

**ASPECTS OF THE RESPIRATORY METABOLISM OF JUVENILE RAINBOW  
TROUT (*Oncorhynchus mykiss*) AT SUSTAINED SWIMMING SPEEDS.**

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## ABSTRACT

A detailed description of the design of an apparatus suitable for investigations into the respiratory physiology of small nektonic organisms at various swimming speeds is presented. Juvenile rainbow trout (*Oncorhynchus mykiss*) were used to evaluate the performance of the apparatus. Twenty-four hour experiments indicated no detectable diurnal variation in the respiration rates of juvenile rainbow trout. Experiments investigating aerobic energy expenditure at sustained swimming speeds, through measurements of oxygen consumption rates, are described. Oxygen consumption was found to increase exponentially with increasing swimming speed. The influence of body size on this relationship was examined.

The contribution of anaerobic metabolism at sustained swimming speeds was investigated. Results of oxygen debt accumulation experiments indicated that this component was negligible at sustained levels of activity. This conclusion was further supported by the results of whole-body lactate accumulation trials. Aerobic energy expenditure data were employed to evaluate the relationship between cost of transport and swimming speed at sustained activity levels. A comparison between the loss of body wet weight and total oxygen consumption is discussed, and compared to generally accepted physiological equivalents. Modifications which could potentially improve the performance of the apparatus are proposed.

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# CHAPTER 1

## INTRODUCTION

Metabolism and energy expenditure in living systems has received widespread attention in the past, and studies investigating this fundamental aspect of physiology are legion. Our understanding of the mechanisms and partitioning of energy gain and expenditure has increased dramatically in the last few years, particularly in the light of recent technological advances. In particular, advances in the field of biochemistry have shed much light on processes which have hitherto been little understood. There are, however, many areas in which research is still required, providing great scope for further investigation.

Studies of metabolism and energy expenditure contribute valuable information to the much broader field of animal energetics, both at the organismal and community level. In the case of fishes of aquacultural importance, an understanding of energy metabolism is essential to develop efficient farming techniques, particularly with respect to dietary energy requirements (Cho, Slinger & Bayley, 1982; Degani, Gallagher & Meltzer, 1989). In attempting to generate an energy budget, researchers aim to achieve an understanding of how organisms obtain and allocate energy in the normal processes of day-to-day living. An energy budget is usually expressed in the form of a thermodynamically balanced energy equation, such as the one presented by Webb (1978):

$$Q_R - (Q_P + Q_N) = Q_S + Q_L + Q_{SDA} + Q_G + Q_P$$

where

$Q_R$  = food consumed

$Q_P$  = faecal loss

$Q_N$  = excretory loss

$Q_S$  = standard metabolism

$Q_L$  = locomotory metabolic cost

$Q_{SDA}$  = apparent specific dynamic action

$Q_G$  = growth

$Q_P$  = reproductive cost of gamete synthesis

In the above equation,  $Q_{SDA}$  is defined as the sum of all energy requirements for processing food. Pandian (1987) considered these to fall into two categories, namely mechanical (physical processing) and biochemical (anabolic and catabolic aspects). These processes are difficult to separate experimentally, hence the term "apparent SDA". Brett and Groves (1979) preferred the use of the term "heat increment" to describe this physiological effect. Several researchers ignore this component in their energy budgets. For example, Diana (1983) presents an energy budget equation for the northern pike (*Esox lucius*):

$$G + R = C - (R_{mct} + F + U + A)$$

where

G = growth

R = reproduction

C = consumption

$R_{mct}$  = respiration (standard metabolism)

F = egestion

U = excretion

A = locomotion

Webb (1978) considered the sum of  $Q_S$ ,  $Q_L$  and  $Q_{SDA}$  in his energy budget equation to be the metabolic component ( $Q_{MET}$ ) of the energy budget.

A great deal of interest has centered on the  $Q_{MET}$  component, and especially on the  $Q_S$  term (e.g. Beamish, 1964a; Beamish & Mookherjii, 1964). Standard metabolism reflects the basal metabolism (i.e the minimum rate of energy expenditure required to keep an animal alive), and is considered to be of great value towards understanding the physiology of an organism. It is important to note that standard (as opposed to basal) metabolic rate is specified in such energy equations, due to the inherent difficulty in obtaining a true measure of this energetic minimum. Since fishes readily elevate their metabolic rate in response to small stimuli, the term standard metabolic rate is applied to describe the minimum observed metabolic rate under conditions as close to complete rest as possible. Measurements of standard metabolic rate provide meaningful insight, particularly with regard to responses to environmental factors (Brett and Groves, 1979).

Winberg (1956), however, called for more research directed on other levels of metabolic requirements in fishes, particularly locomotion. This latter requirement forms a very costly component of the energy budget in view of the viscous medium which fishes inhabit.

The activity physiology of fishes has become the subject of widespread investigation, and there is a growing body of literature concerning this topic. No recent comprehensive review of this information appears to have been published, but discussion of various aspects can be found in the series of volumes entitled "Fish Physiology", edited by W.S.Hoar and D.J.Randall. An interesting perspective is presented by Bennett (1978), with comparisons to the other "lower" vertebrates. The most comprehensive experimental studies reported in the literature to date are those investigating the swimming energetics of the sockeye salmon, *Oncorhynchus nerka* (Brett 1962, 1964, 1965a, 1965b, 1972, 1973, 1976; Brett and Glass, 1973; Brett and Zala, 1975). A brief synopsis of current thinking in terms of animal metabolism, and methods of measuring metabolic rates, with special reference to fish activity physiology and the problems involved, is presented prior to discussion of the specific aims and questions addressed in this study.

In order for any living system to function, energy is required. This energy, derived from one or more of the basic body fuels (carbohydrates, fats and proteins), is released through a series of chemical reactions (collectively referred to as intermediate or cellular metabolism). Details of these reactions and associated processes can be found in any standard biochemical textbook (e.g. Stryer, 1988). Two principle energetic pathways responsible for generating the required energy can be identified:

- (i) Aerobic metabolism - requires oxygen for the complete oxidation of body fuels.
- (ii) Anaerobic metabolism (often referred to as glycolysis) - can progress in the absence of O<sub>2</sub>, and involves incomplete oxidation of body fuels.

An important difference between the two pathways involves their efficiency in terms of the amount of ATP generated per unit of fuel. Aerobic metabolism generates 38 mol ATP.mol glycogen<sup>-1</sup> with carbon dioxide and water as end products. In contrast, anaerobic metabolism (glycolysis) only generates 3 mol ATP.mol glycogen<sup>-1</sup>, with lactic acid as the main end product.

In fish propulsive systems, both pathways may operate. However, their relative contribution to the overall energy production at any given level of activity varies, depending on the intensity and duration of the activity, and hence, energy demand. Since aerobic metabolism is dependent on the cardiovascular and ventilatory physiological support systems for the uptake and transport of  $O_2$ , and the removal of waste products, it is generally restricted to long-term sustainable energy requirements falling within the capacity of these support systems. Anaerobic metabolism, on the other hand, does not immediately depend on these support systems, and can consequently generate relatively large amounts of energy very rapidly. However, the duration of such energy production is limited to the short-term (usually only a few seconds), due primarily to end-product accumulation in the tissues (mainly lactic acid), which adversely affect the physiological functioning of the animal. Accumulation of high concentrations of lactic acid have been shown to affect the blood and muscle pH, as well as disrupting enzymatic function and oxygen transport (Bennett, 1978), and the mobilization of lipid deposits (Goldspink, 1977a). Ultimately, aerobic metabolism is primarily responsible for the removal of excess lactic acid, either through reconversion back to glycogen and glucose, or further oxidation to  $CO_2$  and  $H_2O$ .

The relative contribution of aerobic and anaerobic pathways to overall energy expenditure depends on the energy demand placed on the animal. Aerobic metabolism plays the dominant role in long-term, sustained activity, where efficient use of energy is possible within the limits imposed by the ventilatory and circulatory systems. The necessity for anaerobic metabolism, even considering its relative inefficiency, comes to the fore in situations where the capacity for high levels of activity are vital (e.g. prey capture and escape from predators). In such situations, the demand for a rapid supply of energy to support bursts of activity cannot be met through aerobic pathways, since there is an inherent time lag involved in the mobilization of the circulatory and ventilatory systems to provide aerobic energy. Such demands can be met through anaerobic pathways, and the associated problems (e.g. metabolic acidosis) later rectified via aerobic metabolic pathways during the recovery phase. The occurrence of anaerobiosis and its measurement will be discussed in greater detail later in this chapter.

The relative contribution of the two metabolic pathways at various swimming speeds is reflected in the recruitment of the various populations of muscle fibres comprising the propulsive system. The swimming musculature of fishes is considered to be made up of red, white and pink fibres (the latter two groups are usually intermingled, and are often referred to as "mosaic" muscle - Hudson, 1973). There appears to be a relationship between the speed of contraction of the fibre types, and the swimming speeds at which they are recruited (Johnston, Ward & Goldspink, 1975; Johnston, Davison & Goldspink, 1977), red muscle operating at low speeds, pink muscle at intermediate speeds, and white muscle at fast cruising and burst speeds. The metabolism of the various fibre types has been the subject of several investigations (e.g. Johnston & Goldspink, 1973a, 1973b; Johnston *et al.*, 1975, 1977; Driedzic & Hochachka, 1976). Red muscle is generally accepted to be primarily "aerobic" muscle, while white muscle is primarily anaerobic (Driedzic & Hochachka, 1976). Consequently, slow steady, sustained swimming is achieved mainly by the red muscles, supported by relatively efficient aerobic energy generation. At the other extreme, short duration burst activity is achieved through contraction of the white muscles (which form the bulk of the myotome), supported primarily by anaerobic energy production.

Investigations into activity physiology are usually aimed at obtaining some understanding of the magnitude of energy expenditure (both aerobic and anaerobic) at various levels of activity, i.e. estimating the amount of energy required to support a given work load. There are two commonly utilized lines of approach:

(i) Theoretical studies, employing mathematical models based on principles of hydrodynamics and physics, and incorporating information on such parameters as drag, thrust and other relevant aspects of kinematics (e.g. Webb, 1975; Alexander, 1977).

(ii) Empirical studies estimating energy expenditure through measurement of metabolic heat production, commonly termed calorimetry. Since this line of approach is the one adopted in the present study, the underlying principles and methods involved require some clarification with regard to studies on fishes.

Fishes, like any other living system, have to conform to the first law of thermodynamics (i.e. matter and energy can be converted, but never destroyed). Energy absorbed from food sources is converted, stored, and subsequently lost through catabolic processes supporting maintenance and activity. Catabolism results in the production of various end products (as discussed previously), and heat. Measurement of this biological heat production is the province of calorimetry (Cho *et al.*, 1982), and can be achieved through one of two basic methods:

### **1. Direct Calorimetry:**

This involves the direct measurement of heat loss from the animal. Detailed discussion of the methods employed are discussed by Brett and Groves (1979). This technique has been considered unsatisfactory for studies on fishes due to the difficulties involved with measuring the temperature increment in an aquatic system. These difficulties are a direct result of both the relatively low metabolic rate of fish (hence low rate of heat production) and the high heat capacity of water (Brett & Groves, 1979).

### **2. Indirect Calorimetry:**

This method utilizes calorific determinations of heats of combustion of the various body fuels, and involves indirect calculation of the energy released during metabolism, either from the rates at which body fuels are used, or from a measure of the amount of oxygen consumed in their combustion. Rates of production of metabolic end products such as CO<sub>2</sub>, ammonia and lactic acid, could also be incorporated into the method.

#### **(a) Measurements of body fuel depletion:**

Measurements of this nature involve the application of physiological equivalents determined from heat of combustion data (usually measured in a bomb calorimeter) for each of the three principle body fuels. Usually, values for "typical" carbohydrates, fats and proteins are employed, and although these values involve a certain degree of error, this is generally considered to be insignificant. Heat of combustion values for typical body fuels have been listed by Webb (1975). The physiological equivalents for fats and carbohydrates are considered to be the same as the total heat of combustion figures presented by Webb (1975), since the total heat of combustion of

these two fuels is equal to the total amount of energy liberated through aerobic metabolism (both of these fuels are totally oxidised to  $\text{CO}_2$  and  $\text{H}_2\text{O}$ ). Proteins, however, are only partially oxidised, since they contain various nitrogenous compounds which are transformed and excreted (mainly as ammonia). The physiological equivalent has to take this factor into account, and values considerably lower than the heat of combustion figures are more frequently applied.

A complication in the application of physiological equivalents to measures of body fuel depletion arises from the extent to which the various metabolic pathways contribute to overall energy expenditure (Webb, 1975). This applies particularly to glycogen, which fuels both aerobic and anaerobic metabolic pathways. Webb (1975), however, felt that this complication can be ignored at sustained and burst activity levels, since either one or the other pathway predominates at each of these extremes (viz. aerobic metabolism at sustained levels, and anaerobic metabolism at burst activity levels). At intermediate and prolonged activity levels, where both pathways are contributing significantly to overall energy production, the problem becomes more complex, and the relative proportions of each pathway are not easily separated. Consequently, the application of physiological equivalents to measures of body fuel depletion may result in erroneous estimates if it is not known through which pathway the fuels are being metabolised. This factor should be borne in mind when interpreting data of this nature. Nonetheless, despite these shortcomings, this technique can be a useful tool in gaining an idea of metabolic energy expenditure under various conditions (e.g. Brett, 1973).

#### **(b) Measurement of gaseous exchange:**

This is the most widely applied method of estimating energy expenditure, usually employing measurements of oxygen consumption rates. This latter is the technique which has been adopted in the present study. There is, however, a fundamental constraint on the use of oxygen consumption measurements as an estimate of the magnitude of energy production. By definition, only aerobic metabolism is estimated by this method, any anaerobic contribution is not detected. Consequently, this technique should be restricted to situations involving sustained and prolonged activity levels, where the anaerobic component can be considered as negligible. The concept of an oxygen debt, however, provides an avenue for estimates of any anaerobic contribution

through measurement of excess post-exercise oxygen consumption. This aspect is discussed in more detail in this, and subsequent chapters.

To estimate energy expenditure through measurements of oxygen consumption, these data must be converted to caloric units of energy through application of oxycalorific equivalents, the values of which are dependent upon the particular fuel being oxidised. Values which have been applied in studies on fishes are typically of the order of 3.5 cal.mg O<sub>2</sub><sup>-1</sup> for fat, 3.22 cal.mg O<sub>2</sub><sup>-1</sup> for carbohydrate, and 3.20 cal.mg O<sub>2</sub><sup>-1</sup> for protein (Webb, 1975). It should be noted that these values have been derived from data obtained in studies on mammalian systems. The validity of applying these equivalents to fish has been questioned by Krueger, Saddler, Chapman, Tinsley and Lowry (1968), who demonstrated that in coho salmon, application of these oxycalorific equivalents to projected oxygen consumption rates resulted in a three-fold underestimate of energy expenditure, relative to estimates derived from monitoring the depletion of lipids and fatty acids. It was suggested by Krueger *et al.* (1968) that this discrepancy was due to the accumulation and excretion of partially oxidised products. The fishes used in the investigation of Krueger *et al.* (1968) were exercised to exhaustion, implying a significant activity-induced and stress-induced anaerobic contribution, which could have influenced the accuracy of the oxygen consumption data.

In response to the above findings, Brett (1973) assessed the possibility of error in the application of oxycalorific equivalents to oxygen consumption rates during sustained swimming in fishes. His study compared the two methods of estimating energy expenditure (*viz.* depletion of body substances versus oxygen consumption), assuming that the more accurate estimate would be obtained from the former method. The results obtained by Brett (1973) indicated an acceptable agreement between the two methods, with a certain degree of error attributable to slight anaerobic metabolism not accounted for by oxygen consumption, combined with other minor energy losses such as mucous production and scale loss. The oxygen consumption estimates were on average 19.8% lower than the loss of body substance estimates, but most of this fell within the 95% confidence limits of the oxygen consumption measurements (Brett, 1973 states that the 95% confidence limits for metabolic rate determinations on small samples are of the order of  $\pm$

15%). Brett concluded that the observations of Krueger *et al.* (1968) were unconvincing, and based on several false premises.

Current opinion thus seems to be that the use of oxygen consumption rates (with associated application of oxycalorific equivalents) to estimate energy expenditure is a valid technique, provided the following constraints are recognised and accounted for:

- (i) measurements of oxygen consumption are recorded in the presence of adequate levels of ambient oxygen,
- (ii) the system is totally aerobic, or as close as possible to this state,
- (iii) the application of the oxycalorific equivalents are based on total oxidation of the fuels (or in the case of proteins, the equivalent should account for incomplete oxidation).

In practice, a mean oxycalorific equivalent is generally used, a value of 4.8 kcal.l O<sub>2</sub><sup>-1</sup> having been employed in studies on fishes in the past. Brett and Groves (1979) suggest a value of 4.63 kcal.l O<sub>2</sub><sup>-1</sup> as more appropriate in aerobic, steady state metabolic studies. The general consensus in the literature however, appears to be that the error inherent in the use of mean oxycalorific equivalents can be considered to be negligible.

A recurrent theme in this discussion has involved the relative proportions of aerobic and anaerobic energy production at various activity levels. It is clear that in any study on the metabolism of an animal involving indirect calorimetric methods, it is of fundamental importance to distinguish between these two pathways, and to ascertain their respective contributions to the overall energy production. If simultaneous measurements of CO<sub>2</sub> production and O<sub>2</sub> consumption are conducted, the occurrence of any anaerobic metabolism would be manifested in an RQ (respiratory quotient - defined as the ratio of CO<sub>2</sub> produced to O<sub>2</sub> consumed) value greater than unity (Kutty, 1968b). Since dissolved O<sub>2</sub> is more conveniently measured than dissolved CO<sub>2</sub> (Brett & Groves, 1979), very few studies reported in the literature have involved measurement of the RQ, despite the value of this variable in estimating anaerobic energy production.

Earlier in this chapter, reference was made to the estimation of the anaerobic component by means of oxygen consumption measurements recorded during recovery subsequent to activity, i.e. measurement of the "oxygen debt". The basic rationale underlying this concept can be considered as follows. The occurrence of anaerobic energy production during activity results in a deviation from the stable physiological state of the animal, and the animal accrues an "oxygen debt". Aerobic pathways are ultimately responsible for restoring this stable state, through maintenance of elevated oxygen consumption rates during recovery. The fundamental assumption implicit in this rationale is that the amount of aerobic energy required to restore the pre-activity physiological state is equivalent to the amount of anaerobic energy produced over the duration of the activity. The elevated oxygen consumption subsequent to activity has been termed the excess post-exercise oxygen consumption or EPOC

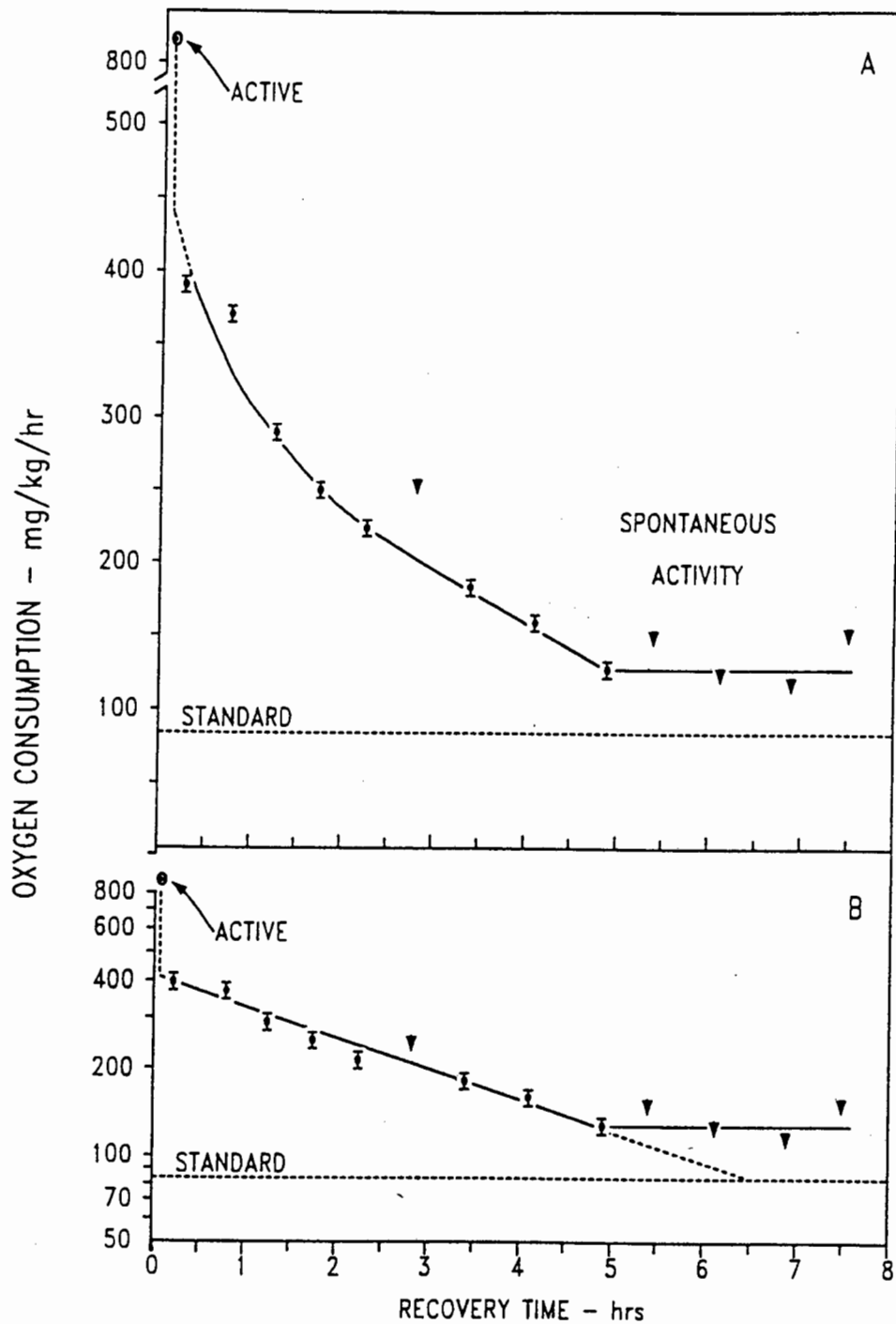
Early propagators of the "oxygen debt hypothesis" (Hill & Lupton, 1923, cited by Scarabello, Heigenhauser & Wood, 1991) considered the debt to be the cost of removing the accumulated waste products of anaerobic metabolism (primarily lactic acid), either through further aerobic oxidation to CO<sub>2</sub> and water, or through conversion back to glycogen or glucose. Some scepticism regarding the use of measurements of EPOC to estimate the oxygen debt has been voiced in the literature. Webb (1975) and Bennett (1978) considered it an unsatisfactory method, basing their observations on the results reported by Black, Robertson, Hanslip & Chiu (1960); Black, Connor, Lam & Chiu (1962) and Brett (1964). These data indicated no relationship between the duration of EPOC, and the duration of elevated lactic acid levels. Black *et al.* (1960, 1962) reported elevated lactic acid levels persisting for a period of 8 to 12 hours subsequent to cessation of strenuous exercise, while Brett (1964) reported an EPOC lasting for a maximum of only 5 hours. Both Webb (1975) and Bennett (1978) advise against attempts to estimate anaerobic energy production through measurements of oxygen consumption alone. It is possible, however, that the basis of these objections is questionable, in view of recent reports (Scarabello *et al.*, 1991) indicating that lactic acid metabolism forms only a minor component of the oxygen debt.

