the product of the completed proportion of the fatigue increment and 20cm s^{-1} (the magnitude of the velocity increment) and adding this value to the water velocity of the last increment completed (Brett, 1964). This value was expressed in body lengths per second (BL s^{-1}) to standardize for small variations in fish length. The U_{crit} was corrected for solid blocking effects of the fish as described by Bell and Terhune (1970). The respirometer was calibrated for water velocity prior to the experiment and did not require recalibration over the course of this study.

When fatigued, the fish were lightly anaesthetized and $700\ \mu\text{l}$ of blood was removed by caudal puncture. Blood was analyzed for Hct, total [Hb], percentage MetHb and organic phosphates as described below. The caudal puncture was used in this series and in series II because cannulation resulted in significantly reduced U_{crit} values and high mortality. In some instances, fish used in series III were used for the experiments of series II, but these fish were allowed to recover for at least 72h after the U_{crit} test. This should be adequate for recovery from exercise (Primmitt *et al.* 1986; Randall *et al.* 1987) and, in preliminary experiments, % MetHb had been restored in less than 24h to the level measured in the blood prior to sodium nitrite injection.

Analytical techniques

Blood samples were immediately analyzed for pH, Hct, total [Hb] and percentage MetHb. Whole-blood pH was measured using a temperature-regulated Radiometer Copenhagen G297/G2 glass capillary electrode in conjunction with a Radiometer PHM71 acid-base analyzer. The haematocrit (ratio of packed red blood cell volume to whole-blood volume) was measured in $60\ \mu\text{l}$ heparinized microhaematocrit tubes

following centrifugation at 11500revsmin^{-1} for 3min in a Damon IEC MB microhaematocrit centrifuge. Total [Hb] was measured using a Sigma total haemoglobin (525-A) assay kit. The relative absorbency was measured at 540nm in a Bausch and Lomb spectronic 20 colorimeter. The mean cellular [Hb] was calculated as $([\text{Hb}]/\text{Hct})\times 100$.

Percentage MetHb was measured by modifying the method of Bartlett *et al.* (1987). Briefly, 50 μl of whole blood was added to 3.0ml of distilled water and centrifuged for 6 min at 3800revsmin^{-1} in a Roto-Uni centrifuge. The supernatant was dispensed equally into two test tubes. The absorbency of the supernatant in test tube 1 was measured at 630nm in the colorimeter prior to (1a) and following (1b) the addition of a crystal of potassium cyanide KCN to the solution. The optical density of the supernatant in test tube 2 was measured initially (2a, and compared with 1a), following the addition of a potassium ferricyanide $[\text{K}_3\text{Fe}(\text{CN})_6]$ crystal (2b) and after subsequent addition of a crystal of (KCN) (2c). Optical density was recorded after the reading had stabilized, which required up to 5min. The percentage MetHb was calculated as follows:

$$\% \text{MetHb} = (1a - 1b)/(2b - 2c) \times 100.$$

Total functional [Hb] was calculated by subtracting MetHb concentration from total [Hb].

Blood used for determining organic phosphate concentration was prepared by mixing 200 μl of blood with 200 μl of 0.6mol l^{-1} trichloroacetic acid (TCA) in a 1.5ml polypropylene Eppendorf micro test tube. The solution was centrifuged for 3min in an Eppendorf centrifuge 3200 and the supernatant was removed and neutralized with 22 μl of 6mol l^{-1} KOH. The samples were frozen in liquid nitrogen and kept frozen until the analyses could be performed.

Adenosine phosphate and guanosine phosphate concentrations were determined from 25 μl samples of the neutralized supernatant (described above) by high pressure liquid chromatography (HPLC) using an LKB-Bromma 2152 HPLC controller and 2150 titanium pump supplied by a 11300 Ultrograd mixer driver. The system was coupled to a Bio-Rad flow-through ultraviolet monitor (model 1306), adjusted to 254nm, and to an LKB-Bromma 2220 recording integrator. The separation was performed on an Aquapore AX-300 (220mm \times 4.6mm) weak ion exchanger (Brownlee laboratories) modified from Schulte *et al.* (1992). Briefly, the first 5min of the elution was isocratic (60mmol l^{-1} KH_2PO_4 , pH3.2), followed by a continuous gradient from 60mmol l^{-1} KH_2PO_4 , pH3.2, to 750mmol l^{-1} KH_2PO_4 , pH3.5, over 9min. The final buffer was eluted for 15min and the column was equilibrated for 5min with the starting buffer prior to the injection of the next sample. For all determinations, the column was regulated at 55°C with a constant flow rate of 2mlmin^{-1} . Analytical reagent grade KH_2PO_4 was purified using a Bio-Rad Econo column, diluted, buffered and filtered as described by Schulte *et al.* (1992).

Solutions of AMP, ADP, ATP, GMP, GDP and GTP from Sigma Chemical Co. were used to construct standard curves which were linear throughout the range required ($r^2=0.97$). The concentrations of all standard solutions were double-checked spectrophotometrically.

Statistics

All data are reported as mean ± 1 standard error of the mean (S.E.M.). Statistical differences between the treatment means were determined by *t*-test or by using a completely randomized design analysis of variance (ANOVA), followed by a Dunnett's multiple-comparison test with a probability level of 5% chosen as the limit of statistical significance. Coefficients of determination were computed by least-squares regression and tested for statistical significance.

Results

Series I: temporal effects of 30mgkg⁻¹ sodium nitrite

Six resting fish were injected intraperitoneally to achieve body concentrations of 30mgkg⁻¹ sodium nitrite to induce the formation of MetHb (Fig. 1). The effect of this treatment was seen very rapidly: MetHb, expressed as a percentage of total [Hb], increased significantly from the control level of 3.25% to 12.6% within 30min of sodium nitrite injection. The percentage MetHb increased to approximately 25% within 3h of the injection.

During this 3h period, there were no statistically significant changes from control values of pH (7.73 \pm 0.081), Hct (26.8 \pm 1.5%), total [Hb] (8.8 \pm 0.28 g dl⁻¹ blood) or mean cell haemoglobin concentration (MCHC: 33.5 \pm 2.67 g dl⁻¹ packedredbloodcells) (data not shown).

Series II: dose-dependent effects of sodium nitrite

Six resting fish were injected intraperitoneally with sodium nitrite to achieve body concentrations of 0, 30, 60, 75 and 90mgkg⁻¹ sodium nitrite. Blood samples were taken 3h after the injection because this was determined to be the time required for MetHb levels to stabilize in resting fish (series I). The percentage MetHb increased with the amount of sodium nitrite injected, from a control level of 17.5% to 51.8% at the highest sodium nitrite dose administered (Fig. 2).

Control values for haematocrit, [Hb] and MCHC are listed in Table 1. Total [Hb] remained constant, but there was a significant decrease in Hct from a control value of 39.3 to between 30.7 and 32.9 at sodium nitrite concentrations of 60mgkg⁻¹ or greater. The significant increase in MCHC from 27.7 to 35.3 g dl⁻¹ packedredcells at 75mgkg⁻¹ sodium nitrite is indicative of red cell shrinkage (data not shown).

Adenosine and guanosine phosphate concentrations were measured in whole blood and are expressed relative to the volume of packed red blood cells, and [ATP] and [GTP] are expressed relative to [Hb] (Table 1). There were no statistically significant differences between red cell adenylate and guanylate concentrations measured in resting animals and those in animals treated with sodium nitrite concentrations (data not shown).

Series III: effect of methaemoglobinaemia on U_{crit}

The 90mgkg⁻¹ sodium nitrite intraperitoneal injection was the only dose to reduce U_{crit} significantly (Fig. 3) and was associated with the greatest proportion of MetHb

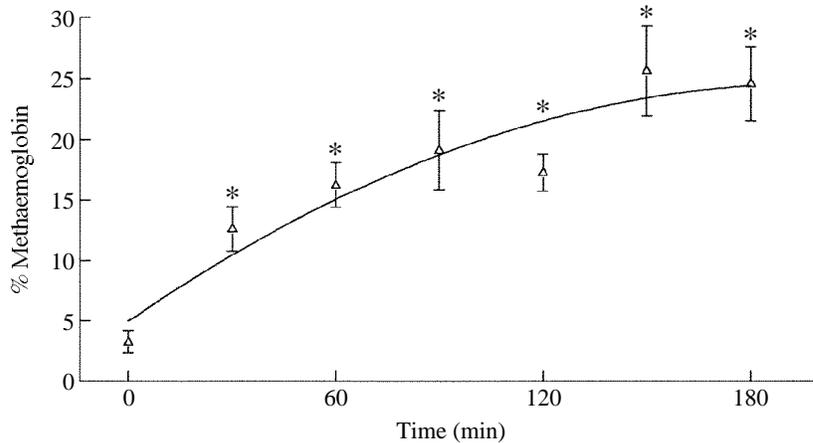


Fig. 1. The change in percentage methaemoglobin over time in the group of large resting chinook salmon injected intraperitoneally with 30mgkg^{-1} sodium nitrite. Asterisks indicate statistically significant differences from the time 0 measurement. Error bars represent one standard error ($N=6$; $r^2=0.992$).

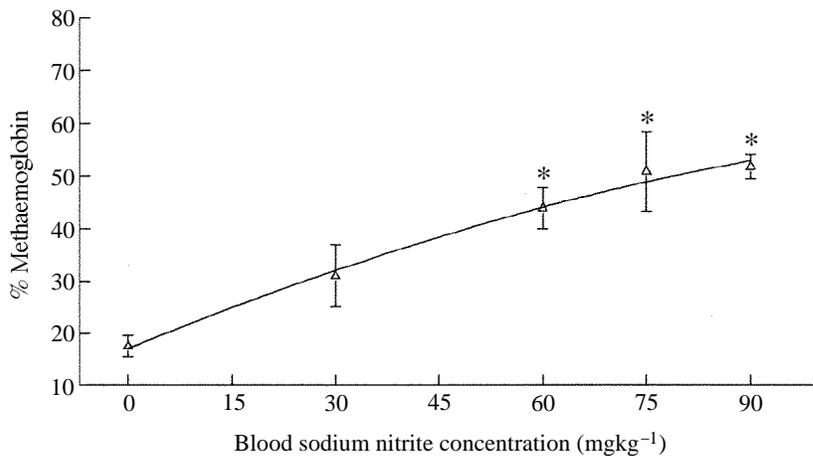


Fig. 2. The effect of different sodium nitrite concentrations on percentage methaemoglobin 3 h following intraperitoneal injection in the group of small resting chinook salmon. Asterisks indicate statistically significant differences from the control value. Error bars represent one standard error ($N=6$; $r^2=0.906$).