Considering the experiments of Brett (1964) and Black *et al.* (1960, 1962) as the basis of the criticisms of Webb (1975) and Bennett (1978), the experiments referred to were conducted on

different species (rainbow trout in the case of Black and colleagues, sockeye salmon in the case of Brett), on a range of fish sizes, and at different temperatures. In addition, neither of the studies referred to combined simultaneous lactic acid measurements with estimations of EPOC. Black *et al.* (1960, 1962) only considered the duration of elevated lactic acid levels, while Brett (1964) only recorded elevated oxygen consumption rates. Variability in the measurements of such physiological variables between species as well as individuals is a well documented phenomenon (Brett, 1962 discusses this in more detail, with reference to the variability in data reported by different authors). Thus, comparisons between studies of different species such as those discussed above should be treated with caution.

Brett (1964) documents several points pertinent to the present discussion. Firstly, recovery after strenuous exercise was considered to be manifested by the onset of spontaneous activity on the part of the fish (the initial stages of recovery were characterized by an absence of any activity). Secondly, an exponential decrease in oxygen consumption rates during the recovery period was observed, until the onset of spontaneous activity resulted in elevated oxygen consumption rates (see Figure 1). To obtain an estimate of the total oxygen debt, Brett (1964) extrapolated the recovery oxygen consumption curve to the estimated standard metabolic rate level (see Figure 1 B), and integrated the area under the curve from the cessation of exercise to the estimated time to complete recovery (where the extrapolated recovery curve reached standard metabolic rate levels).

Thirdly, on the basis of his results, Brett (1964) concluded that the fish was capable of carrying a sustained oxygen debt, which was still present after recovery from exercise. The oxygen debt repaid by the onset of spontaneous activity was referred to by Brett (1964) as the "overload debt", and should not be equated with the total oxygen debt. The difference between the total and the overload debts was considered to be representative of the tolerable load readily sustained by the fish. This supports the conclusion reported by Hochachka (1961), who deduced that when the oxygen debt has disappeared, the accumulated lactic acid need not necessarily have been completely oxidised, but that lactate levels bear a certain relationship to pyruvate levels, permitting metabolic oxidations to return to normal. This concept was discussed by Webb (1975)



**FIGURE 1:** Oxygen debt replacement in a yearling sockeye (21.5 cm, 87.9 g, 15°C) at rest following fatigue. Last recorded metabolic rate when swimming indicated by Active rate. Arithmetic plot in A transformed to semi-log relation in B. Extrapolation to zero time gives maximum non-swimming rate of oxygen consumption; extrapolation to standard rate gives expected time to complete recovery. Limits are shown equivalent to  $\pm 0.04$  ppm, involving two values (subtracted). Arrow indicates that a lower value would be expected because of observed restless behaviour. After Brett (1964).

as the basis for a more accurate technique of measuring oxygen debt, where estimates of anaerobic energy expenditure could be calculated from the amount of lactic acid produced in excess of pyruvic acid. Both metabolites would increase by similar amounts through aerobic metabolism, while anaerobic metabolism would elevate lactic acid relative to pyruvic acid.

The extrapolation of the oxygen consumption recovery curve to standard levels by Brett (1964) to estimate the total oxygen debt, can be taken further in view of the modifications to the oxygen debt hypothesis proposed by Margaria, Edwards & Dill (1933), who included two components into the EPOC:

- an initial fast component, attributed to the recovery of ATP, CP (creatine phosphate) and internal oxygen stores, which was termed the "alactacid" oxygen debt.

- a second slow component, attributed to the metabolism of accumulated lactic acid, termed the "lactacid oxygen debt".

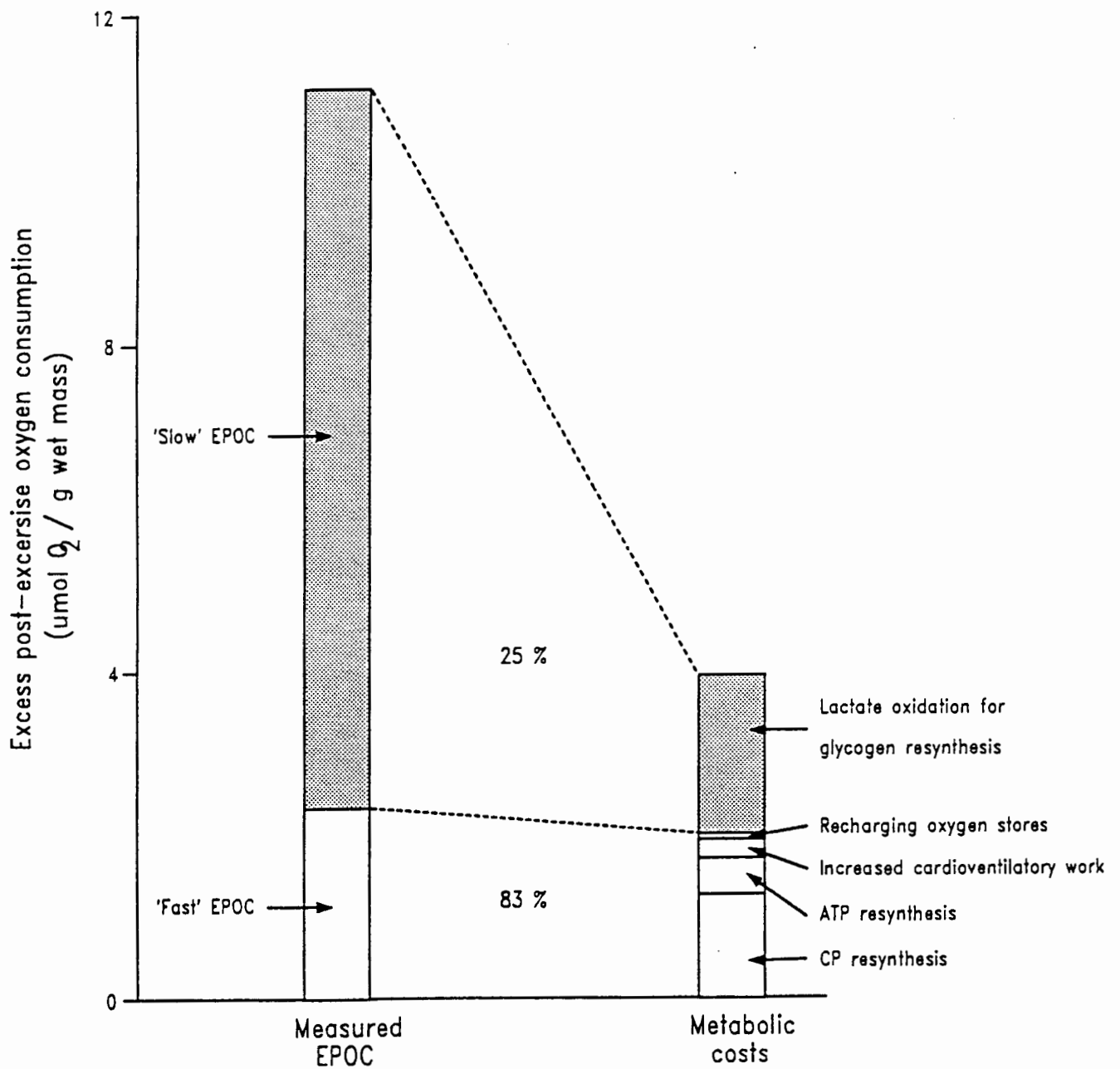
Recent research on mammals suggests that the "oxygen debt hypothesis" considered above, cannot be the sole explanation for EPOC (Scarabello *et al.*, 1991). This would appear to apply to fishes as well, in view of the results reported for young rainbow trout by Wieser, Platzer and Hinterleitner (1985), which indicated that post-exercise energy generation exceeded the apparent energy demand. Wieser *et al.* (1985) concluded that the recovery oxygen consumption need not be the same as the oxygen debt.

An examination of the oxygen debt hypothesis in juvenile rainbow trout after exhaustive exercise was conducted by Scarabello *et al.* (1991). Oxygen consumption recovery curves were separated into the fast and slow components through the curve-stripping technique of Riggs (1963) (cited by Scarabello *et al.*, 1991). EPOC was compared to changes in the whole-body levels of lactic acid, glycogen, ATP, ADP, AMP and CP. The results indicated that the bulk (83%) of the fast component (comprising about 20% of the total EPOC) could be accounted for by standard components of the "alactacid oxygen debt", i.e. resynthesis of ATP and CP, restoration of body oxygen stores, and the cost of increased ventilatory and cardiac energy expenditure. However, no agreement between the EPOC and lactic acid metabolism was evident. The measured slow component of the EPOC (about 80% of the total EPOC) was found to be much greater than could

be accounted for by the standard pathways of either lactic acid oxidation, or glycogen resynthesis. Only about 25% of the slow component of EPOC could be accounted for by either of these two scenarios of lactic acid metabolism (see Figure 2). These results are in direct contrast to the observations of Webb (1975) and Bennett (1978) discussed earlier, where the implication was that post-exercise energy expenditure was not as great as apparent energy demand. Scarabello *et al.* (1991) suggested several possible factors contributing to the discrepancy between the measured slow component of the EPOC and lactate metabolism, including 'futile' substrate cycling subsequent to exercise, as well as the mobilization of catecholamines. The latter was considered to be of probable significance in fish due to almost 100-fold increases in fish plasma levels of adrenaline and noradrenaline after an exhaustive exercise-stress protocol (Butler, Metcalfe & Ginley, 1986).

A detailed discussion of probable factors contributing to this discrepancy is presented by Wood (1991), with reference to a number of physiological responses considered to distinguish exhaustive exercise from steady state aerobic exercise. Responses characterizing exhaustive exercise included significant depletion of glycogen reserves with associated build-up of white muscle lactate levels; substantial degradation of muscle ATP and CP stores; marked depression of blood pH as a result of combined respiratory ( $PCO_2$ ) and metabolic acidosis ( $H_m^+$ ); profound disturbance of the ionic, osmotic and fluid volume homeostasis (as a result of increased intracellular lactate levels, with associated osmotic gradient); and rapid release of catecholamines (adrenaline and noradrenaline), particularly at high stress levels. In view of these responses, Wood (1991) considers the possible contributions to EPOC to be as follows:

- (i) The metabolic costs of altered membrane transport processes associated with re-establishing the ion, acid-base and fluid volume homeostasis.
- (ii) The energy expenditure required to retain lactate and  $H_m^+$  within the white muscle for apparent metabolism *in situ*. Available evidence presented by Wood (1991) suggests that lactate and  $H_m^+$  are retained inside the cells out of electrochemical equilibrium, an energetically costly process.



**FIGURE 2:** The measured 'fast' and 'slow' components of excess post-exercise oxygen consumption (EPOC) in juvenile rainbow trout after exhaustive exercise stress (5 min chasing) at 15°C, and their possible origins. Most (83%) of the fast component is accounted for by creatine phosphate (CP) and ATP resynthesis, increased cardioventilatory work and the recharging of body O<sub>2</sub> stores. However, only 25% of the slow component is explained by the metabolism of lactate back to glycogen. Based on data from Scarabello *et al* (1991). After Wood (1991).

(iii) Post-exercise hyperventilation with associated energetic costs (crucial if the demands of EPOC are to be met). The current view appears to be that the increased ventilatory response is stimulated by post-exercise acidosis.

(iv) Although increased catecholamine mobilization has been implicated in many physiological responses during recovery from exhaustive exercise, Wood (1991) tentatively concludes that catecholamine effects on oxygen consumption rates are not an important contributor to EPOC in fishes. Further research is required to substantiate this conclusion.

From the factors discussed above, it is evident that the metabolism of lactate is only one of several processes requiring the support of metabolic energy generation to restore a stable physiological state subsequent to intensive activity. However, it should be emphasised that the responses examined above are characteristic of exhaustive-stress activity on the part of the fish. The methods employed to generate activity at this level range from manual chasing of the fish (Milligan & Wood, 1986), to application of low voltage electric shocks (Wieser *et al.* 1985; Pearson, Spriet & Stevens, 1990; Scarabello *et al.* 1991), all involving high levels of stress. It may be argued that the cumulative magnitude of the physiological responses observed under these artificial circumstances, leading to the observed discrepancy between measured EPOC and lactate metabolism, is purely a function of these high levels of stress. Increased mobilization of catecholamines (which appears to bear some relationship to the level of stress) have been implicated in many of these responses. The stimulus for catecholamine mobilization remains unclear, but possible mechanisms mentioned by Aota, Holmgren, Gallagher & Randall (1990) and Wood (1991) include elevated acidosis, the reduced oxygen content of the blood, and the psychological factor ('panic') acting through sympathetic innervation of the chromaffin cells (catecholamine releasing cells). This latter mechanism has been mentioned by Scarabello, Heigenhauser & Wood (1992) as a possible influence on improved rates of metabolic recovery in juvenile rainbow trout after sequential bouts of exhaustive exercise.

In the light of these observations, it is perhaps somewhat injudicious to assume that the physiological responses involved with this level of activity have the same significance in more realistic situations where the stress factor is not as pronounced. If the experimental protocol employed minimises the stress level to which the fish is exposed, measurement of lactic acid

accumulation could well provide, at least, an index of anaerobic metabolism, if not a direct measure. Results obtained through such experimental protocols may be more applicable to the natural environment. Obviously, any experimental protocol imposing a given level of activity on the fish will incorporate a certain degree of stress, but not to the extent of the exhaustive-stress protocols employed by Wieser *et al.* (1985) and Scarabello *et al.* (1991).

In conclusion, it is felt that the indirect calorimetric techniques of estimating energy expenditure through measurement of oxygen consumption rates during and after given levels of activity are valid, provided the limitations of the technique are recognised, and the relevant precautions applied. The application of this technique will be discussed in Chapter 2, where the experimental approach adopted in the present study is discussed.

The primary objective of this study was to develop an apparatus suitable for investigating the energy expenditure of small nektonic organisms at various levels of swimming activity. The emphasis of this study was on the design and construction of the apparatus. The experiments which will be described in this thesis were aimed at establishing whether the apparatus was capable of generating useful information, comparable to data reported in the literature. Rainbow trout (*Oncorhynchus mykiss*) were selected as the test species, primarily because a great deal of research has centered on the energetics of this species, providing sufficient data for comparative purposes. Further, this species is readily obtainable, and relatively simple to maintain. Various aspects of the respiratory metabolism of rainbow trout which were felt to be of interest were investigated during the course of this work.

The fundamental questions addressed in this study are as follows:

1. What are the energy requirements of rainbow trout at sustained swimming speeds? This was the key question in this study, the other aspects investigated all have bearing on this central theme. For the purposes of this study, three levels of swimming activity were characterized in the manner proposed by Webb (1975):

(a) **Sustained** - activity levels maintained for periods longer than 200 minutes.

(b) **Prolonged** - activity levels maintained for periods of between 200 minutes and 15 seconds.

(c) **Burst** - high activity levels maintained for less than 15 seconds.

2. At what levels of activity does anaerobic metabolism (as estimated through measurement of EPOC) begin to play a significant role in terms of its contribution to overall energy expenditure?

3. Can glycolytic lactic acid production be used as an index of anaerobic metabolism?

4. Does the metabolic rate (as measured by the rate of oxygen consumption) of rainbow trout exhibit any consistent patterns or rhythms of diurnal variation, and if so, is this variation of a magnitude sufficient to influence oxygen consumption rate measurements at a given swimming speed recorded at different times of day?

The first two questions are intimately linked, since the proportion of overall energy expenditure supported by anaerobic metabolism increases with elevated activity levels, and any estimate of total energy expenditure must incorporate this component. Data obtained from assessment of the anaerobic component at various activity levels could also be used to obtain an indication of the limits of sustained activity in juvenile trout.

With regard to the first question, various additional aspects which are felt to be of interest will be considered in the analysis. Briefly:

(a) The nature of the relationship between  $VO_2$  (oxygen consumption rate, usually expressed in units of  $\text{mg O}_2 \cdot \text{kg body weight}^{-1} \cdot \text{hr}^{-1}$ ) and swimming speed (expressed in units of  $\text{body lengths} \cdot \text{second}^{-1}$ ).

(b) The influence of body size on this relationship.

(c) The influence of swimming speed on the cost of transport (in terms of the total amount of oxygen required for the fish to move through a set distance) - this could provide an indication of the most "efficient" swimming speed.

(d) Whether a relationship exists between total oxygen consumption and loss in body weight, and whether this relationship is comparable to the conventionally applied oxycaloric equivalents. The fundamental assumption involved here is that any manifested loss in body weight is due to depletion of internal energy stores through aerobic energy generation.

The rationale behind the third question requires some clarification, considering the observed discrepancies between EPOC and lactate metabolism discussed earlier. The viewpoint adopted in this study is that this discrepancy is perhaps due to high levels of stress involved in the experimental protocols employed. Increased levels of circulating catecholamines, which appear to be related to stress (Wood, 1991), have been implicated in many of the physiological responses which have been proposed as major contributors to this discrepancy. Many of these physiological responses appear to be intimately linked with, if not a direct result of, lactic acid accumulation. It is felt that experimental protocols minimising stress could lead to a better agreement between lactate metabolism and EPOC, to the extent that levels of accumulated lactic acid could be considered as an index of the level of anaerobic metabolism (e.g. Duthie, 1982). In the experimental procedures adopted in this study, every effort was made to minimise stress to the fish. An assumption implicit in this approach is that lactate is the only end product of anaerobic metabolism in rainbow trout. Several species of fishes, particularly those with good anaerobic capacities, have a modified metabolism that is characterized by non-acidic end products. For example, in the goldfish, ethanol is an anaerobic end product (Van der Thillart & Verbeek, 1991). Such metabolic adaptations do not appear to have been demonstrated for rainbow trout, which are considered to have poor anaerobic capacities (Kutty, 1968a). For the purposes of this investigation, lactate was considered to be the only end product of anaerobic metabolism in rainbow trout.