formation (Fig. 4). In a few instances, fish injected with 90mgkg^{-1} sodium nitrite fatigued near the end of the 40cm s^{-1} introductory period, prior to the first velocity increase of the U_{crit} test. Although significant elevations in percentage MetHb were detected in the fish injected with 60 and 75mgkg^{-1} sodium nitrite, their U_{crit} was not significantly affected. It is interesting that at 60 and 90mgkg^{-1} sodium nitrite the percentage MetHb of swimming fish was significantly higher than that of resting fish (compare Figs 2 and 4).

Table 1. Control haematological variables in non-swimming (series II) and swimming (series III) chinook salmon

Variable	Non-swimming (N=10)	Swimming (N=6)
Haematocrit (%)	39.3±1.5	37.0±1.8
Hb (gdl ⁻¹ blood)	10.8±0.35	10.8±0.59
MCHC (gdl ⁻¹ redbloodcells)	27.7±0.69	29.0±0.74
ATP:Hb	0.506±0.026	0.591±0.035
GTP:Hb	0.123±0.008	0.099±0.006
ATP	2.08±0.086	2.54±0.161
ADP	0.317±0.026	0.321±0.021
AMP	0.273±0.037	0.287±0.020
GTP	0.511±0.038	0.424±0.022
GDP	0.064±0.007	0.021±0.002
GMP	0.523±0.034	0.575±0.042

Values are mean ± S.E.M.

Organic phosphate concentrations are expressed as NTP:Hb ratio and μmolml^{-1} packed red bloodcells, respectively.

MCHC, mean red cell haemoglobin concentration.

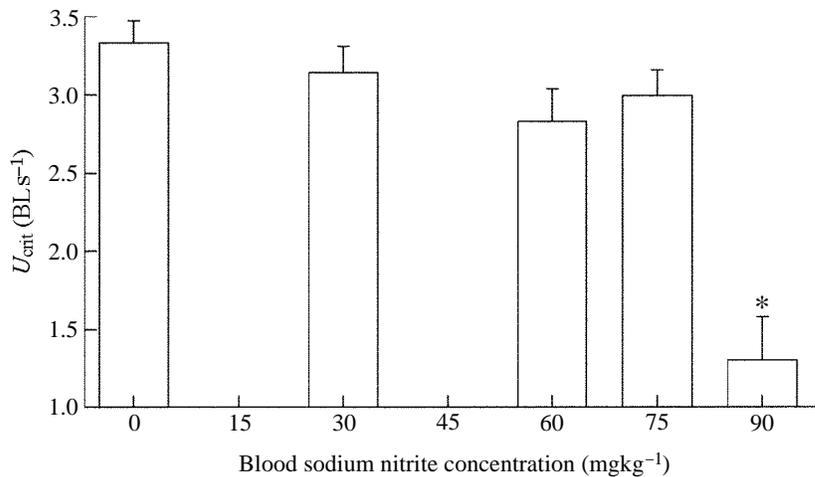


Fig. 3. The effect of sodium nitrite injections on the critical swimming velocity (U_{crit}) in the group of small chinook salmon. (See Fig. 2 for further explanation.)

Individual measurements of U_{crit} and total functional [Hb] measured at fatigue are shown in Fig. 5. The coefficient of determination for a best-fitting second-order regression (solid line) is highly significant ($r^2=0.50$). The shape of this regression indicates that, for a given reduction in total functional [Hb], there is a much greater decrease in U_{crit} in fish possessing low levels of functional Hb than in those with near control levels.

Control values for haematocrit, [Hb] and MCHC in swimming fish are listed in

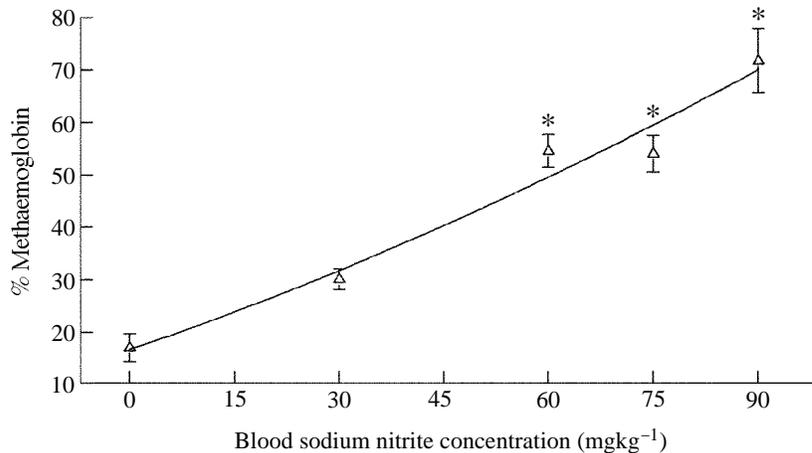


Fig. 4. The effect of sodium nitrite injections on percentage methaemoglobin measured at fatigue in fish swum to U_{crit} ($r^2=0.968$). (See Fig. 2 for further explanation.)