The rationale behind question 4 rests on preliminary observations where measurements of oxygen consumption rates recorded at the same level of activity, but at different times of the day, exhibited substantial differences. It was felt that this necessitated a more detailed investigation. The occurrence of some form of rhythmic fluctuation or circadian rhythm in the metabolic rate of the fishes could introduce a substantial degree of error into the experiments estimating total energy expenditure. The causes or stimuli involved with the observed variation were not examined, since an investigation into the possible operation of an endogenous physiological "clock" regulating the fishes' behaviour or metabolism requires precisely controlled conditioning experiments which were beyond the scope of the present study.

The central question of the present study was addressed by adopting the approach of Brett (1964) (after Blazka *et al.*, 1960), involving measurement of oxygen consumption rates of fishes forced to swim at known velocities. The next chapter provides a detailed description of the design of the apparatus developed for the present study, prior to discussion in subsequent chapters of the various experiments conducted.

## CHAPTER 2

### THE APPARATUS

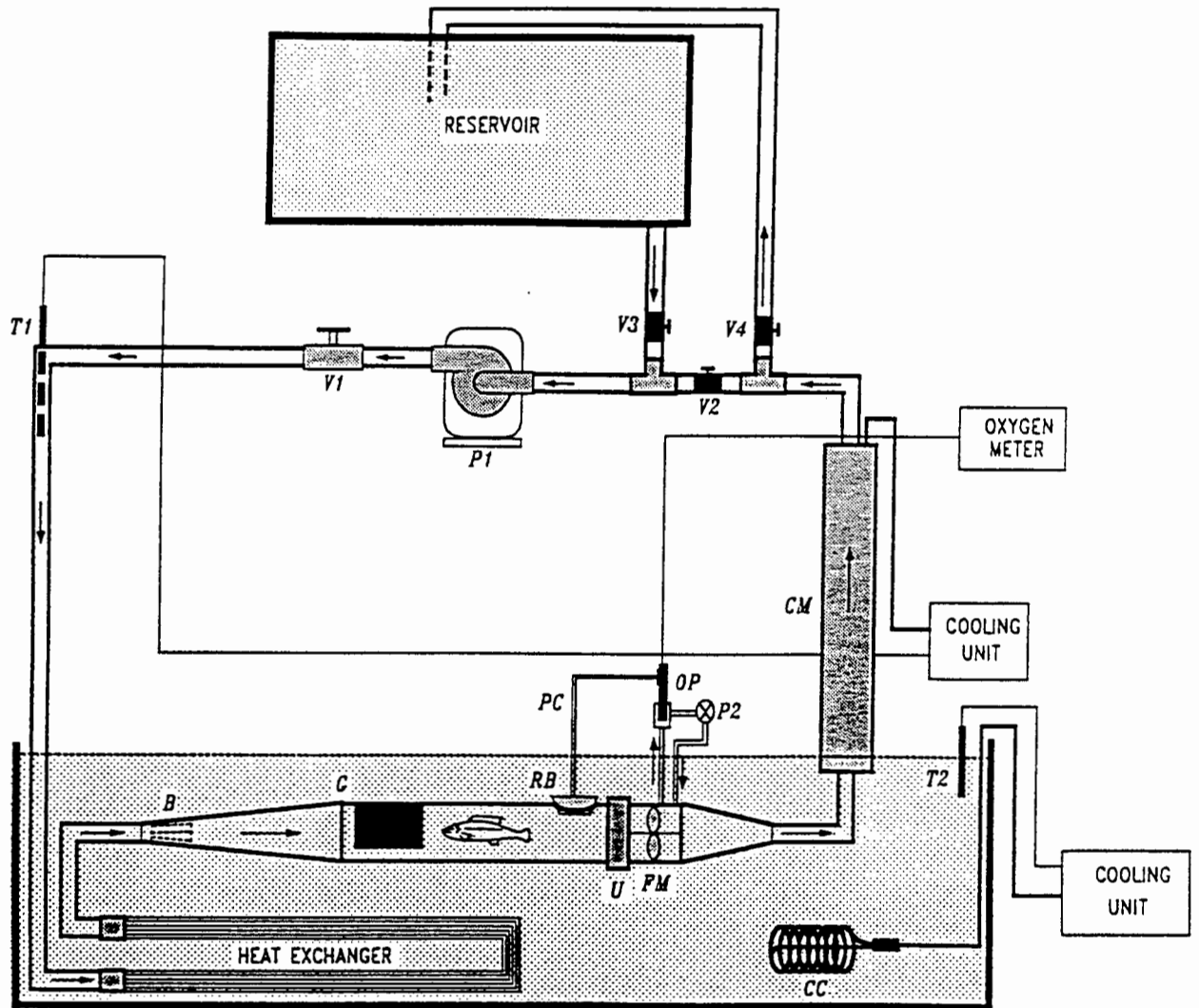
The nature of the experimental approach adopted in this study imposes several constraints on the design of the apparatus. These are referred to in the description of the apparatus presented below. Students of this field are advised to refer to the discussion of Bell & Terhune (1970) with regard to the design of water tunnels for fish research. Their elaboration of the potential problems and techniques involved could save much time, effort and cost.

#### **A. Respirometer Design:**

The design of the respirometer (illustrated in Figure 3), was based on the apparatus developed by Brett (1964), the basic format of which was a recirculating water tunnel, incorporating a flow chamber connected to a pump via contraction and expansion cones. This basic format has been employed in a number of studies (e.g. Stevens & Randall, 1967; Kruger & Brocksen, 1978; Priede & Holliday, 1980; Steffensen, Tufts & Randall, 1987; Bårdgard, Salhus & Brix, 1989; and Facey & Grossman, 1990). In this study, several modifications were made to the design of Brett (1964), as will be clarified during the course of the following description.

#### **1. Flow Generation and Regulation.**

A fundamental requirement of the adopted approach is that a water current is induced through the flow chamber, the velocity of which can be accurately regulated and maintained for prolonged periods, over the entire range of velocities dictated by the experimental protocol. Inherent in this regulation is the concomitant accurate measurement of velocity. Water flow is usually induced by means of either a pump (e.g. Brett, 1964; Webb, 1971a, 1971b; Priede & Holliday, 1980; Steffensen, Johansen & Bushnell, 1984), or propellor attached to a variable speed motor (Smit, 1965; Beamish, 1968; Smith & Newcomb, 1970; Hartwell & Otto, 1978; Bårdgard *et al.*, 1989). The nature of the flow-generating and regulating mechanisms may contribute to the stress experienced by the experimental animal, through the transmission of pressure pulses through the



**FIGURE 3:** Diagram illustrating the design of the respirometer developed for the present study.

See text for detailed description. Arrows indicate the direction of water flow.

*P1* - main circulating centrifugal pump; *V1* - flow velocity regulating ball valve; *T1* & *2* - thermostats; *B* - honeycomb baffle; *G* - grid; *RB* - rubber bung; *PC* - tube to pressure compensator; *OP* - oxygen sensor; *P2* - centrifugal pump circulating water past oxygen sensor; *U* - union joint; *FM* - flow meter assembly; *CC* - cooling coil; *CM* - cooling mantle; *V2,3* & *4* - ball valves regulating the flushing procedure.

water as a result of the rotation of the propellor or impeller. Variable speed pumps and motors are often employed to permit regulation of flow rate. It is felt that the associated variation in the frequency of pressure pulses could significantly add to the stress factor, since the fish would be exposed to widely different pulse regimes, depending on the imposed velocity. Constant speed pumps, on the other hand, generate a constant pulse frequency, no matter what the velocity. It can be argued that the stress factor would perhaps be reduced if the fish were allowed sufficient time to acclimate to a constant pressure pulse regime.

With this argument as a basis, a constant speed centrifugal pump (capable of generating flow rates of up to  $90 \text{ l.min}^{-1}$ , corresponding to a maximum velocity of about  $40 \text{ cm.s}^{-1}$  in the flow chamber) was used ("P1" in Figure 3) in an attempt to minimise the effect of the pressure pulses. Regulation of flow rate was achieved by means of a PVC ball valve ("V1" in Figure 3) situated immediately downstream of the pump. Operation of this valve altered the pressure head applied to the pump, with a resultant alteration in the flow rate. Velocity was monitored by means of a simple flow meter mounted at the downstream end of the flow chamber (see next section). Water flow between the flow chamber pump was carried through 32 mm diameter rigid PVC piping. Two lengths of flexible 32 mm diameter rubber tubing were connected to the PVC piping at each end of the flow chamber, in an attempt to minimise vibrations resulting from the pump motor.

## **2. The Flow Chamber:**

The design of the flow chamber is of crucial importance, and involves two major factors which are discussed in depth.

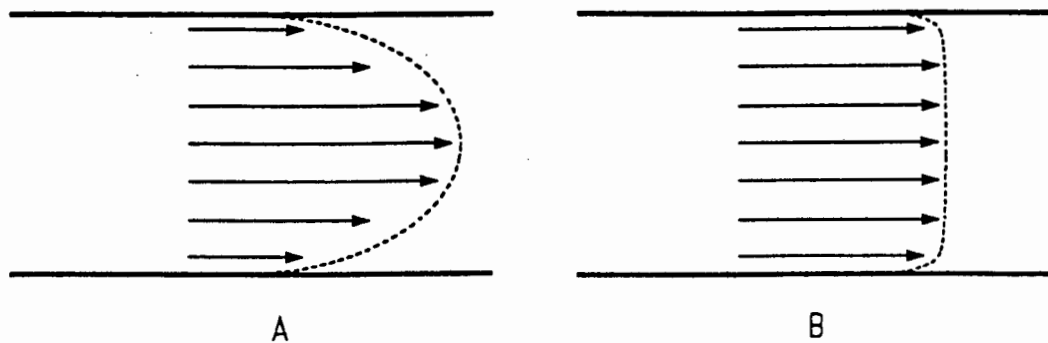
### **(a) Size:**

The chamber dimensions should be sufficient to allow a reasonable degree of free movement by the fish (i.e. the fish should not be confined to the extent that stress-induced excitement could seriously influence results). On the other hand, the volume of the system should not be so great as to reduce the sensitivity of the respirometer, as is discussed below. The flow chamber was constructed from a clear perspex cylinder, allowing visual monitoring of the fish, of dimensions

440 mm length and 70 mm internal diameter. This was considered to be satisfactory for the size range of the fishes used in these experiments (lengths of about 50 mm to 120 mm).

**(b) Velocity profile:**

The nature of water flow through the flow chamber is of central importance in this experimental design. The basic assumption inherent in this approach is that the fish maintains its position in the flow chamber by actively swimming against the water flow. Hence, swimming speed is equivalent to the velocity of the water flow, which should be the same at all points in the flow chamber. Goldspink (1977b) and Bårdgard *et al.* (1989) have stated that flow as close as possible to laminar is the required state. The classical view of laminar flow implies a curved velocity gradient across a cylindrical pipe (see Figure 4 A), ranging from zero velocity at the walls of the pipe, to a maximum velocity in the centre. A fish will consequently be exposed to a range of flow velocities, depending on its position in the velocity gradient. Since velocity was only measured at one point in the flow chamber (see Figure 3), calculation of a mean velocity would be virtually impossible.



**FIGURE 4:** Schematic representation of the velocity profile of viscous fluid flow through enclosed circular pipes. A - laminar flow; B - microturbulent flow. The length of the arrows represent the magnitude of the flow velocity. After Webb (1975).

What is in fact required is microturbulent flow (Webb, 1975). This involves the formation of micro-scale turbulence patterns, and a resulting rectilinear velocity gradient across the pipe (see

Figure 4 B). An important factor is that major inconsistencies in the flow (e.g turbulence effects and eddies), which might influence swimming behaviour and contribute to stress, are to be avoided. In order to promote an even, microturbulent flow through the flow chamber, several features were incorporated into the system. Expansion and contraction cones constructed from moulded fibreglass were mounted at the inflow and outflow ends of the chamber respectively. The expansion cone diverged at an angle of about  $6^\circ$ , which has been proposed as the critical angle for even divergence of water flow (Bell and Terhune, 1970). The contraction (outflow) cone converged at an angle of about  $10^\circ$ . The characteristics of flow downstream of the flow chamber were of no importance to the design, and by increasing the angle of convergence the total volume of the flow chamber was substantially reduced (see below). It has been suggested (Farmer & Beamish, 1969 and Steffensen *et al.*, 1984) that a more effective layout is to converge water flow into the flow chamber, a more efficient process than the one adopted in this study. While the author agrees with this, the implications of such a layout (in terms of substantially increasing the total volume of the system) are considered to outweigh the advantages.

Additional features incorporated into the system were a honeycombe baffle ("B", Figure 3), mounted into the expansion cone, and a 2.5 mm mesh plastic grid ("G", Figure 3) mounted across the inflow end of the chamber, separating the chamber from the expansion cone. The effectiveness of such grids and baffles in promoting microturbulent flow is well documented in the literature (Brett, 1964), and proved very effective in the present study. Finally, the choice of a cylindrical shape for the flow chamber has relevance to the nature of flow. Rectangular chambers have been used in the past (Webb, 1971a; Soofiani & Priede, 1985; Kaufmann, 1990), but required flanges mounted across the corners to eliminate "dead space" (Priede & Holliday, 1980). It was felt that a cylindrical flow chamber would be more effective, as no corners with potentially low velocity areas would be available to the fish. Tests to establish the effectiveness of these measures in promoting microturbulent water flow through the chamber were conducted by observing the motion of air bubbles in supersaturated water flowing through the chamber, a technique of flow visualization employed by Brett (1964). No major eddies or areas of discernable turbulence were observed. Further, no detectable differences in the speed of the bubbles in the various regions of the flow chamber were noticeable.

A 120 mm long section of the upper surface at the inflow end of the chamber was covered with opaque black plastic (Figure 3), the purpose of which was to provide the fish with a point of reference. During experimental runs, it was observed that the fish spent most of the time in this section of the chamber, presumably due to the apparent concealment offered by the black covering. Webb (1971b) reported improved station-holding by rainbow trout in a flow chamber partially covered with opaque black plastic sheeting. The downstream end of the chamber comprised the flow meter assembly ("FM", Figure 3), separated from the rest of the flow chamber by another plastic grid (not illustrated in Figure 3), which served to confine the fish to the flow chamber. Several studies reported in the literature (Brett, 1964; Smit, Amelink-Koutstaal, Vijverberg & von Vampel-Klein, 1971; Webb, 1971a; Steffensen *et al.*, 1984), have incorporated an electrified grid, to ensure that the fishes did not "rest" against the grid. This measure has been criticised by Thomas, Poupin, Lykkeboe & Johansen (1987) as a source of stress, and was considered to be unnecessary in the present study, since the trout usually avoided contact with the grid, maintaining their position under the opaque covering.

The flow meter assembly comprised a light-weight, multi-bladed black plastic propellor, mounted across the chamber normal to the direction of flow. The propellor occupied virtually the entire cross-sectional area of the chamber, hence providing a measure of velocity that was representative of the water flow across the entire chamber. One of the blades of the propellor was painted white to facilitate measurement of the propellor's rotation rate, either by eye (using a stopwatch) at low speeds, or by means of a variable frequency stroboscope at high velocities. The principle involved in the use of the flow meter rested on the assumption that the rotation rate of the propellor was directly proportional to the velocity of the water current flowing across the blades of the propellor. The validity of this assumption was confirmed during the calibration procedure (described later in this chapter).

A 70mm PVC threaded union joint ("U" in Fig. 3) was fitted to the end of the section of the chamber to which the fish was confined. This joint could be unscrewed, and the flow chamber separated from the rest of the system to permit introduction and removal of the fish. A 40mm diameter circular port was situated on the upper surface of the downstream end of the chamber.

This port could be sealed by means of a rubber bung which incorporated a flexible tube connected to the pressure compensator on the oxygen sensor. The port also allowed for the removal of any air bubbles which might accumulate in the chamber, the presence of which could introduce errors into the measurements of oxygen consumption rates.

### **3. Respirometer volume:**

The respirometer developed for this study is a closed-volume system. Although a flow-through system is preferable in fish metabolism studies (O'Hara, 1971; Caulton, 1978), the implications of such a system in terms of measurement of gaseous exchange preclude its application to the present study. The use of a flow-through system involves measurement of dissolved O<sub>2</sub> concentration in the inflow and the outflow water, the difference between the two readings being a measure of the amount of oxygen consumed by the fish. However, since fish (particularly the juveniles tested in this study) exhibit extremely low metabolic rates (with concomitant low rates of oxygen consumption), there is an inherent problem associated with the detection of any differences in O<sub>2</sub> concentration between the inflow and outflow water. This problem is a result of the combined effect of slow depletion of dissolved O<sub>2</sub>, coupled with the relatively high flow rates associated with experiments on fish propulsive systems. For these reasons, it was decided that the respirometer design would have to follow the closed volume format.

Since the experimental approach involves the measurement of dissolved oxygen concentration, the larger the volume of water in the system, the longer the period required for detectable decreases in dissolved oxygen to occur. In other words, the sensitivity of the respirometer to oxygen consumption by the fish decreases with increased volume of the system. Although a system with the lowest possible volume would be the most efficient, this is in direct opposition to the factors mentioned earlier, namely minimising the stress level of the fish by maximising the size of the flow chamber to which it is confined. The final design of the apparatus must, therefore, involve a compromise between these two constraints (Kaufmann, 1990).

A further important implication in the use of a closed volume respirometer regards water quality. Since the water in the system contains a finite amount of dissolved oxygen, the ultimate effect of the ongoing metabolic processes of the fish is to decrease the dissolved oxygen content of the water with a concomitant increase in the concentration of waste metabolites, specifically carbon dioxide and ammonia. The effects of this degradation of water quality on the metabolism of fish have been well documented in the literature (see Fry, 1971 for a review), and are to be avoided. Apart from the effects of hypoxia on metabolic rates, another consideration which has bearing on the use of EPOC as a measure of anaerobic metabolism has been raised by Brett (1962). This is the possibility that oxygen debt (anaerobic metabolism) resulting from deprivation of  $O_2$  may involve different pathways and rates of repayment to that resulting from high levels of activity.

The lower the volume of the system, the more pronounced the decline in water quality in terms of the time required to lower it to levels adversely affecting the fish. The final volume of the respirometer used in this study was 5.01 liters. In view of this low volume, the problem of rapid degradation of water quality was a very real one. Monitoring of water quality, and a facility allowing the replacement of the water in the system with fresh water was therefore crucial.

#### **4. The Flushing System:**

The flushing facility incorporated into the design of the apparatus relied on a 100 l reservoir of fresh water mounted above the respirometer (see Figure 3). Water in the reservoir was continuously aerated (ensuring a supply of water saturated with oxygen), and maintained at a temperature  $0.5^{\circ}C$  above that in the respirometer. This prevented supersaturation of the water and the formation of air bubbles when the respirometer was flushed. The flushing procedure was regulated by three PVC ball valves ("V2", "V3" and "V4" in Figure 3). Simultaneous opening of valves 3 and 4, with closure of valve 2 introduced fresh water from the reservoir into the respirometer, placing the apparatus in the "open circuit" condition. When the water quality had reached satisfactory levels, the reverse procedure returned the respirometer to the "closed circuit" condition. Water quality in the system was assessed by measuring the concentration of dissolved oxygen. This variable was never permitted to decline to levels below 90% saturation.

The adverse effects of low oxygen concentrations on the swimming performance (Bushnell, Steffensen & Johansen, 1984) and metabolic rates of fishes (Kutty, 1968a; Fry, 1971), appeared to become increasingly pronounced as oxygen content decreased below 90% saturation. Hence the precaution mentioned above.

During preliminary trials, it was found that a flushing period of about ten minutes at the lowest flow rates, and about two minutes at the highest, was sufficient to restore the oxygen content of the water in the system to saturated levels. The flushing period was usually prolonged to allow for equilibration of the oxygen sensor. The advantage of this system was that water in the respirometer could be replaced with minimal disturbance to the fish in the flow chamber. The only potentially disturbing factor might be the fluctuations in pressure associated with the flushing procedure (Fry, 1971 mentions mere operation of the valves as a disturbing factor). Since the pressure fluctuation was less than that which the fish would experience while swimming from the bottom to the surface of the holding tank in which the trout were maintained, this factor was considered to be negligible (this was supported by later observations of oxygen consumption rates). If it was necessary to prolong an experiment for several days (as was the case in the oxygen debt tests), the water in the reservoir was replaced with fresh, uncontaminated water (again, with minimal disturbance to the fish in the chamber). Operation of the flushing procedure was found to have a negligible effect on the velocity of water flow through the flow chamber (flow velocities measured during the flushing procedure varied by less than 1% from those measured when the respirometer was in the closed circuit condition).