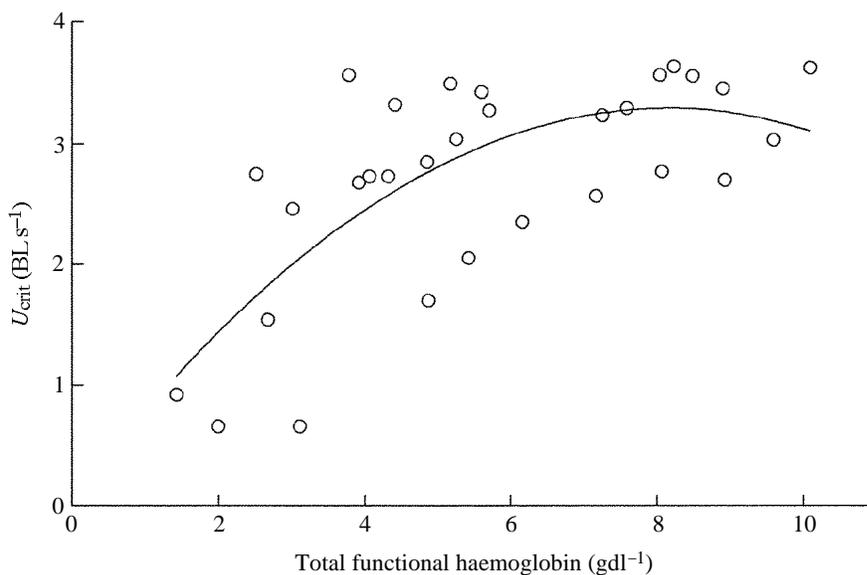


Fig. 5. The relationship between U_{crit} and total functional haemoglobin concentration measured at fatigue. Symbols represent individual fish and the line is the best-fitting second-order regression of these data ($r^2=0.50$, $y=0.03547+0.80145x-0.04934x^2$).

Table 1. Total [Hb] of fatigued fish did not differ from control values in any treatment (data not shown). Haematocrit was significantly reduced from a control value of 37% to 29% at 75mgkg^{-1} sodium nitrite and MCHC increased significantly from a control value of 29.0 to 33.6g Hbdl^{-1} packed red blood cells at 90mgkg^{-1} sodium nitrite, indicating that elevated blood nitrite concentration is associated with shrinkage of the red blood cells, as seen in resting fish of series II (data not shown).

Adenosine and guanosine phosphate concentrations in control swimming fish are listed in Table 1. There were no significant changes in the adenosine or guanosine phosphate levels with increasing MetHb levels in swimming fish (data not shown).

Discussion

Swimming performance

Total functional [Hb] was manipulated in chinook salmon by intraperitoneal injections of sodium nitrite to induce methaemoglobinaemia. The only condition to reduce U_{crit} significantly was the injection of 90mgkg^{-1} sodium nitrite (Fig. 3), which induced a mean MetHb level of 72%. The 60 and 75mgkg^{-1} sodium nitrite treatments induced 52% and 53% MetHb respectively, but did not significantly affect U_{crit} . The data are best described by a second-order regression which reveals that U_{crit} is significantly correlated with the functional [Hb] in the blood ($r^2=0.50$, Fig. 5), but there is no statistically significant effect of functional [Hb] on U_{crit} until functional [Hb] is reduced to less than 51% of the control level. This is consistent with the result of Gallagher *et al.* (1992), who were unable to find a difference in U_{crit} between splenectomized and sham-operated rainbow trout. In an earlier study, however, Pearson and Stevens (1991) reported that splenectomy significantly reduced U_{crit} and they were able to demonstrate a weak, but significant, relationship between U_{crit} and blood [Hb].