Another aspect of water quality which should be discussed is that of the release of toxic substances into the water from the apparatus itself. Construction of the respirometer involved the use of several compounds (sealants, adhesives, and the materials of the fibreglass expansion and contraction cones) which could exert a profound influence on the metabolic rates of the fish. To avoid this complication, once the entire respirometer had been assembled and was being tested, fresh water was circulated through it for a period of at least a month (with daily water changes) before any animals were introduced into the chamber. It was assumed that this period would be sufficient to allow any potentially harmful substances to leach from the materials of the

apparatus. This assumption appeared to be substantiated by later measurements of metabolic rates. To overcome the potential problem of metal ions released into the water from the main circulating pump (which was constructed of cast iron), the interior surface of the impeller chamber was commercially coated with a glass-epoxy compound ("CORROGLASS"), preventing any corrosion. All other metal components of the apparatus were constructed from stainless steel.

## 5. Temperature Control and Measurement:

All experiments in this study were, without exception, conducted at 15°C. Maintenance of constant temperature is of vital importance in any study examining metabolic rates, particularly in the case of an ectothermic organism such as a fish, where ambient temperature is probably the most important environmental variable influencing metabolic rate (Brett, 1964). Studies documenting the effect of temperature on fish metabolism are well represented in the literature (e.g. Dickson & Kramer, 1971; Bernatchez & Dodson, 1985; Eccles, 1985; Paul, 1986), and have been reviewed by Fry (1971) and Brett & Groves (1979). Further, the solubility of oxygen in water decreases with increasing temperature, so care must be taken that temperature fluctuations in the system do not attain the level where the water becomes oversaturated, with resultant formation of air bubbles in the water. Ideally, one would prefer no temperature fluctuations whatsoever, but this is virtually impossible to achieve in a dynamic system. Fluctuations of the order of  $\pm 0.1$  to  $0.5^\circ\text{C}$  are commonly reported in the literature (Brett, 1964; Webb, 1971a; Priede & Holliday, 1980; Steffensen *et al.*, 1984; Bårdgard *et al.*, 1989; Facey & Grossman, 1991). The temperature regulating system described below was capable of maintaining a constant temperature of  $15 \pm 0.3^\circ\text{C}$ . Despite such a low temperature fluctuation some variability in the readings of the oxygen meter were occasionally observed, but did not cause any significant variation in the data obtained. Intimately involved with temperature regulation, is the facility to accurately monitor temperature. A thermistor probe incorporated into the oxygen sensor provided a digital reading of temperature when required (with a precision  $\pm 0.1^\circ\text{C}$ ). The accuracy of the probe was checked against a standardized mercury thermometer, and was found to fall within the limits mentioned above.

Temperature was found to be the most difficult variable to control, since the circulating pump generated large amounts of heat, particularly at high velocities. This problem was largely overcome through the incorporation of a heat exchanger into the system, and the addition of a cooling unit connected to the circulating pipes (Figure 3). The heat exchanger comprised twelve thin-walled, flexible plastic tubes (5 mm diameter, 1.5 m long), which were coupled to the apparatus immediately upstream of the flow chamber (see Figure 3), increasing the surface area available for heat exchange. The entire heat exchanger and flow chamber were then submerged in a recirculating water bath which was maintained at 15°C by a thermostatically controlled cooling unit. Water circulation in the bath was induced by means of a small centrifugal pump (not illustrated in Figure 3), further promoting heat exchange.

A section of the pipe downstream from the flow chamber incorporated a cooling mantle (a 400 mm length of PVC pipe of diameter 70 mm, "CM" in Figure 3), enclosing a second refrigeration coil. This coil was controlled by a thermostat ("T1") situated downstream from the circulating pump. Using this layout, water from the flow chamber was pre-cooled before entering the pump, where it was subsequently heated through the friction associated with rotation of the pump's impeller, the conflicting processes of heating and cooling being regulated by the thermostat.

## **6. Measurement of Dissolved Oxygen Concentration:**

Several methods of measuring oxygen concentration are available. A polarographic oxygen sensor, capable of providing a continuous record of dissolved oxygen content, was considered to be preferable for the purposes of this study (a detailed discussion of such sensors and their applications can be found in Gnaiger & Forstner, 1983). A SYLAND "Simclair-F" oxygen meter, which incorporated an automatic temperature-compensation facility, was consequently employed. This meter provides readings of dissolved oxygen concentration ( $\text{mg O}_2 \cdot \text{l}^{-1}$  or ppm), with a precision of  $\pm 0.1 \text{ mg} \cdot \text{l}^{-1}$ . Since the probe ("OP", Figure 3) effectively consumed oxygen, resulting in a local depletion of dissolved oxygen, a continuous flow of water had to be induced over the tip of the probe in order to obtain representative readings of dissolved oxygen. The manufacturers recommended a flow of about  $30 \text{ cm} \cdot \text{s}^{-1}$  past the tip of the probe. An additional

feature which had to be accounted for was the sensitivity of the probe to pressure fluctuations. Alterations in flow rate resulted in changes in the kinematic pressure of the water flow, causing aberrant readings to be registered by the meter.

These requirements were fulfilled by mounting the probe into a small plastic cuvette, connected to the downstream end of the flow chamber (the region of the apparatus where changes in kinematic pressure would be at a minimum) by two flexible 10 mm diameter rubber tubes. Water movement through the tubes and over the tip of the probe was induced by means of a small centrifugal pump ("P2", Figure 3) situated downstream of the probe. Water was drawn directly from the flow chamber and immediately across the tip of the probe, before being returned to the flow chamber, ensuring that the probe was supplied with a continuous water flow. A 5 mm diameter flexible tube ("PC") leading from the flow chamber (sealed into the rubber bung) was connected to the pressure compensating facility on the body of the sensor. This arrangement appeared to nullify any problems of pressure fluctuation (constant readings of oxygen concentration were obtained over the entire range of flow velocities of which the apparatus was capable).

The entire apparatus was housed in a controlled-environment cell in the Zoology Department at the University of Cape Town. The temperature and humidity in the cell could be controlled, as could the photoperiod, which was identical to that in the aquarium where the experimental fishes were maintained (see Chapter 3).

## **B. Calibration and Correction Procedures:**

### **1. Calibration of the oxygen meter:**

An advantage of the particular oxygen meter used in this study was that it only required calibration to saturated conditions in order to provide satisfactory readings. This was achieved by placing the respirometer onto the open circuit condition, inducing continuous circulation of 15°C oxygen-saturated water from the reservoir across the tip of the probe. The dissolved oxygen

reading on the meter was then set to the relevant figure, obtained from the standard tables of water saturation values (at 15°C the concentration of oxygen in 100% saturated water is 10.07 mg.l<sup>-1</sup>). To check the accuracy of the calibration procedure, water samples were taken from the respirometer at various levels of oxygen concentration, analysed by means of the Winkler determination, and the results compared to the readings provided by the oxygen meter. The measurements obtained from the oxygen meter were found to be within the range of  $\pm 0.1$  mg.l<sup>-1</sup> of the values obtained from the Winkler determination. Since these values fell within the precision range of the oxygen meter, the calibration was considered to be satisfactory, and the data obtained to be robust. Over the period during which experiments were conducted, it was found that the meter rarely required recalibration, since very little "drift" in the readings was observed.

## **2. Calibration of the flow meter:**

The validity of the assumption that the rotation rate of the propellor was directly proportional to the velocity of the water flow was tested during the calibration procedure. The flow meter was calibrated using volumetric measurements of flow rate. The measured flow rates were converted to velocities by applying the Law of Continuity, which states that the flow rate through all sections of an enclosed pipe system is constant (Webb, 1975). This can be expressed by the following equation:

$$Q = V \times A$$

where:

Q = flow rate (units of volume per unit time)

V = velocity of flow (units of distance per unit time)

A = cross-sectional area of the pipe (units of area)

(after Webb, 1975).

During the calibration procedure, the flow rate (cm<sup>3</sup>.second<sup>-1</sup>) at various settings of the velocity regulating valve ("V1") was measured by diverting the water flow through the apparatus into a volumetrically graduated container, and measuring the time required (by means of a stopwatch) for a set volume of water to flow into the container. The rotation rate of the propellor

( $\text{revolutions}\cdot\text{min}^{-1}$ ) was measured simultaneously. Since the cross-sectional area of the flow chamber was known ( $38.48\text{ cm}^2$ ), and a measure of flow rate had been obtained, the resultant velocity ( $\text{cm}\cdot\text{s}^{-1}$ ) of the water across the flow meter could be calculated from the above equation. This procedure was repeated for a range of velocity settings. The relationship between flow velocity (independent variable) and flow meter rotation rate (dependent variable) was then established by means of a simple least-squares linear regression analysis carried out on these data. The resulting regression equation (illustrated in Figure 5) was found to be

$$Y = -6.212 + 8.857 X \quad (n = 10 ; P < 0.001)$$

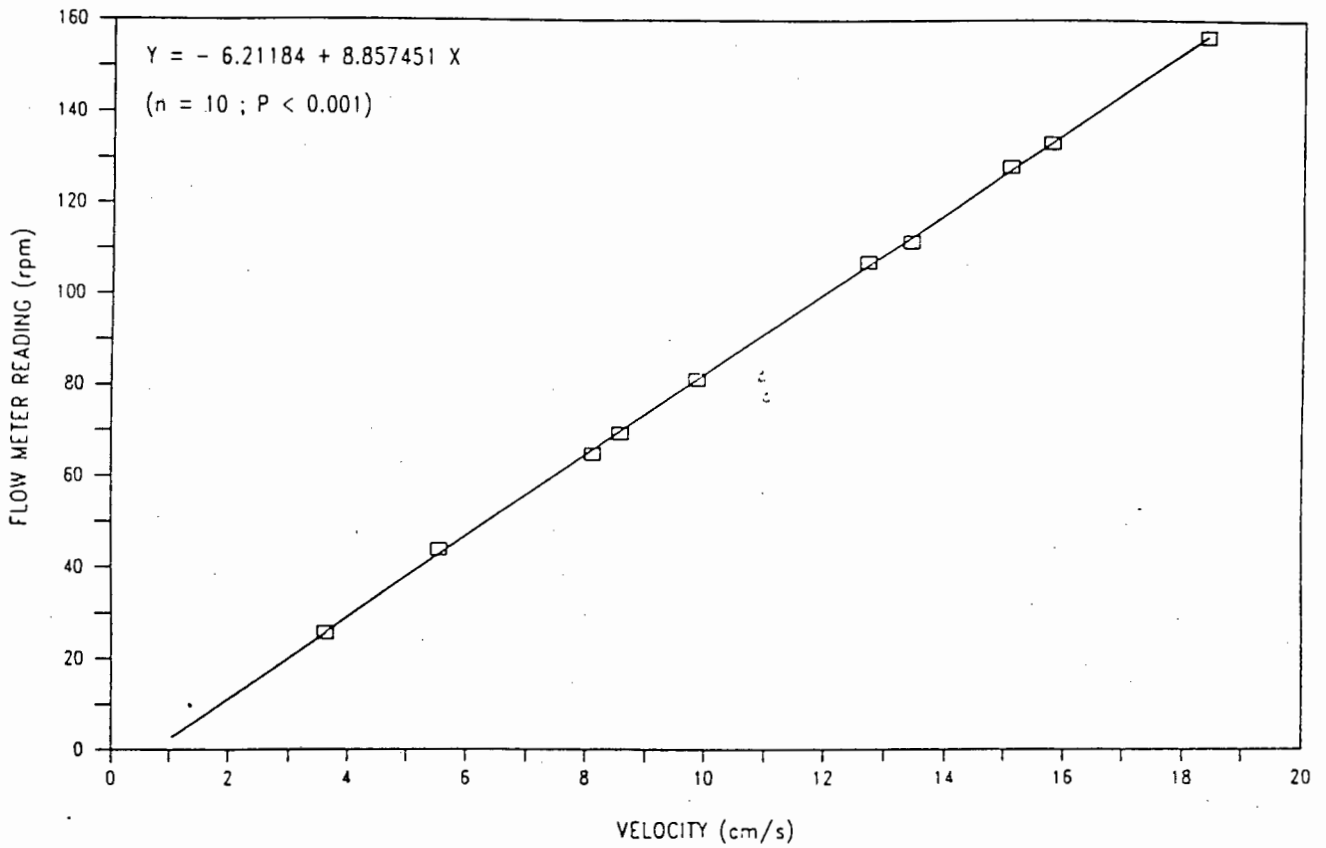
where  $Y$  = the flow meter reading (rpm).

$X$  = flow velocity ( $\text{cm}\cdot\text{s}^{-1}$ ).

The fact that the regression line does not pass through the origin can perhaps be explained by the construction of the flow meter. A water current of a certain velocity (approximately  $0.6\text{ cm}\cdot\text{s}^{-1}$ ) would be required to overcome the friction inherent in the system, and thus turn the propellor. The assumption inherent in the principle of the flow meter was, however, considered to be valid, justifying the application of the flow meter.

### 3. Correction of the velocity measurements:

The measurements of flow velocity obtained from the flow meter have to be subjected to various correction procedures before they can be considered to be a representative estimate of the swimming speed of the fish in the flow chamber. These correction factors are a direct result of the confined nature of the flow chamber. The restrictions of the walls of the chamber on water flow, with concomitant pressure changes, influence the drag on the fish in the chamber (detailed discussion is given by Bell & Terhune, 1970 and Webb 1975). Four corrections have to be considered; horizontal buoyancy, wake blocking, solid blocking and propellor correction. Wake blocking is usually considered to be negligible in systems such as the one described (Webb, 1975), and can be discounted. The effect of horizontal buoyancy is to increase drag on the fish, while the propellor correction is of the same magnitude but opposite in effect (by adding to the thrust of the caudal propellor). These two corrections tend to cancel out (Webb, 1975). Thus only solid blocking remains as an important correction.



**FIGURE 5:** Flow meter calibration curve. Open squares are flow meter readings (revolutions.min<sup>-1</sup>) corresponding to measurements of flow velocity (cm.s<sup>-1</sup>). The solid line is the best-fit regression line, described by the equation included in the figure.

Correction for solid blocking arises from the increased velocity of the fluid around any solid object in a tunnel through which the fluid is moving. This is a direct result of the Law of Continuity discussed previously. Since the flow rate in an enclosed pipe system must be constant, the flow velocity varies inversely with the cross-sectional area through which the fluid is passing. The physical presence of the fish in the flow chamber represents a restriction of the cross-sectional area, and hence a concomitant increase in velocity of the fluid over the body of the fish. This velocity is greater than the free-stream velocity in the unrestricted tunnel measured by the flow meter. The equation for the Law of Continuity presented previously can be rewritten as follows:

$$Q = V_t \times A_t = V_f \times (A_t - A_f)$$

where

$Q$  = flow rate

$V_t$  = flow velocity in the tunnel

$A_t$  = cross-sectional area of the tunnel

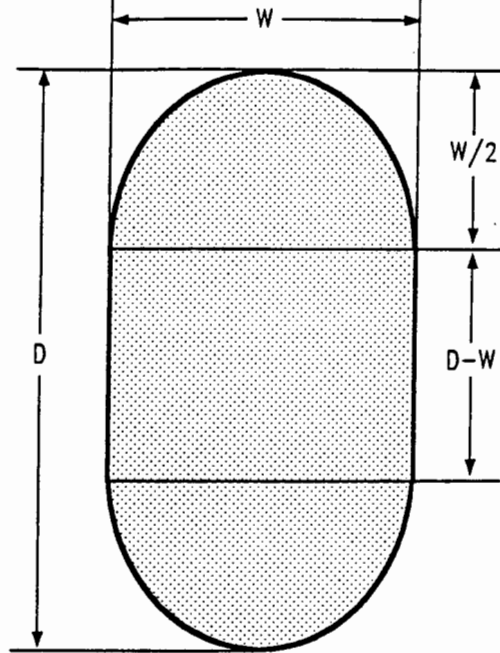
$V_f$  = velocity at the surface of the body

$A_f$  = cross-sectional area of the body

Hence, the correction for a body of uniform cross-sectional area can be calculated as follows:

$$V_f = (A_t \times V_t) \div (A_t - A_f)$$

The body of a fish does not have a uniform cross-sectional area, but is a fusiform shape, with varying sectional area along its length. A common practice, adopted in the present study, employs the maximum sectional area of the fish's body. Webb (1975) has stated that this will result in an over-estimate of the swimming speed of the fish, but it is felt that the effect of any over-estimation will be negated by not allowing for the wake blocking correction, as mentioned earlier. To calculate the maximum cross-sectional area of the fish, the body of the fish was assumed to have an oval cross-section composed of a central rectangle with upper and lower semi-circles, of dimensions as illustrated in Figure 6.



**FIGURE 6:** Schematic representation of the cross-section of a juvenile rainbow trout employed for the solid blocking velocity correction procedure. Dimensions are in terms of maximum dorso-ventral body depth (D) and maximum body width (W).

The area of the oval was calculated as follows:

$$A_f = (D \times W) - 0.215 W^2$$

where

$A_f$  = maximum cross-sectional area

D = maximum dorso-ventral depth

W = maximum lateral width

The cross-sectional area of a fish will not be perfectly described by this equation, but the margin of error is felt to be negligible. Since the cross-sectional area of the flow chamber was known (38.48 cm<sup>2</sup>), the correction for solid blocking was calculated as follows:

$$V_f = V_t [38.48 \div (38.48 - DW + 0.215 W^2)]$$

#### 4. Correction for respirometer volume:

The fish, by physically occupying space in the flow chamber, displaced a volume of water equivalent to its body volume. The volume of water in the respirometer was therefore not the same as the measured volume of the respirometer. This factor had to be accounted for in the calculation of oxygen consumption rates. The volume of each fish (ml) was therefore considered to be equivalent to its wet weight (g), assuming that the fishes had a specific gravity of unity. This assumption was subsequently substantiated by measurements of the volume to weight ratio of five living trout, the average of the five ratios being 0.98 ( $SD = 0.02$ ).

#### 5. Correction for oxygen consumption not attributable to the fish:

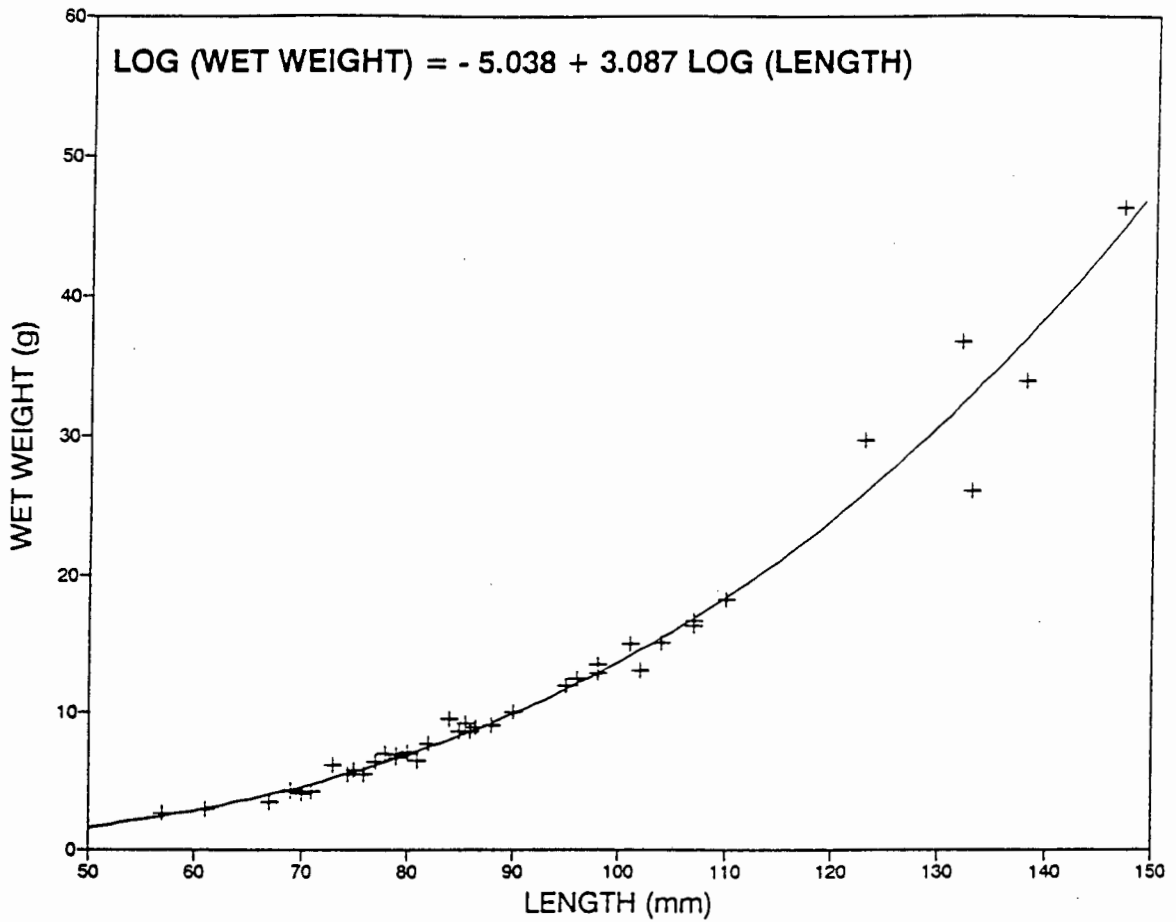
This is a vital correction which must be applied to the measurements of oxygen consumption rates, since not all the measured oxygen depletion can be attributed to the respiration of the fish. Firstly, the oxygen probe itself consumes oxygen, and secondly, there is usually a microbial component to measured oxygen consumption rates resulting from respiration of bacteria, algae and other microbes resident in the apparatus. To gain an estimate of this source of error, control oxygen consumption rates (oxygen consumption rates measured without the fish in the respirometer) were obtained before and after each experiment. Control oxygen consumption was assumed to increase linearly from the pre-experimental to the post-experimental measurements. Oxygen consumption rates measured with the fish in the respirometer were then corrected for control oxygen consumption increasing as a function of time, a procedure employed by Wieser *et al.* (1985). Before an experiment was initiated, the respirometer was cleaned, and sterilized by introducing about 2 g of a commercially available dry granular chlorine into the water, and circulating the solution for about 10 minutes. The respirometer was then flushed with fresh water several times to ensure that none of the chlorine remained in the system. This method was found to be very effective at reducing the control oxygen consumption to acceptable levels. These procedures were carried out for all of the experiments which were conducted in the course of this study.