There have been many studies in fish designed to examine the whole-animal metabolic cost of swimming at different velocities. Generally, the rate of oxygen consumption of a fish increases exponentially with swimming velocity (Brett, 1964; Webb, 1971) because the power required to overcome hydrodynamic drag increases as the square of the swimming velocity (Webb, 1978). The power required to overcome hydrodynamic drag is supplied by the propulsive musculature, which is fuelled by oxygen when swimming is wholly aerobic. Assuming that the maximum aerobic swimming velocity is determined by the maximal rate of oxygen consumption, which is in turn determined by the amount of functional Hb in the blood, one might expect the shape of the curvilinear relationship between the rate of oxygen consumption and submaximal swimming velocity also to describe the relationship between functional [Hb] and U_{crit} . One would therefore expect a given reduction in functional [Hb] to have the greatest effect on U_{crit} when functional [Hb] was low, as is seen in Fig. 5. Conversely, reduction in functional [Hb] will have little effect on U_{crit} until functional [Hb] has been substantially reduced. Given these assumptions, the shape of the relationship in Fig. 5 can be partly accounted for by the exponential increase in power (and therefore in rate of oxygen consumption) required to overcome hydrodynamic drag with an increase in swimming velocity. These assumptions, however, greatly oversimplify the relationship between U_{crit} and functional [Hb] because (i) the maximal rate of oxygen consumption might not be determined by functional [Hb] and, (ii) the U_{crit} test is not entirely aerobic.

Maximal oxygen consumption rate is probably not proportional to functional [Hb] for several reasons. Jensen *et al.* (1987) observed a reduction in the oxygen saturation of functional Hb in carp following 48h of exposure to aqueously dissolved nitrite. The oxygen-carrying capacity of the blood is, therefore, not necessarily proportional to the

amount of total functional Hb. In addition, fish may compensate for a reduction in functional [Hb] at a given swimming velocity by increasing arterial–venous oxygen content difference, or cardiac output, or both. The degree to which blood is redistributed from the gut may also be an important compensation to maintain oxygen delivery to the active muscles when functional [Hb] is reduced (Thorarensen *et al.* 1993). Therefore, the relationship between functional [Hb] and maximal rate of oxygen consumption may be complex. This relationship is further complicated by the possibility that there may be a difference in the anaerobic contribution to U_{crit} in animals with low functional [Hb]. This cannot be ruled out because oxygen consumption rate was not measured in this study.

The U_{crit} test was used to quantify swimming performance in this study. Metabolism during the U_{crit} test has traditionally been thought to be aerobic (Brett, 1964), but there is evidence for a significant anaerobic component. Jones (1982) has estimated that maximum aerobic power output is sufficient to maintain speeds up to approximately 80% of U_{crit} in salmonids but, as a fish approaches fatigue, there is a sequential recruitment of anaerobic muscle fibre types (Bone, 1978; Bone *et al.* 1978). Bushnell *et al.* (1984) found no difference in the oxygen consumption rate between rainbow trout swimming at the same submaximal velocity in normoxic or hypoxic water, but U_{crit} was reduced in the hypoxic environment as a result of the lower maximal rate of oxygen uptake. Therefore, although there is undoubtedly some contribution of anaerobic metabolism to U_{crit} , the critical swimming velocity test is at least a rough estimate of the maximal aerobic swimming velocity in fish.

The insensitivity of U_{crit} to functional Hb concentrations greater than 51% of control levels might not be fully accounted for by the exponential increase in power (and therefore in rate of oxygen consumption) required to overcome hydrodynamic drag with an increase in swimming velocity, as described above. The concentration of haemoglobin found in fish may, therefore, be important for processes other than maximizing oxygen transport capacity during prolonged swimming. Many of these functions have been described in detail in the literature (Jensen, 1991; Randall, 1990; Perry and Wood, 1989) and will only briefly be mentioned here. With respect to oxygen transfer across the gills, the oxygen-carrying capacity may be secondary to the ability of Hb to maintain conditions for oxygen uptake. The driving force for oxygen flux from the environment to Hb is determined by the oxygen partial pressure (P_{O_2}) gradient across the gills. The greater the [Hb], the longer the P_{O_2} gradient can be maintained for a given gill ventilation and blood perfusion rate, and therefore the greater the flux of oxygen across the gills.

Haemoglobin is the most important nonbicarbonate buffer of the blood. Blood haemoglobin content may, therefore, be partly related to the importance of blood buffering capacity during exercise or recovery from exercise. In addition, the role of Hb in CO_2 excretion cannot be ignored. Almost all excreted CO_2 diffuses across the gills as molecular CO_2 , but it is carried as HCO_3^- in the plasma (Perry *et al.* 1982). HCO_3^- must therefore enter the red cell, where it can be dehydrated at the catalysed rate to CO_2 , provided that sufficient protons are available. The rate of CO_2 excretion is therefore dependent on the release of protons from Hb upon oxygenation (the Haldane effect). In addition, CO_2 excretion is dependent upon the rate of HCO_3^- entry into the red cell by way of the slow Cl^-/HCO_3^- exchanger in the band 3 protein. An increase in the number

of red cells increases the number of $\text{Cl}^-/\text{HCO}_3^-$ exchanger sites and thus CO_2 excretion rate (Perry *et al.* 1982). Total CO_2 was not measured in this study so the degree to which methaemoglobinaemia affected CO_2 excretion is not known.