### FISH MAINTENANCE AND HANDLING

#### **Fish Maintenance:**

Juvenile *Oncorhynchus mykiss*, (40 individuals of length about 30 mm) were obtained from the University of Stellenbosch's trout research station at Jonkershoek in August 1991, and again in February 1992. The fishes were transported to the aquarium at the University of Cape Town's Zoology department in 10 l plastic bags half filled with water and inflated with pure oxygen. Following a temperature equilibration period, the fishes were introduced into a 2000 liter PVC "PORTAPOOL" containing fresh dechlorinated tap water at 15°C. Undergravel filtration systems in the holding tanks maintained the water quality in the tanks at a satisfactory level (assessed through observations of water colour and oxygen content) for up to 3 weeks at a time. The fishes were periodically (every 2 weeks) transferred to a tank containing fresh uncontaminated water. The first tank was then drained, cleaned, refilled with fresh water and allowed to stand for at least a week to stabilize at the desired temperature. No water flow through the holding tanks was induced, but the water was continuously aerated.

From first introduction to the holding tanks, the fishes were exposed to a constant photoperiod regime, which involved a 12 hour light : 12 hour dark cycle. The photoperiod also included 10 minute "dawn" and "dusk" phases, where the light intensity gradually increased and decreased respectively. The identical regime was applied during all experiments conducted in this study. The fishes were fed a commercially available trout pellet feed ("MEADOW" trout growth and breeding feed) the proximate composition of which was stated by the manufacturers to be 38% protein, 6% fat, 4% fibre and 12% moisture. The fishes were fed to satiation once every two days (satiation was taken to be when the fishes no longer responded to further introduction of feed into the tank). This feeding regime was selected so as to minimise fouling of the tanks, as well as to correspond to the starvation periods imposed during the various experiments described in later chapters. Figure 7 illustrates the relationship between the lengths and the wet weights of



**FIGURE 7:** The length (mm) - wet weight (g) relationship of the rainbow trout used in the present study. Symbols are measured data points, while the solid line is the best-fit regression line described by the equation presented in the figure. Note: both axes are scaled arithmetically.

the trout used in this study. The regression equation describing the relationship between these two variables was found to be highly significant ( $P < 0.001$ ). The fishes were allowed an acclimation period of at least one month to aquarium conditions, before experiments were initiated. With the exception of one instance where the air supply to the holding tanks was interrupted overnight, no mortalities were observed in the holding tanks.

### **Fish Handling:**

All experiments conducted were carried out on individual fishes (it was felt that data obtained from individuals could provide valuable information in terms of individual variation). Fishes to be used in the experiment were starved for at least 48 hours before being introduced to the flow chamber. This precaution was adopted in an attempt to avoid the complicating effects associated with post-ingestion elevation of metabolic rate (Saunders, 1963; Wissing & Hasler, 1971; Beamish, 1974). Reports in the literature vary with respect to the pre-experimental starvation periods applied. Brett (1964) (after Beamish, 1964a) ensured a fasting period of at least 36 hours prior to any determination of metabolic rate, while the rainbow trout used in experiments by Webb (1971a) were starved for a period of 3 days. Facey and Grossman (1990) starved rainbow trout for 20 hours prior to introduction to the respirometer, but did not assess whether the fish were in a post-absorptive state on initiation of the experiments. A fasting period of 48 hours prior to introduction into the respirometer was adopted in this study (some exceptions to this precaution did occur, as will be referred to in later discussion). A minimum acclimation period of 12 hours was imposed before any measurements of oxygen consumption were recorded. The total fasting period in most cases was therefore at least 60 hours.

For all experiments conducted in this study, measurements of fork length (measured from the tip of the upper jaw to the base of the notch in the caudal fin - Priede & Secombes, 1988), maximum dorso-ventral body depth, maximum lateral body width, and wet weight of each fish were recorded prior to introduction into the respirometer. Wet weights were obtained after first gently blotting the fish dry with absorbent tissue paper. These measurements were repeated when the fish was removed from the respirometer after each experiment. Mean values were then calculated

for application to the various correction calculations discussed previously, as well as to calculation of specific values of oxygen consumption ( $\text{mg O}_2 \cdot \text{kg body wet weight}^{-1} \cdot \text{hr}^{-1}$ ) and swimming speed ( $\text{bl} \cdot \text{s}^{-1}$ ). The latter unit allows for comparison of fishes of different lengths (Brett, 1964), and has been shown by Bainbridge (1958) to be a useful basis for more general comparisons.

Following introduction into the respirometer, the fishes were subjected to a very low imposed velocity (usually of the order of  $0.5 \text{ bl} \cdot \text{s}^{-1}$ ), and left overnight (for at least 12 hours) to acclimate to conditions in the respirometer and recover from handling, the effects of which have been demonstrated by Barton & Schreck (1987) and Ladu & Ross (1992). Oxygen consumption rates immediately subsequent to introduction into the respirometer were often observed to be an order of magnitude greater than those measured after the fish had settled down. Similar observations have been reported in the literature (e.g. Beamish & Mookherjee, 1964; Smit, 1965; Kutty, 1968b; Steffensen *et al.*, 1984; Paul, 1986). During the acclimation phase, the respirometer was left in the open circuit condition, ensuring a constant supply of freshly aerated water to the fish. Water used in all the experiments conducted in this study was obtained from the holding tanks in which the fishes were maintained, ensuring that the fish in the respirometer was exposed to water of a familiar chemical milieu. Fry (1971) suggested that this precaution could possibly have a role in reducing the disturbance experienced by the fish. Once the experiment had been completed, the fish was removed from the respirometer, measured, and returned to a holding tank. Fishes which had been used in an experiment were kept separate, ensuring that no fish was used in more than one experiment.

Before and after each experiment, control runs were carried out with no fish present in the chamber. Once the final control run had been completed after each experiment, the respirometer was cleaned, flushed several times, and refilled with fresh water from the holding tanks in preparation for the next experiment. No mortalities resulting from any of the experimental procedures were observed.

## DIURNAL VARIATION EXPERIMENTS

In Chapter 1, a possible circadian rhythm in the metabolic rate of the rainbow trout used in this study was discussed. Preliminary measurements of oxygen consumption rates ( $VO_2$ ) indicated that a diurnal rhythm of this nature may have been operative, and could introduce a substantial source of error into later estimates of energy expenditure. Experiments aimed at estimating the energy costs of locomotion assumed that oxygen consumption rates in excess of the estimated standard rate could be ascribed purely to the imposed level of activity, and that the time of day at which  $VO_2$  data were recorded had no influence on the magnitude of the measurements. This assumption would not be valid if a circadian rhythm was present. The series of experiments described in this chapter were conducted in an attempt to test this assumption.

**Experimental Procedure:**

The experimental protocol followed the same initial format as described in the previous chapter. Following introduction to the flow chamber, each fish was immediately subjected to the test velocity, which was maintained for the duration of the experiment. Once the overnight acclimation phase was complete,  $VO_2$  measurements were recorded virtually continuously for the following 24 hours. Subsequent to the 24 hour period, the fish was removed, measured, and returned to the holding tanks.

To permit continuous recording of  $VO_2$  during the dark phase of the photoperiod, a low intensity desk lamp was placed in the cell. To ensure that the fish was in complete darkness, the flow chamber was covered with an opaque cover. During the initial experiments, this cover was periodically partially removed in order to monitor the behaviour of the fish. This practice was discontinued when it was observed to result in a visually-induced behavioural response on the part of the fish, with concomitant elevation of the metabolic rate.

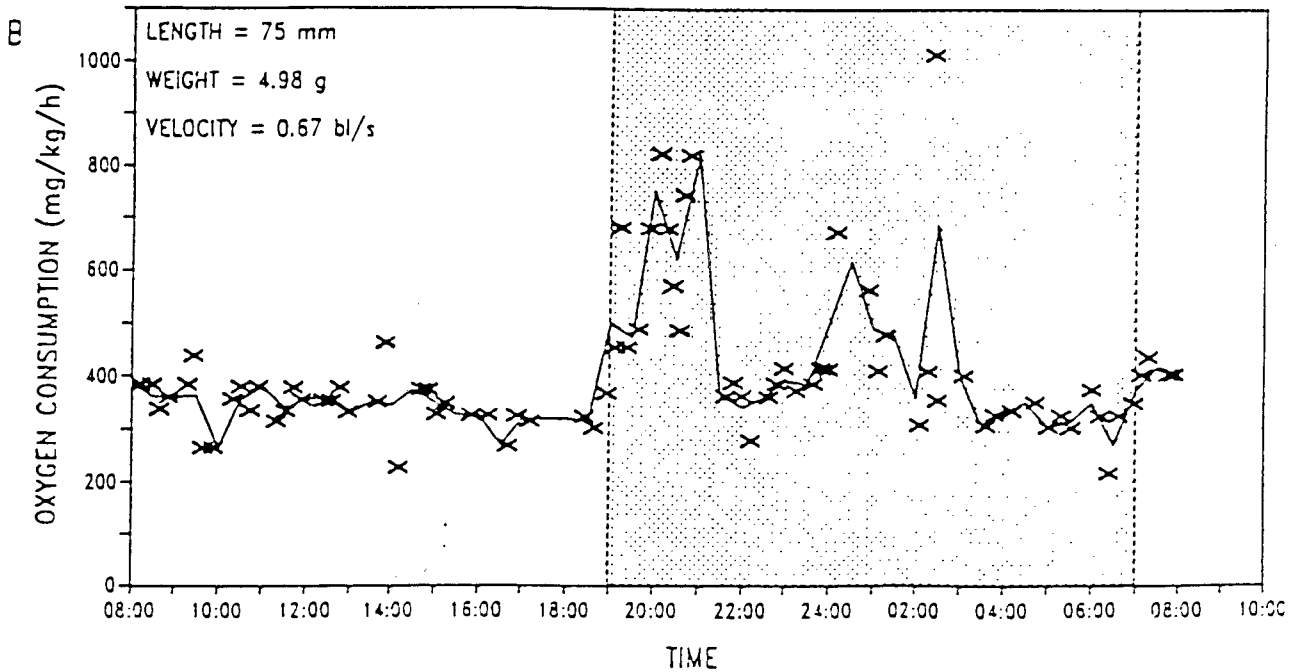
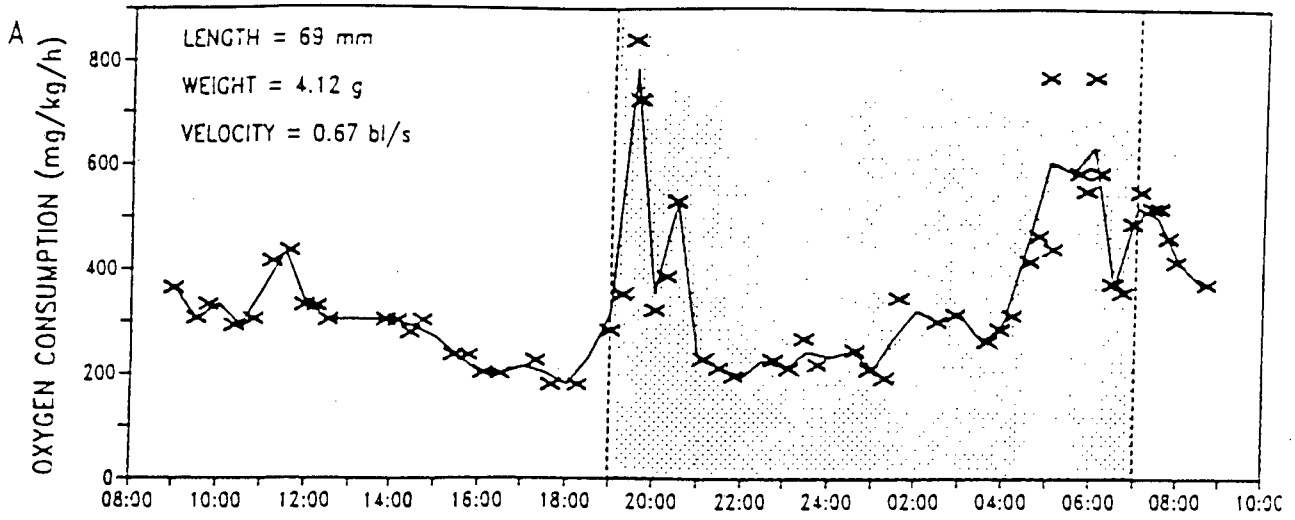
The test velocities imposed during these experiments were maintained at relatively low levels ( $\leq 1.0 \text{ bl}\cdot\text{s}^{-1}$ ) for two reasons. Firstly, in order to obtain representative data, it was crucial that the fish was capable of sustaining a constant level of activity for the duration of the experiment, without the occurrence of fatigue-related stress with associated elevated  $\text{VO}_2$  and oxygen debt accumulation. Secondly, to avoid obscuring any diurnal variation in metabolism which may have occurred. Since the magnitude of this potential variation was the factor under investigation, it was essential that all other metabolic contributions be kept to a minimum.

### **Data Analysis:**

The results were analysed by means of a time series analysis procedure employing an autoregressive, moving average model (Crawford, Siegfried, Shannon, Villacastin-Herrero & Underhill, 1990). The procedure identified any consistent trends and patterns evident in the data. Prior to analysis, the data were standardized in order to comply to the format required by the time series procedure, namely a series of values spaced at equal time intervals. The raw data collected during the experiments did not exhibit this basic format, since gaps occurred in the series (as a result of flushing periods). In addition, the time between discrete measurements of oxygen consumption varied considerably, dependant upon the magnitude of the metabolic rate of the fish at any given time. A time interval of 30 minutes was selected for the analysis. All data points falling within a given 30 minute interval were averaged, yielding a single mean value for that interval. Where insufficient data were available for any particular time interval, the mean of the immediately preceding and subsequent values was included. The use of a 30 minute sampling interval was found to result in an acceptably representative data series. A shorter interval would have involved an unacceptably high percentage of missing values, while a longer sampling interval would have resulted in a loss of resolution in the standardized data series.

### **Results and Discussion:**

A series of six experiments investigating the possible presence of a diurnal rhythm were conducted. The results are illustrated in Figure 8 A to F, where each graph corresponds to a test



**FIGURE 8:** Results of the diurnal variation experiments. Each diagram presents measured oxygen consumption rates of an individual rainbow trout (of indicated length and width) subjected to the indicated velocity over a 24 hour period. Symbols are measured data points, while the solid line joins the standardized data points (see text). Shaded areas indicate the dark phase of the applied photoperiod. For comparative purposes, the scaling of the X and Y axes is kept constant through all the diagrams. All tests at 15°C.

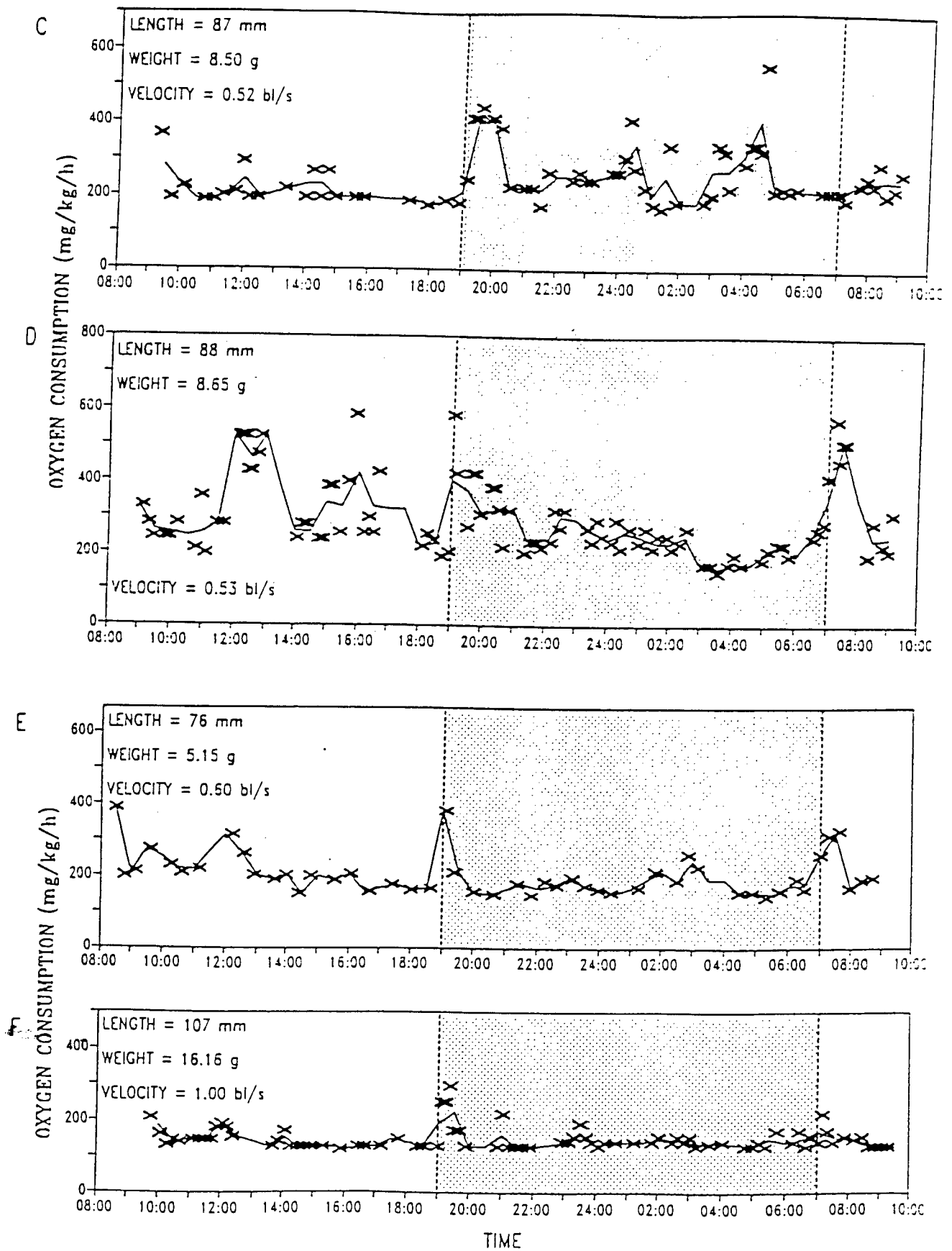


FIGURE 8: (continued).

on an individual fish. The length and wet weight of each fish are indicated, as is the velocity imposed during each experiment. The symbols illustrated in Figure 8 correspond to measured data points, while the solid line connects the standardized data points obtained through the procedure described above.

A high degree of variability was evident in the data, both within and between experiments, with no clear pattern discernable. The variability manifested in these data was to be expected, in view of the relatively low imposed velocities permitting a certain degree of spontaneous activity on the part of the fish, with associated elevated  $VO_2$ . Where the behaviour of the fishes were monitored, it was observed that the occurrence of elevated  $VO_2$  was always associated with periods of excess activity on the part of the fish (i.e. when the fish was not maintaining a constant position in the flow chamber, but was moving around within the confines of the chamber). Further, the intensity of the excess activity (ranging from slow swimming back and forth in the chamber, to bursts of rapid swimming) appeared to be reflected in the magnitude of the associated  $VO_2$  elevation (a similar observation has been reported by Evans, 1972). Presumably, this was also the case during the periods where activity was not monitored.