Fish may be exposed to nitrite in natural aquatic systems and in aquacultural facilities (Kiese, 1974; Collins *et al.* 1975). If MetHb levels become severely elevated in fish exposed to nitrite, then swimming performance may be impaired. The relationship between functional [Hb] and U_{crit} in fish is complex. Large reductions in functional [Hb] are required to reduce U_{crit} significantly, indicating that U_{crit} is probably not a very sensitive indicator of maximal rate of oxygen consumption in fish. Insight into the effect of various treatments on aerobic metabolism should therefore be quantified by measuring that rate of oxygen consumption rather than U_{crit} .

Methaemoglobin

The proportion of MetHb measured in the untreated resting chinook was found to vary from a mean of 3.3% in series I (Fig. 1) to 17.5% in series II (Fig. 2). Although seemingly high, these values are not uncharacteristic of fish: Cameron (1971) reported values as high as 17% in rainbow trout in fresh water and Graham and Fletcher (1986) found interspecific differences of 7–27% in marine teleosts. In mammals, MetHb rarely exceeds 1–2% of total [Hb] (see Kiese, 1974, for a review), and similar levels have been reported for reptiles and birds (Board *et al.* 1977). It is not clear why fish possess such high MetHb levels, because they, like most animals, possess an enzyme, NADH-dependent MetHb reductase, which limits accumulation of MetHb by reducing it back to Hb (Freeman *et al.* 1983).

The highest sodium nitrite dose used in this study (90mgkg^{-1}) induced a MetHb level of 51.8% of total [Hb] in resting chinook salmon (Fig. 2). Bartlett *et al.* (1987) observed a maximum of 80–100% MetHb, 2–3h following an intraperitoneal injection of 30mgkg^{-1} sodium nitrite in *Semaprochilodus insignis* and *Brycon melanopterus*, two species of freshwater migratory fish from the Amazon. The time course of nitrite-induced MetHb formation following intraperitoneal administration of sodium nitrite is therefore similar in different species, but there are differences in the proportion of MetHb produced for a given dose of sodium nitrite.

In this study, MetHb levels appeared to stabilize 3h following intraperitoneal injection, but the time course of MetHb formation was only investigated for 30mgkg^{-1} sodium nitrite. It is possible that higher sodium nitrite concentrations require longer for MetHb levels to stabilize in resting fish. At 60 and 90mgkg^{-1} sodium nitrite, % MetHb was greater in swimming fish than in resting fish, indicating that, at the higher sodium nitrite concentrations, forced swimming enhanced MetHb formation. Jensen (1990) observed that the rate of nitrite entry *in vitro* was much more rapid in deoxygenated than in oxygenated red cells. The red cells of exercising fish undergo a greater degree of deoxygenation than those of resting fish (Jones and Randall, 1978; Kiceniuk and Jones, 1977). Therefore, for a given blood nitrite concentration, the rate of nitrite entry into the erythrocytes of active fish will be greater than the rate of entry into the cells of resting fish, thus leading to the higher MetHb formation observed in swimming fish.

In addition to inducing MetHb formation, nitrite will also oxidize the iron in myoglobin

to create metmyoglobin, which will affect oxygen transport to both skeletal and cardiac muscle (Bailey *et al.* 1990). The extent to which this occurred and influenced the results in our study is unknown.

Whole-blood characteristics

Nitrite-induced formation of MetHb is associated with a whole-blood alkalosis that is a direct result of Hb oxidation and nitrite reduction (Kiese, 1974). We found that, in resting cannulated fish, 30mgkg⁻¹ sodium nitrite had no significant effect on arterial whole-blood pH up to 3h following injection. The effect of larger sodium nitrite doses on arterial whole-blood pH was not determined. Resting and exercising chinook subjected to high doses of sodium nitrite displayed a slight shrinkage in red cell volume, as indicated by an increase in MCHC. Red cell shrinkage has been observed in resting carp exposed to environmental nitrite (Jensen, 1990; Jensen *et al.* 1987) but is less pronounced in our study. This can be partly explained by the use of the caudal puncture technique to sample blood in series II and III. This method undoubtedly disturbs the fish and may be associated with the release of catecholamines and subsequent adrenergically mediated red cell swelling, which would oppose the dehydrating effects of nitrite on the red cells noted by Jensen (1990) and Jensen *et al.* (1987). In addition, the degree to which the caudal puncture technique affects Hct, [Hb] and MCHC may differ between resting and exercising fish. Because of this, conclusions about the effect of nitrite exposure on these variables in this study must be made with caution.

Red cell organic phosphates

Red blood cell organic phosphates are powerful modulators of the affinity of Hb for oxygen in most vertebrates. In fish, the most potent of these is GTP, followed by ATP (Bartlett, 1978). Red blood cell organic phosphate levels are modulated during both hypoxia (Tetens and Lykkeboe, 1985; Boutilier *et al.* 1988) and anaemia. Anaemia induced by red cell removal results in an increase in the NTP:Hb ratio after only a few hours (A. L. Val, unpublished data). In our study, we observed no such changes in the levels of organic phosphates, consistent with the observations of Jensen *et al.* (1987), indicating that MetHb formation is not associated with modulation of organic phosphate concentrations. The reason why organic phosphate concentrations are modulated when blood oxygen-carrying capacity is reduced by red cell removal but not when it is reduced by an increase in MetHb levels is unknown and is worthy of further investigation.