No consistent periodicity appeared to be evident in the incidence of excess activity, with the exception of the periods immediately subsequent to "dusk" and "dawn" (i.e. the switching phases of the applied photoperiod). It should be stressed that these periods of excess activity (with associated  $VO_2$  elevation) were almost exclusively evident after the lights had switched either off or on. No anticipatory response appeared to be evident, suggesting that these periods of excess activity were purely a behavioural response to the visual stimulus of the relatively rapid changes in light intensity, and not the manifestation of an endogenous circadian rhythm. The intensity of the excess activity occurring during these periods varied to a large extent, ranging from rapid, intense bursts, to relatively minor swimming activity within the confines of the chamber.

The data recorded during the dark phases of the experiments illustrated in Figures 8 A, B, & C exhibited a much higher degree of variability than do the light phase data of each experiment. This is very likely a direct result of the practice mentioned previously, namely the periodic

partial removal of the flow chamber cover during the dark phases of these experiments. As was discussed, this practice resulted in a direct behavioural response on the part of the fish, with concomitant  $VO_2$  elevation. In contrast, the experiments illustrated in Figures 8 D, E & F (where activity was not monitored during the dark phase) exhibit the reverse pattern. It is therefore concluded that the dark phase variability evident in the first three experiments was purely an experimental artefact, and not representative of any endogenous biological rhythm.

The data presented in Figure 8 F are of interest, in that they display the least amount of variability of all these experiments. This particular fish was subjected to the highest imposed velocity ( $1 \text{ bl.s}^{-1}$ ), which would appear to support the earlier argument that very low velocities have to be imposed to avoid obscuring any possible diurnal variation. The low variability in this case could also, however, be a function of size (this individual was the largest of the trout tested in this series of experiments).

Visual inspection of the data consequently revealed no consistent patterns of diurnal variation (in  $VO_2$  or activity) in the results of these experiments. This was tested statistically by means of the time series analysis described previously. The results of the analysis indicated that no significant rhythms or trends were evident in the data. The only consistent feature was a significant correlation ( $P < 0.05$ ) between data points at successive 30 minute sampling intervals. In other words, the variation in any one particular interval could be significantly accounted for ( $P < 0.05$ ) by the variation evident in the previous interval. This result implies that at the activity levels observed in these experiments, a period of 30 minutes was required for the  $VO_2$  to recover from any activity which might have occurred.

In conclusion, no significant rhythms or cycles were evident in these data, and this potential source of error was consequently discounted in subsequent experiments conducted in this study.

## INCREMENTAL VELOCITY TRIALS

**Experimental Procedure:**

These experiments were based on the incremental velocity protocol developed by Brett (1964), in which the fish was subjected to each velocity level for a period of 75 minutes, after which the velocity was increased to the next level. Two measurements of oxygen consumption were recorded at each velocity, extending over a period of 60 minutes after the test velocity had been imposed. The remaining 15 minutes were used to flush the respirometer with fresh water. This protocol has been adopted by several other researchers, with various modifications (e.g Tytler, 1969; Webb, 1971b; Facey & Grossman, 1990). In all these studies, the period during which the measurements of oxygen consumption were recorded always fell within one hour following the increase in velocity.

During preliminary trials, it was observed that the  $VO_2$  of the fishes often varied considerably for up to two hours or more, following an increase in imposed velocity. Further, a gradual decline in  $VO_2$  was often evident over this period. Given these observations, it was felt that the test durations employed by Brett (1964) and others (mentioned above), were too short to provide any valid estimates of the energy requirements associated with a given level of activity, and that values obtained from such procedures would probably be over-estimates. This argument is not supported by the observations of Webb (1971b), where rainbow trout exhibited a limited period of unsteady swimming (about 5 minutes) after each velocity increment. Webb (1971b) concluded that the 60 minute test period employed was sufficient for the fish to settle down. However, no  $VO_2$  data were provided to substantiate this conclusion.

In accordance with this argument, each test velocity was maintained for a minimum of 3 hours (or longer, if the  $VO_2$  had not stabilized by this time). In all of the experiments presented in this chapter,  $VO_2$  measurements were only recorded during the day. Further, at no stage during these

experiments were the fishes exercised to exhaustion. The fishes were always capable of maintaining the imposed level of swimming activity for the duration of the test.

### **Data Analysis:**

Data collected during this series of experiments were subjected to two selection criteria prior to analysis. Firstly, all data points associated with excess activity on the part of the fish were discarded. Estimates of swimming speed calculated from the flow meter readings assume that the fish is maintaining a constant position in the chamber (see Chapter 2). Where this assumption was not valid, corresponding measurements of oxygen consumption were not representative of the measured swimming speed, and were therefore excluded from the analyses. Secondly, all data recorded during a two hour stabilization period following each increase in velocity were discarded. It was felt that stress resulting from increases in velocity, as well as the complication of anaerobiosis, could influence these data.

The least-squares regression analysis proposed by Sokal and Rohlf (1981) for cases where a sample distribution of Y-values corresponding to each X-value is obtained, was performed on the  $VO_2$  - swimming speed data. This model has an advantage over the more commonly used simple linear regression analysis, as it allows separation of the two sources of variation in the data, namely the error among the Y values, as well as the deviations from the regression. To establish the nature of the relationship between  $VO_2$  and swimming speed, the data were tested against both linear and exponential (where the  $VO_2$  data were logarithmically transformed) models. To assess the influence of body size on this relationship, the line of "best fit" for each fish (the regression showing the highest *F* statistic) was then used to predict the  $VO_2$  corresponding to a series of set swimming speeds, increasing from the predicted standard  $VO_2$  in increments of 1  $bl.s^{-1}$ . In practice, it was found to be very difficult to regulate the imposed velocity with this level of precision, resulting in data which were not comparable across experiments. The prediction procedure overcame this problem. In order to obtain  $VO_2$  values for progressive increments of 1  $bl.s^{-1}$ , several of the predictions involved a certain degree of extrapolation, either to zero swimming speed to estimate the standard  $VO_2$  (a technique used by Brett, 1964, and

supported by the results of Brett & Sutherland, 1965), or to slightly above the maximum imposed velocity where this was not an increment of 1 bl.s<sup>-1</sup>. Although such extrapolation involves a high level of uncertainty, it was felt that the margin of error in the cases where such extrapolation was performed would not significantly influence the results. The VO<sub>2</sub> predictions for each velocity increment were plotted against the wet weight of the fishes, and a least-squares linear regression analysis conducted on the data. The data were tested against linear, exponential and logarithmic models through the transformation procedures mentioned above. For these regression analyses to be statistically valid, a weighting factor had to be incorporated into the analysis. A fundamental assumption inherent in such Model 1 regressions is that the level of variance in the dependent variable remains more-or-less constant over the range of the independent variable. The predicted VO<sub>2</sub> data did not fulfil this requirement. Each prediction of VO<sub>2</sub> against wet weight at any given swimming speed was calculated from a different regression equation, with a different degree of error, and hence a different variance. In order to overcome this problem, each data point was assigned a weighting factor expressing the degree of confidence associated with the particular prediction. For the purposes of these analyses, the reciprocal of the 95% prediction limits of each estimate was employed. This statistic was calculated as follows:

$$95\% P.L. = Y \pm t_{0.05 (2) n-2} (s_Y)$$

where  $Y$  = predicted value

$s_Y$  = standard error of the  $Y$  estimate (after Zar, 1984)

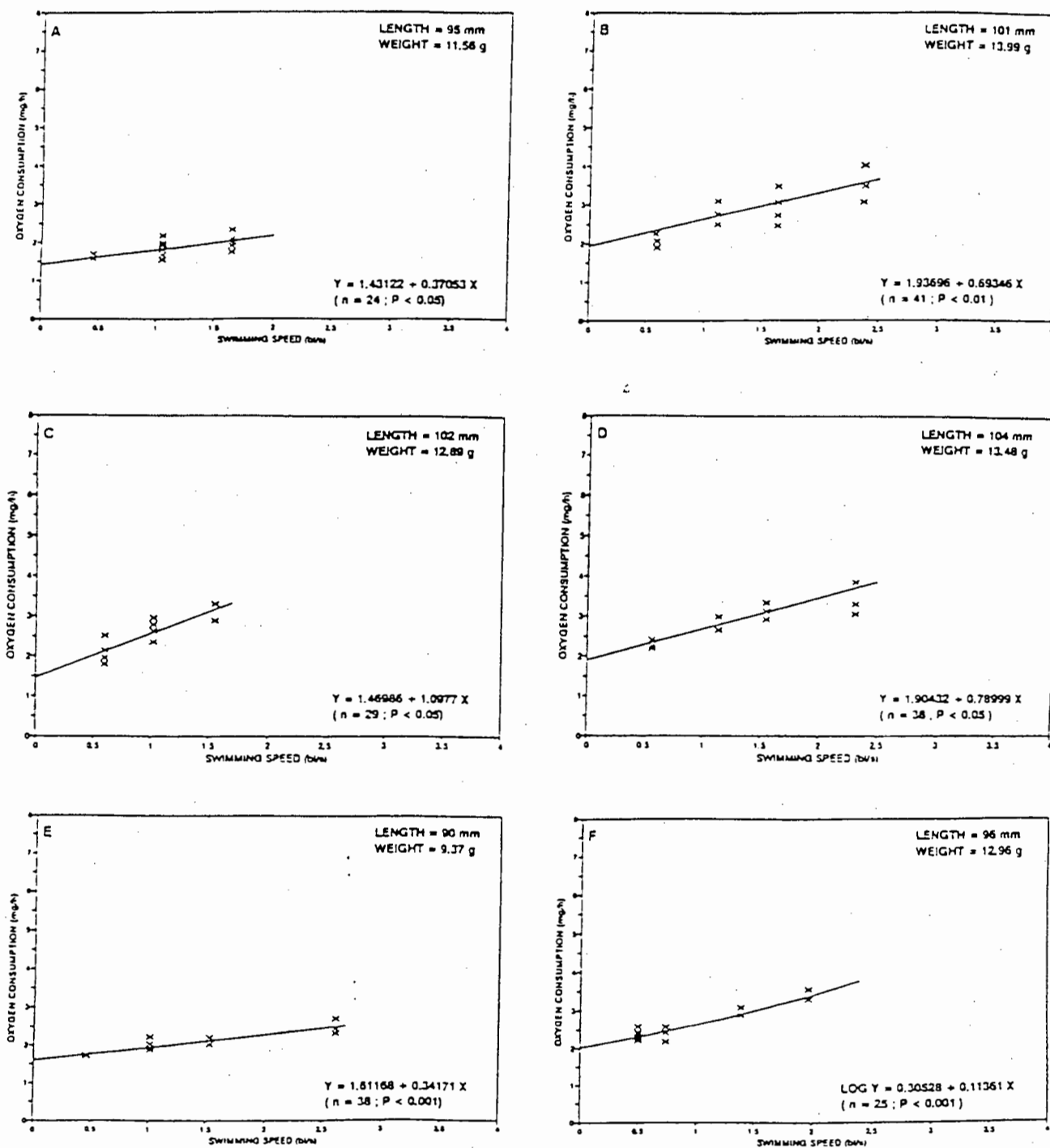
Once the upper and lower prediction limits ( $L1$  and  $L2$  respectively) had been calculated for each estimate, the weighting factor was taken to be the reciprocal of  $[L1 - L2]$ . Where a regression was carried out on the logarithmically transformed data, the weighting factor of each prediction was then calculated as the reciprocal of  $[\text{LOG}(L1) - \text{LOG}(L2)]$ . Predictions involving a high level of uncertainty (with a high inherent standard error) resulted in wide prediction limits, and hence very low weighting factors.

The regression analyses were carried out using the Statistical Graphics Corporation's "STATGRAPHICS" software package, which allowed the application of a weighting factor to the residuals during the least squares analysis. The results of these analyses provided a series of regression equations describing the relationship between oxygen consumption and wet weight

over a range of swimming speeds. These equations could then be used to estimate the aerobic energy expenditure of a fish of given body weight at any swimming speed within the range of velocities imposed during the course of these experiments. In the early stages of this work, preliminary data were examined by multiple regression in an attempt to set up a relationship between all three variables (swimming speed,  $VO_2$  and weight). This analysis did not provide a statistically significant result, and was therefore not used for later data analyses.

## Results and Discussion:

The results of incremental velocity tests conducted on 13 juvenile rainbow trout are shown in Figure 9. The fishes ranged in length from 57 mm to 138 mm, and in wet weight from 2.49 to 32.63 g. The symbols in each figure are measured absolute  $VO_2$  values ( $\text{mg O}_2 \cdot \text{h}^{-1}$ ) plotted against swimming speed ( $\text{bl} \cdot \text{s}^{-1}$ ). Each figure (9 A to M) presents the results of a single experiment on an individual fish. The weight indicated is the average of the measurements recorded at the beginning and end of each experiment (body weight was observed to decrease in all of the experiments). The results of the least-squares regression analyses conducted on the illustrated data are also included in each diagram. The "best-fit" regression line (described by the indicated equation) and significance level of the regression are shown. The relationship between oxygen consumption and swimming speed in fishes is generally best described by an exponential model, where a linear relationship is observed between the logarithm of  $VO_2$ , and swimming speed (Brett, 1964, 1972; Tytler, 1969; Beamish, 1970; Smit *et al.*, 1971; Webb, 1975; Goldspink, 1977b; Kiceniuk & Jones, 1977; Facey & Grossman, 1990). This was found to be the case in only five of the data sets presented in Figure 9, the other experiments indicating a linear increase in  $VO_2$  with increasing swimming speed. These latter data, however, were recorded during experiments where relatively low levels of activity were imposed. Data indicating an exponential relationship between these two variables were collected over a wider range of imposed velocities, extending to relatively higher activity levels, and as such, are probably more representative of the relationship. It could be postulated that the data indicating a linear relationship corresponded to the initial, almost linear section of an exponential curve, and if higher levels of activity had been imposed, an exponential model may have resulted.



**FIGURE 9:** Results of the incremental velocity tests. Each diagram illustrates the absolute oxygen consumption rate ( $\text{mg O}_2 \cdot \text{h}^{-1}$ ) swimming speed ( $\text{bl} \cdot \text{s}^{-1}$ ) relationship in an individual rainbow trout of indicated length and wet weight at  $15^\circ\text{C}$ . Symbols are measured data points fulfilling the criteria specified in the text. Solid lines are the best-fit regression lines. The regression equation, number of data points included in the analysis, and significance level of the regression are shown. (NS - not significant at the 95% level).

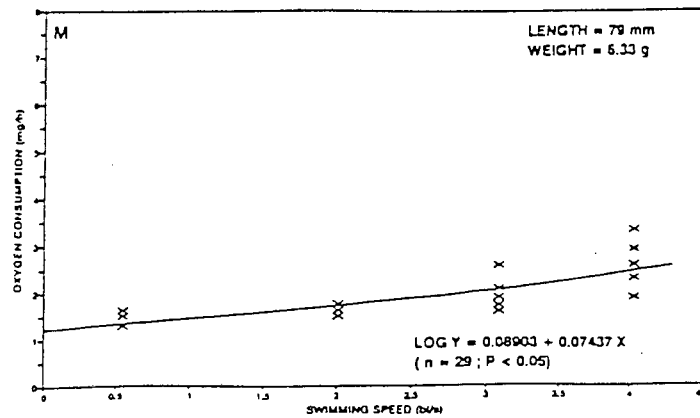
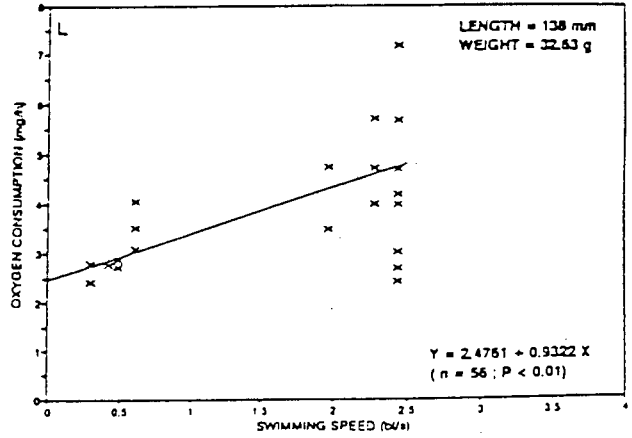
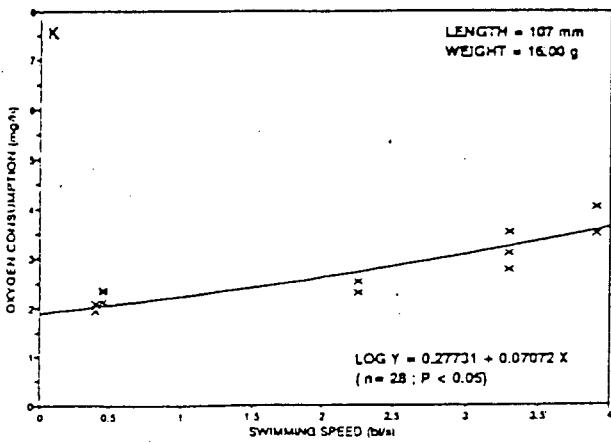
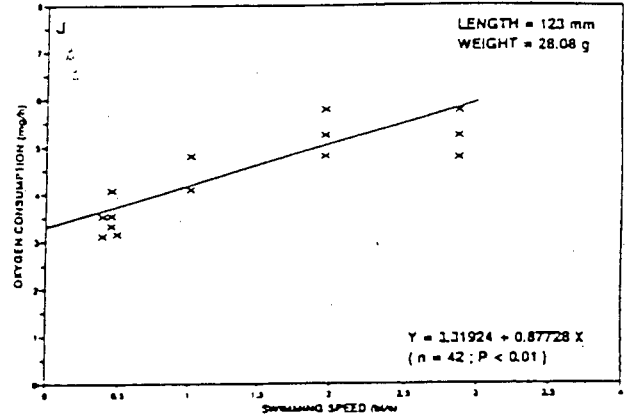
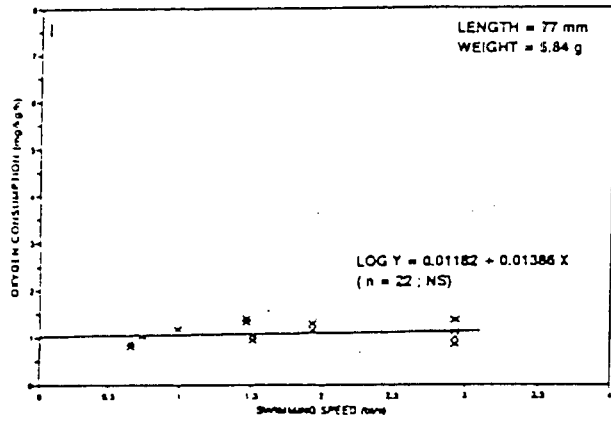
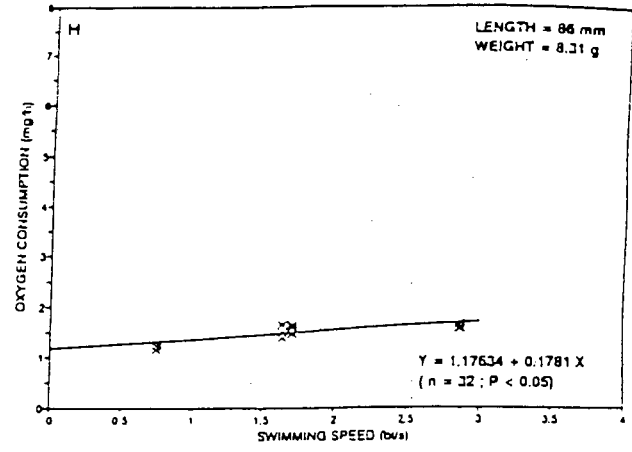
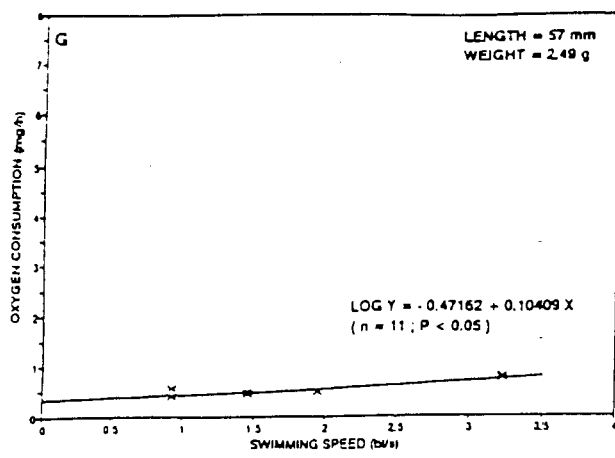
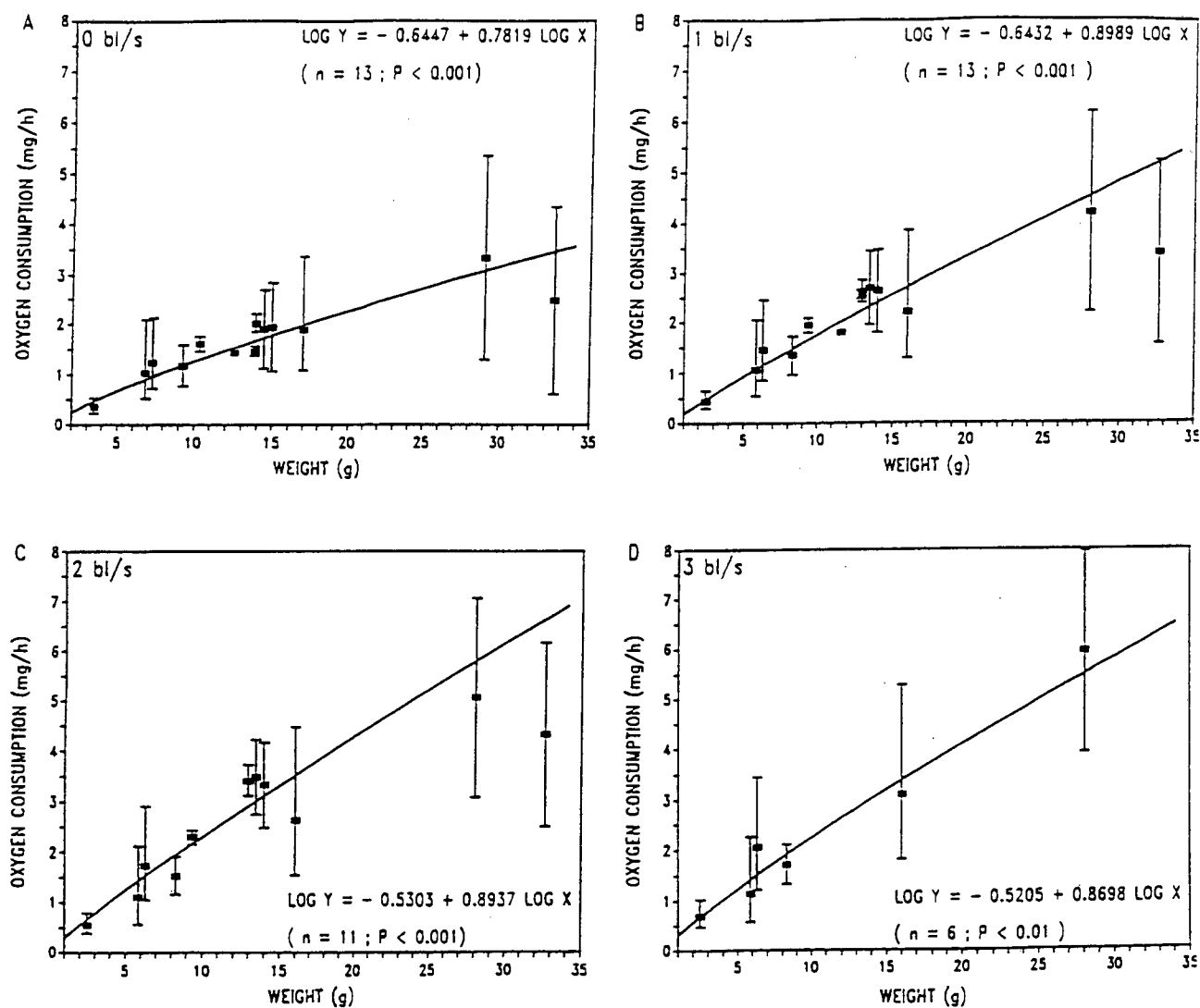


FIGURE 9: (continued).

An aspect of the data presented in Figure 9 which warrants further discussion is that of the variability evident both within and between the fishes investigated. This feature has been well documented in the literature (e.g. Brett, 1964; Fry, 1971; Smit *et al.*, 1971; Goldspink, 1977b), and is to be expected in results of such experiments. Apart from the size effect on the variability between individuals, natural biological variation coupled with several additional factors plays a major role. These include varying starvation periods and degree of stress experienced by the fish. Considering that the trout used in these experiments were juveniles, it is unlikely that the sex of the individuals had any influence on the results.

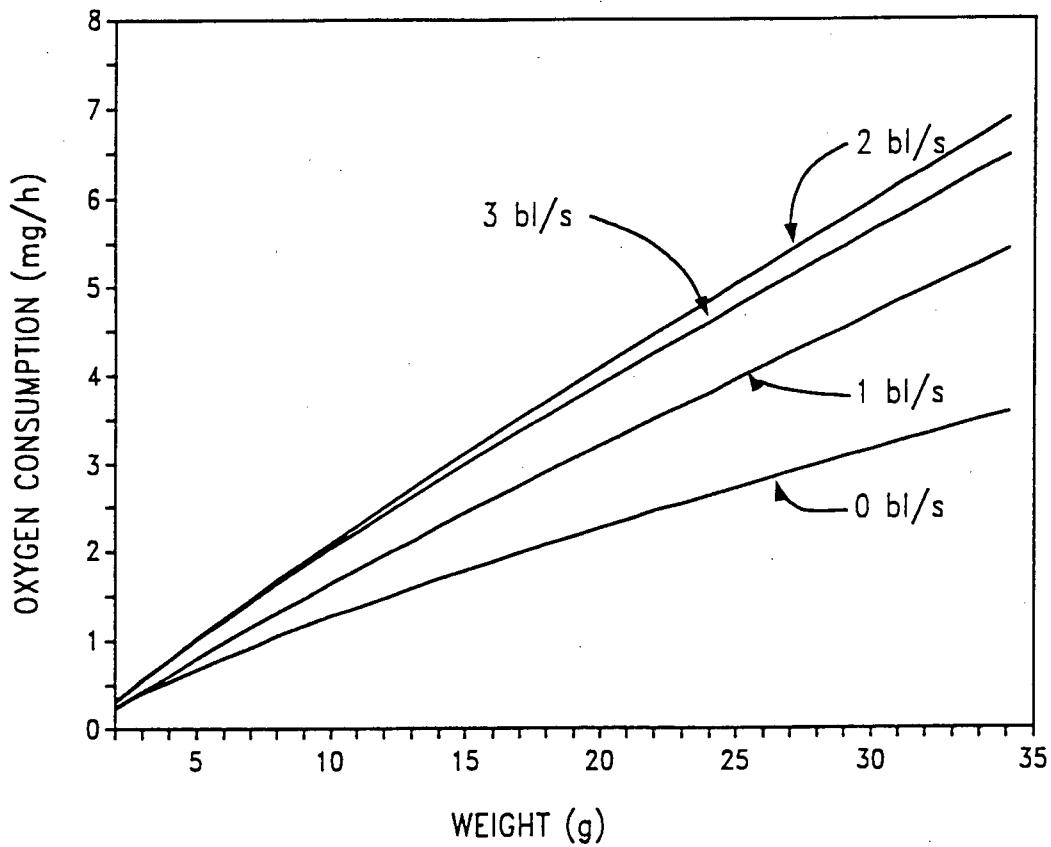
The variability in  $VO_2$  within each individual fish is of particular interest. Apart from natural biological variability, the primary cause of this feature was felt to be stress resulting from the experimental procedure, the influence of which (manifested by highly variable and elevated  $VO_2$ ) often lasted for several hours after an increase in imposed velocity. Usually, this was associated with excess activity on the part of the fish, particularly at the lower imposed velocities (where there was presumably more scope for spontaneous activity). In contrast, activity of this nature at the higher swimming speeds was not observed to the same extent, if at all. Similarly, Brett (1965) reported a decreasing variability in  $VO_2$  measurements with increasing imposed velocity. The opposite trend, however, is apparent in several of the data sets illustrated in Figure 9. This is a direct result of the criteria governing the selection of the data, specifically the elimination of data associated with excess activity. Since  $VO_2$  fluctuations at the lower imposed velocities were usually associated with excess activity, and these data consequently discarded, the higher degree of variability at these swimming speeds is not reflected in Figure 9. In contrast,  $VO_2$  fluctuations at higher swimming speeds were rarely associated with excess activity, and were attributed to excitement of the fish. However, it was very difficult to characterize this factor on either a behavioural or physiological basis, and no objective criterion could be established justifying the exclusion of such data from the analyses. Since these data are included in the plots in Figure 9, an apparent increase in  $VO_2$  variability with increasing swimming speed is evident in some cases (e.g. Figure 9 L).



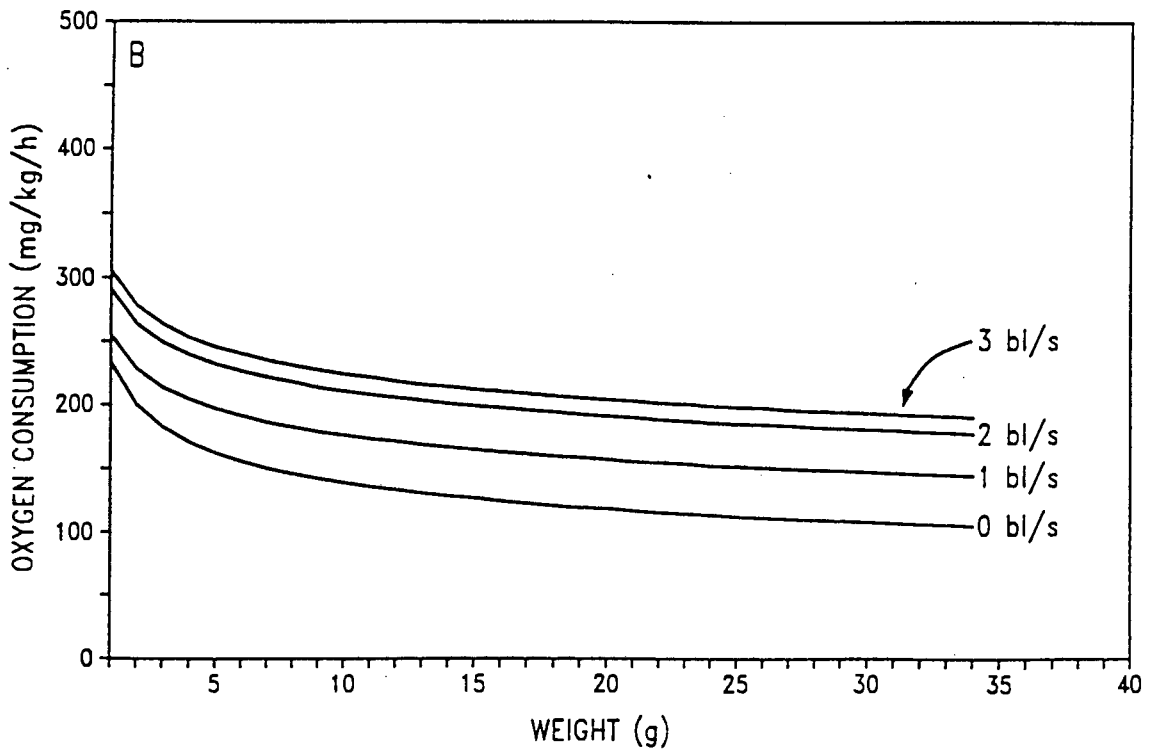
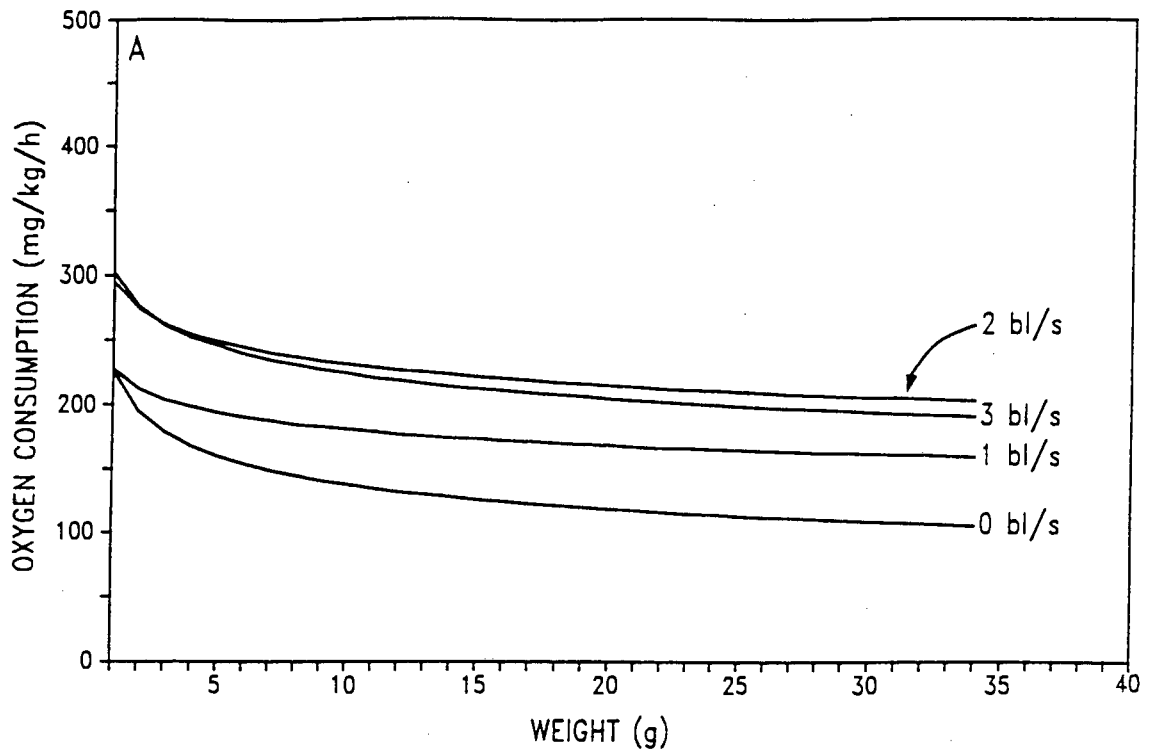
**FIGURE 10:** The oxygen consumption ( $\text{mg O}_2 \cdot \text{h}^{-1}$ ) wet weight relationship in juvenile rainbow trout at swimming speeds of A -  $0 \text{ bl} \cdot \text{s}^{-1}$ ; B -  $1 \text{ bl} \cdot \text{s}^{-1}$ ; C -  $2 \text{ bl} \cdot \text{s}^{-1}$  and D -  $3 \text{ bl} \cdot \text{s}^{-1}$ . Solid squares are predicted oxygen consumption rates calculated from the regressions illustrated in Fig. 9. Vertical bars represent the 95% prediction limits of each estimate. Solid lines are the regression lines obtained through the weighted regression procedure, described by the equation presented in each figure. The number of data points and the significance level of the regression are also indicated. Note that the axes are scaled arithmetically.

In spite of the variability in the data, all but one of the regressions (see Figure 9 I) were found to be statistically significant ( $P < 0.05$ ). Absolute  $VO_2$  estimates (and 95% prediction limits) for each individual fish at swimming speeds of 0, 1, 2 and 3  $bl.s^{-1}$ , are plotted against the corresponding wet weights in Figure 10. The "best-fit" regression lines obtained from the weighted regression procedure are shown, as are the number of data points included in the analysis and the significance level of the regression. For comparative purposes, the curves illustrated in Figure 10 are combined in a single diagram in Figure 11. In addition, the variables in Figures 10 and 11 are converted in Figure 12 A to describe the relationship between weight-specific  $VO_2$  ( $mg\ O_2.kg^{-1}.h^{-1}$ ) and wet weight over the swimming speeds investigated. Very few experiments were extended to swimming speeds in excess of 3  $bl.s^{-1}$ , with a resulting paucity of data at these higher swimming speeds. Only 6 experiments provided valid data at 3  $bl.s^{-1}$ , and although the regression on the data at this level was statistically significant, the validity of the established relation is open to question.

As is evident in Figures 10 and 11, the relationship between these two variables at all the swimming speeds under consideration was found to be best described by a logarithmic model. Comparing the curves illustrated in Figure 10, an increase in the slope of the absolute  $VO_2$  - weight relation is evident with increasing swimming speed, although this trend breaks down at the higher swimming speeds (above 2  $bl.s^{-1}$ ). Similar observations have been reported in the literature. Brett (1965) and Brett & Glass (1973), investigating the relation between body size and metabolic rate in sockeye salmon (*Oncorhynchus nerka*), documented a linear increase in the relationship between the logarithm of oxygen consumption and the logarithm of body weight. A similar result was reported by Madan Mohan Rao (1971) for rainbow trout. Regarding the relationship between the two variables at increasing levels of activity, Brett & Glass (1973) reported a slope of 0.846 for the standard  $VO_2$ , increasing to 0.963 for the active  $VO_2$  (corresponding to the maximum swimming speed) in sockeye salmon at 15°C. This implies that as activity increases to maximal levels, weight-specific  $VO_2$  ( $mg\ O_2.kg^{-1}.h^{-1}$ ) tends towards weight-independence (i.e. small fishes use the same amount of oxygen per unit body weight as do large fishes). Complete weight independence would be manifested by a slope of unity in the absolute  $VO_2$  - weight relation. Madan Mohan Rao (1971) describes a similar increase in slope



**FIGURE 11:** The absolute oxygen consumption rate - wet weight - swimming speed relationship in juvenile rainbow trout at sustained swimming speeds. The curves are those illustrated in Fig 10, combined on a single set of axes for comparative purposes.



**FIGURE 12:** The weight-specific oxygen consumption rate ( $\text{mg O}_2 \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ) - wet weight (g) - swimming speed ( $\text{bl} \cdot \text{s}^{-1}$ ) relationship in juvenile rainbow trout at sustained swimming speeds.

A - curves are converted from those illustrated in Fig. 11, obtained through the weighted regression procedure.

B - curves obtained from a regression analysis where no weighting factor was employed in the regression (see text).

with increasing swimming speed for rainbow trout at 15°C, ranging from 0.7834 at 0 cm.s<sup>-1</sup> (standard rate), to a value in the region of 0.9 at 72 cm.s<sup>-1</sup>. The implications of this trend to the concept of the "scope for activity" (introduced by Fry, 1947) have been discussed by Wieser (1985).

The slopes observed in the present study are comparable to those of Brett & Glass (1973) and Madan Mohan Rao (1971), ranging from 0.7819 at 0 bl.s<sup>-1</sup> (standard rate) to 0.8989 at 1 bl.s<sup>-1</sup>. The general trends, however, exhibit several inconsistencies, specifically the decrease in the slopes of the relationships above 1 bl.s<sup>-1</sup>. It is suggested that this feature is an artefact of the statistical procedures employed, combined with a lack of data for the larger fish at the higher swimming speeds, rather than a reflection of the true relationship. In support of this argument, Figure 12 B illustrates the relationships obtained when no weighting factor was incorporated into the regression analyses. Although the statistical validity of these curves is questionable, they do serve to illustrate what may well be a more representative reflection of the relationship than those presented in Figure 12 A. The slopes of the curves illustrated in Figure 12 B, when converted into terms of absolute  $\dot{V}O_2$ , were found to range from 0.773 at 0 bl.s<sup>-1</sup>, to 0.866 at 3 bl.s<sup>-1</sup>, with a consistent increase over the intermediate swimming speeds. This is probably a more realistic result than that provided by the weighted regression procedures.

## OXYGEN DEBT TRIALS

**Experimental Procedure:**

In contrast to the incremental velocity tests, each oxygen debt experiment involved three trials at different imposed velocities, each trial consisting of an exercise phase followed by a recovery phase. Following the overnight acclimation period, the trials were initiated with a gradual increase of velocity (over a 10 minute period) to the test velocity, which was then maintained for the entire exercise phase. The duration of each exercise phase was initially 3 hours, but in later experiments this was prolonged for periods of up to 5 hours. The reasons for prolonging this period were twofold. Firstly, to ensure that the respiration rates during the exercise phase had stabilized. Secondly, to minimise the contribution of potential anaerobic metabolism associated with the increase in imposed velocity at the beginning of the exercise phase, to the overall oxygen debt and EPOC.

Following each exercise phase, the imposed velocity was immediately reduced to a minimum (less than  $0.5 \text{ bl.s}^{-1}$  in all of the experiments), marking the beginning of the recovery phase.  $\dot{V}O_2$  was monitored virtually continuously during the recovery period. Recovery was considered to be complete when the  $\dot{V}O_2$  had decreased to a minimum, followed by a consistent elevation above this minimum. Once each trial had been completed, the fish was left overnight (a minimum of 12 hours) with the apparatus in the open circuit condition before the next trial was initiated. During this "rest" period, the imposed velocity was maintained at the low level characterizing the previous recovery phase to ensure that any remaining oxygen debt not detected during a trial was not carried over to the next trial. The test velocities of each exercise phase were imposed at levels as close as possible to increments of  $1 \text{ bl.s}^{-1}$  to permit comparisons of the data between experiments.