We thank the Department of Fisheries and Oceans Canada at the Pacific Biological Station for donating the fish used in the study. In addition, we thank the staff of the Bamfield Marine Station for assistance during the study. Financial support for this study was provided by an NSERC operating grant to D.J.R. and an NSERC international exchange grant to D.J.R. and A.L.V. A.L.V. was supported at University of British Columbia by a CNPq/Brasil fellowship. Finally, we would like to thank the anonymous referees for their comments.

References

- BAILEY, J. R., SEPTON, D. H. AND DRIEDZIC, W. R. (1990). Oxygen uptake by isolated perfused hearts with differing myoglobin concentrations under hypoxic conditions. *J. molec. cell. Cardiol.* **22**, 1125–1134.
- BARTLETT, G. R. (1978). Water-soluble phosphates of fish red blood cells. *Can. J. Zool.* **56**, 870–877.
- BARTLETT, G. R., SCHWANTES, A. R. AND VAL, A. L. (1987). Studies on the influence of nitrite on methaemoglobin formation in Amazonian fishes. *Comp. Biochem. Physiol.* **86C**, 449–456.
- BEAMISH, F. W. H. (1978). Swimming capacity. In *Fish Physiology*, vol. 7 (ed. W. S. Hoar and D. J. Randall), pp. 101–187. New York: Academic Press.
- BELL, W. M. AND TERHUNE, L. D. B. (1970). Water tunnel design for fisheries research. *Tech. Rep. Fish. Res. Bd Can.* **195**, 69.
- BOARD, P. G., AGAR, N. S., GRUCA, M. AND SHINE, R. (1977). Methaemoglobin and its reduction in nucleated erythrocytes from reptiles and birds. *Comp. Biochem. Physiol.* **57B**, 265–267.
- BONE, Q. (1978). Locomotor muscle. In *Fish Physiology*, vol. 7 (ed. W. S. Hoar and D. J. Randall), pp. 361–417. New York: Academic Press.
- BONE, Q., KICENIUK, J. AND JONES, D. R. (1978). On the role of the different fibre types in fish myotomes at intermediate swimming speeds. *Fishery Bull. Fish Wildl. Serv. U.S.* **76**, 691–699.
- BOUTLIER, R. G., DOBSON, G., HOEGER, U. AND RANDALL, D. J. (1988). Acute exposure to graded levels of hypoxia in rainbow trout (*Salmo gairdneri*): metabolic and respiratory adaptations. *Respir. Physiol.* **71**, 69–82.
- BRETT, J. R. (1964). The respiratory metabolism and swimming performance of young sockeye salmon. *J. Fish. Res. Bd Can.* **21**, 1183–1226.
- BUSHNELL, P. G., STEFFENSEN, J. F. AND JOHANSEN, K. (1984). Oxygen consumption and swimming performance in hypoxia-acclimated rainbow trout *Salmo gairdneri*. *J. exp. Biol.* **113**, 225–235.
- CAMERON, J. N. (1971). Methaemoglobin in erythrocytes of rainbow trout. *Comp. Biochem. Physiol.* **40A**, 743–749.
- COLLINS, M. T., GRATZKE, J. B., SHOTTS, E. B., DANE, D. L., CAMPBELL, L. M. AND SENN, D. R. (1975). Nitrification in an aquatic recirculating system. *J. Fish Res. Bd Can.* **32**, 2025–2031.
- DAVIS, E. D., FOSTER, J., WARREN, C. E. AND DOUDOROFF, P. (1963). The influence of oxygen concentration on the swimming performance of juvenile Pacific salmon at various temperatures. *Trans. Am. Fish. Soc.* **92**, 111–124.
- EDDY, F. B., KUNZLIK, P. A. AND BATH, R. N. (1983). Uptake and loss of nitrite from the blood of rainbow trout, *Salmo gairdneri* Richardson and Atlantic salmon, *Salmo salar* L. in fresh water and in dilute sea water. *J. Fish Biol.* **23**, 105–116.
- FREEMAN, L., BEITINGER, T. L. AND HUEY, D. W. (1983). Methemoglobin reductase activity in phylogenetically diverse piscine species. *Comp. Biochem. Physiol.* **75B**, 27–30.
- GALLAUGHER, P., AXELSSON, M. AND FARRELL, A. P. (1992). Swimming performance and haematological variables in splenectomized rainbow trout, *Oncorhynchus mykiss*. *J. exp. Biol.* **171**, 301–314.
- GRAHAM, M. S. AND FLETCHER, G. L. (1986). High concentrations of methemoglobin in five species of temperate marine teleosts. *J. exp. Zool.* **239**, 139–142.
- JENSEN, F. B. (1990). Nitrite and red cell functions in carp: control factors for nitrite entry, membrane potassium ion permeation, oxygen affinity and methaemoglobin formation. *J. exp. Biol.* **152**, 149–166.
- JENSEN, F. B. (1991). Multiple strategies in oxygen and carbon dioxide transport by haemoglobin. In *Physiological Strategies for Gas Exchange and Metabolism* (ed. A. J. Woakes, M. K. Griesenhaber and C. R. Bridges), pp. 55–78. Cambridge: Cambridge University Press.
- JENSEN, F. B., ANDERSEN, N. A. AND HEISLER, N. (1987). Effects of nitrite exposure on blood respiratory properties, acid–base and electrolyte regulation in the carp (*Cyprinus carpio*). *J. comp. Physiol B* **157**, 533–541.
- JONES, D. R. (1971). The effect of hypoxia and anaemia on the swimming performance of rainbow trout (*Salmo gairdneri*). *J. exp. Biol.* **55**, 541–551.
- JONES, D. R. (1982). Anaerobic exercise in teleost fish. *Can. J. Zool.* **60**, 1131–1134.
- JONES, D. R. AND RANDALL, D. J. (1978). The respiratory and circulatory systems during exercise. In *Fish Physiology*, vol. 7 (ed. W. S. Hoar and D. J. Randall), pp. 425–501. New York: Academic Press.
- KICENIUK, J. W. AND JONES, D. R. (1977). The oxygen transport system in trout (*Salmo gairdneri*) during sustained exercise. *J. exp. Biol.* **69**, 247–260.

- KIESE, M. (1974). *Methemoglobinemia: a Comprehensive Treatise*. Cleveland, Ohio: CRC Press Inc. 260pp.
- PEARSON, M. P. AND STEVENS, E. D. (1991). Splenectomy impairs aerobic swim performance in trout. *Can. J. Zool.* **69**, 2089–2092.
- PERRY, S. F., DAVIE, P. S., DAXBOECK, C. AND RANDALL, D. J. (1982). A comparison of CO₂ excretion in a spontaneously ventilating blood-perfused trout preparation and saline-perfused gill preparations: contribution of the branchial epithelium and red blood cell. *J. exp. Biol.* **101**, 47–60.
- PERRY, S. F. AND WOOD, C. M. (1989). Control and coordination of gas transfer in fishes. *Can. J. Zool.* **67**, 2961–2970.
- PRIMMETT, D. R. N., RANDALL, D. J., MAZEAUD, M. AND BOUTILIER, R. G. (1986). The role of catecholamines in erythrocyte pH regulation and oxygen transport in rainbow trout (*Salmo gairdneri*) during exercise. *J. exp. Biol.* **122**, 139–148.
- RANDALL, D. J. (1990). Control and coordination of gas exchange in water breathers. In *Advances in Comparative and Environmental Physiology*, vol. 6 (ed. R. G. Boutilier), pp. 253–278. Berlin, Heidelberg: Springer-Verlag.
- RANDALL, D. J., MENSE, D. AND BOUTILIER, R. G. (1987). The effects of burst swimming on aerobic metabolism in chinook salmon (*Oncorhynchus tshawytscha*). *Mar. Behav. Physiol.* **13**, 77–88.
- SCHULTE, P. M., MOYES, C. D. AND HOCHACHKA, P. W. (1992). Interpreting metabolic pathways in post-exercise recovery of white muscle. *J. exp. Biol.* **166**, 181–195.
- SMITH, C. E. AND RUSSO, R. C. (1975). Nitrite-induced methemoglobinemia in rainbow trout. *Progve Fish Cult.* **37**, 150–152.
- SMITH, C. E. AND WILLIAMS, W. G. (1974). Experimental nitrite toxicity in rainbow trout and chinook salmon. *Trans. Am. Fish. Soc.* **103**, 389–390.
- SMITH, L. S. AND BELL, G. R. (1967). Anaesthetic and surgical techniques for Pacific salmon. *J. Fish. Res. Bd Can.* **24**, 1579–1588.
- SOIVIO, A., NYHOLM, K. AND WESTMAN, K. (1975). A technique for repeated sampling of the blood of individual resting fish. *J. exp. Biol.* **63**, 207–217.
- TETENS, V. AND LYKKEBOE, G. (1985). Acute exposure of rainbow trout to mild and deep hypoxia: O₂ affinity and O₂ capacitance of arterial blood. *Respir. Physiol.* **61**, 221–235.
- THORARENSON, H., GALLAUGHER, P. E., KIESSLING, A. K. AND FARRELL, A. P. (1993). Intestinal blood flow in swimming chinook salmon (*Oncorhynchus tshawytscha*) and the effects of haematocrit on blood flow distribution. *J. exp. Biol.* **179**, 115–129.
- WEBB, P. W. (1971). The swimming energetics of trout. II. Oxygen consumption and swimming efficiency. *J. exp. Biol.* **55**, 521–540.
- WEBB, P. W. (1978). Hydrodynamics: nonscombroid fish. In *Fish Physiology*, vol. 7 (ed. W. S. Hoar and D. J. Randall), pp. 189–237. New York: Academic Press.
- WILLIAMS, E. M. AND EDDY, F. B. (1986). Chloride uptake in freshwater teleosts and its relationship to nitrite uptake and toxicity. *J. comp. Physiol. B* **156**, 867–872.