## Data Analysis:

Two sets of data were obtained from each trial. Firstly,  $VO_2$  measurements recorded during each exercise phase, and secondly,  $VO_2$  measurements recorded during the post-exercise recovery phase. Exercise phase  $VO_2$  data were selected and analysed in the same manner as the incremental velocity data described in the previous chapter.  $VO_2$  data recorded during the recovery phase were never included in the regression analyses, since these values could have incorporated a certain amount of unpaid oxygen debt. The results of these regression analyses were pooled with those of the incremental velocity tests described in the previous chapter. The primary purpose of these regressions, however, was the prediction of the baseline  $VO_2$  corresponding to the velocity imposed during the recovery phase of each trial. To calculate EPOC from recovery  $VO_2$  measurements, the underlying energy expenditure supporting the level of exercise imposed during the recovery phase had to be established (EPOC, by definition, is that proportion of the total measured  $VO_2$  in excess of this baseline value).

To estimate the oxygen debt corresponding to the exercise phase of each trial, the EPOC was considered to be the sum of all of the post-exercise oxygen consumption measurements recorded until recovery had occurred, corrected for the baseline  $VO_2$ . It was observed in a few of the trials that the opening and closing of the valves during the flushing procedure induced a brief burst of activity on the part of the fish, with concomitant sharp elevations in  $VO_2$ . Since these data most probably over-estimate the recovery oxygen consumption, data associated with excess activity were excluded from the calculation of EPOC. Where gaps in the data record occurred, either through exclusion of these data, or as a result of the flushing procedure, an average of the values immediately before and after the gap were used in the calculations.

Once a value of total EPOC had been obtained for each trial, this value was divided by the duration of the exercise phase, yielding an estimate of the oxygen debt accumulation ( $\text{mg O}_2 \cdot \text{h}^{-1}$ ) at the level of activity imposed during the exercise phase. This procedure assumes that the observed EPOC can be equated with the oxygen debt incurred during the exercise (see Chapter

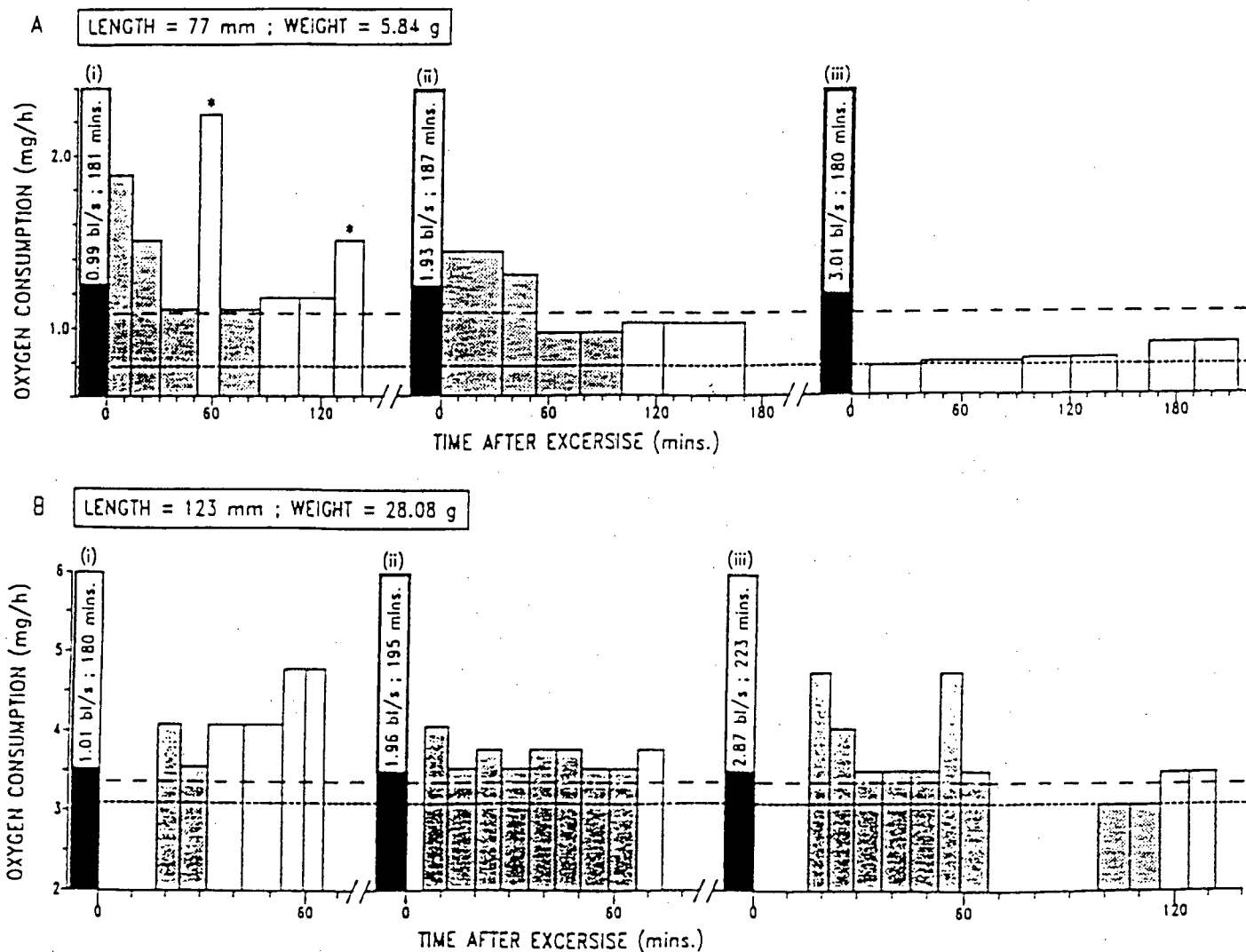
1). Values of oxygen debt accumulation were then converted to specific rates ( $\text{mg O}_2 \cdot \text{kg body weight}^{-1} \cdot \text{hour of exercise}^{-1}$ ).

## **Results and Discussion:**

The results of five experiments investigating oxygen debt accumulation during sustained exercise in juvenile rainbow trout are presented in Figures 13 and 14. The length and weight of each fish are indicated in the relevant figure. The duration and intensity of the exercise phase of each trial are indicated in the solid bars in Figure 13.

The results of the linear regression analyses conducted on the exercise phase  $\text{VO}_2$  values are shown in Figure 9 J to M (these data correspond to those presented in Figure 13 A to E respectively). Once the data analysis had been completed, however, it was apparent that recovery  $\text{VO}_2$  in all of the trials decreased to levels below the predicted baseline  $\text{VO}_2$  values, and with the exception of one experiment, to levels below the predicted standard  $\text{VO}_2$ . It is interesting to note that the minimum post-exercise  $\text{VO}_2$  values were all recorded during the third trial following the most intense exercise phase of each experiment. This observation is discussed in more detail later. Since the predicted baseline values were such apparent over-estimates, they are not included in the diagrams illustrated in Figure 13.

The over-estimation of the baseline and standard  $\text{VO}_2$  levels can be explained by the observed behaviour of the fishes during the recovery phase, combined with the selection criteria of the data included in the regression analyses which provided these predictions. Firstly, it was observed that the recovery phases of virtually all of the trials were characterized by an almost complete absence of any swimming activity on the part of the fish. Following the cessation of exercise, the fishes tended to rest virtually motionless on the bottom of the flow chamber, only minor movements of the pectoral fins, and periodic arching movements of the body coupled with strong opercular action being evident. This "non-activity" appeared to become more pronounced after the more intense exercise phases. In view of these observations, it is probable that the flow velocities imposed during the recovery phases were of a sufficiently low magnitude to enable the



**FIGURE 13:** Results of the oxygen debt trials. Each diagram shows the measured recovery oxygen consumption rates ( $\text{mg O}_2 \cdot \text{h}^{-1}$ ) from three trials on an individual trout of indicated length and wet weight. Solid bars (not to scale) represent the exercise phase of each trial, the duration and intensity of which are indicated. Each vertical bar after time zero on the X-axis represents a single discrete measurement of oxygen consumption. The shaded bars indicate the measurements employed for calculation of EPOC. Measurements associated with excess activity are indicated with an asterisk. The horizontal dashed line indicates the predicted standard oxygen consumption rate calculated from the relevant regression lines illustrated in Fig. 9. The horizontal dotted line indicates the minimum observed recovery oxygen consumption employed for calculation of EPOC (see text). All experiments at  $15^\circ\text{C}$ . Note: scaling of the axes is not constant in all the diagrams.

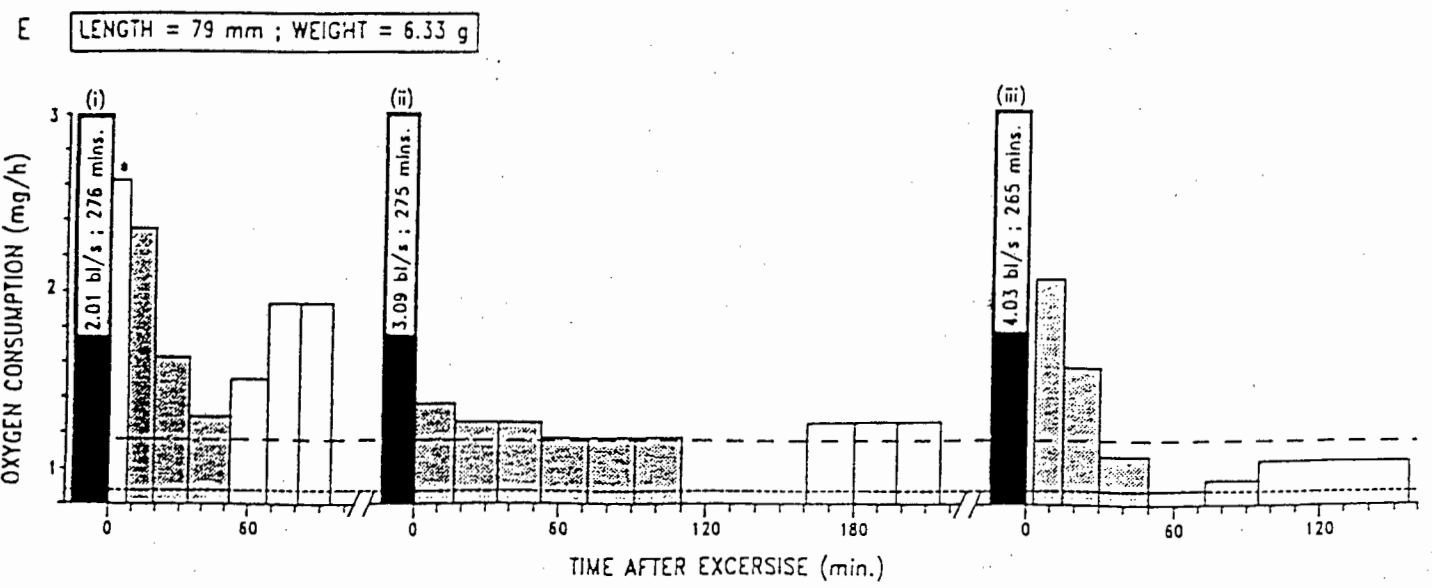
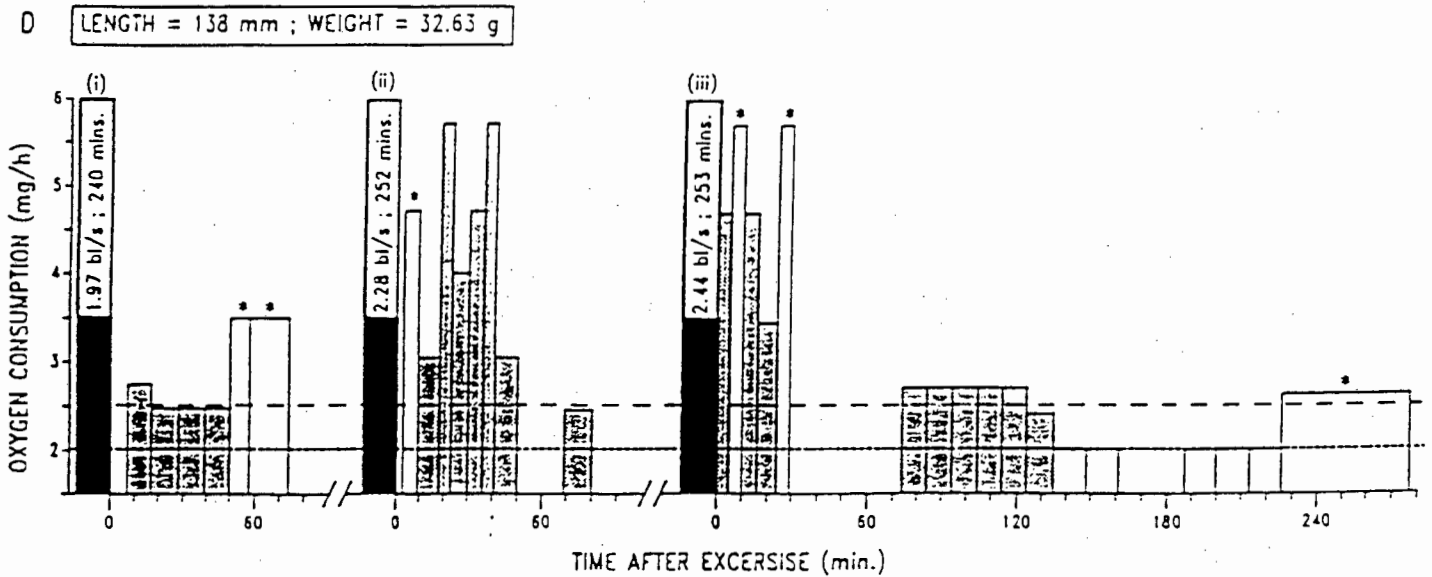
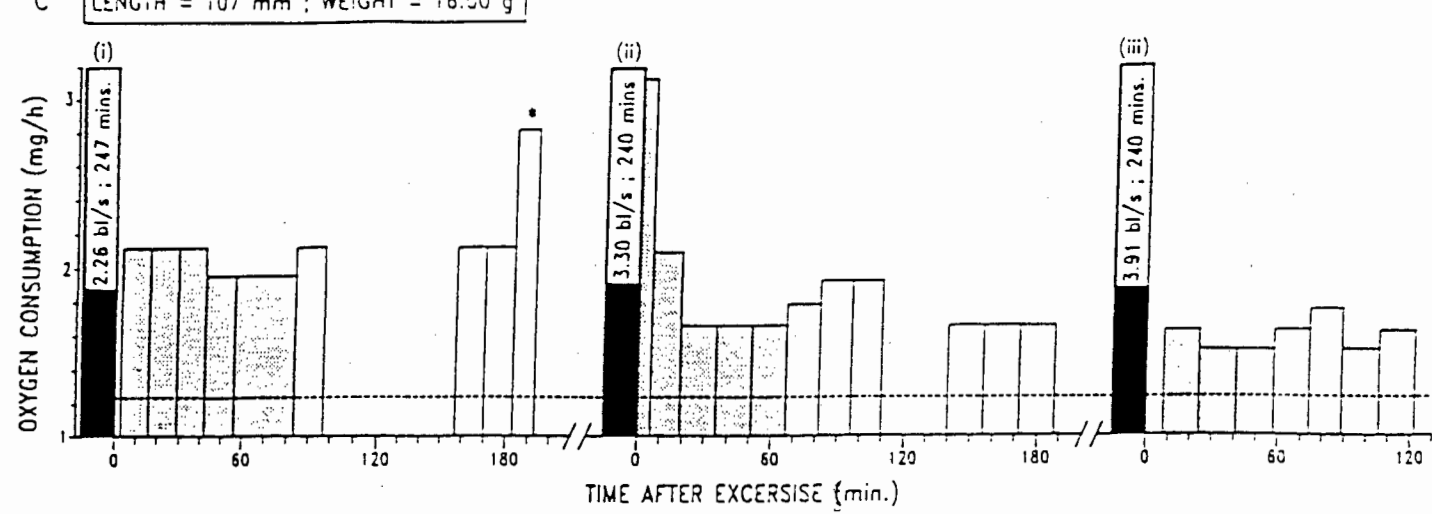
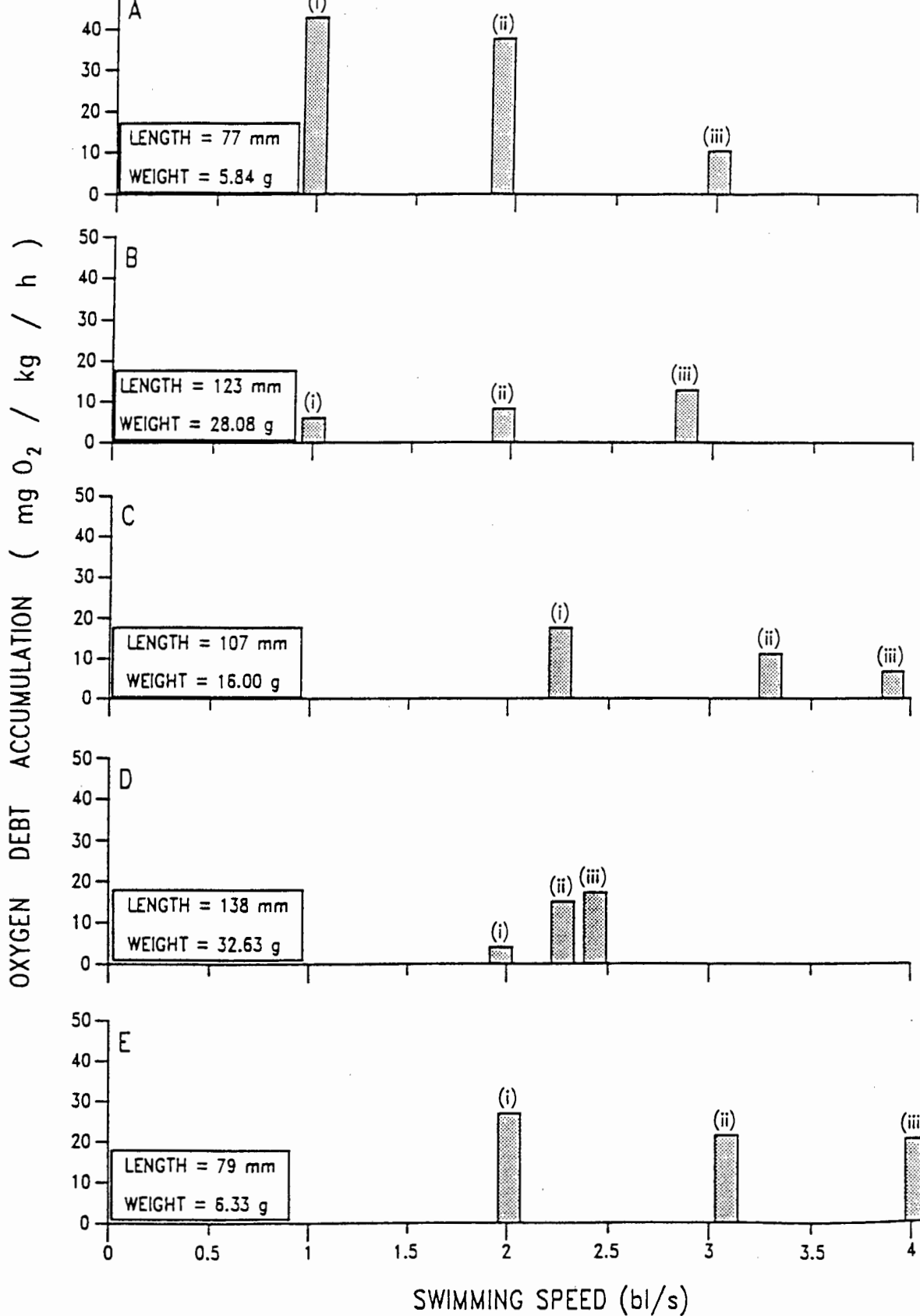


FIGURE 13: (continued).



**FIGURE 14:** Oxygen debt accumulation versus swimming speed in juvenile rainbow trout. Data calculated from the EPOC estimates obtained in the oxygen debt trials. Each diagram A to E corresponds to the oxygen debt experiments illustrated in Fig. 13 A to E respectively.

fish to maintain its position in the flow chamber without having to resort to any locomotory activity at all. It may consequently be argued that the minimum observed  $VO_2$  values evident under these circumstances are in fact a reflection of the true standard metabolic rate of the fish.

Coupled to this observation, the criteria excluding recovery phase  $VO_2$  data from the regression analyses would contribute to an over-estimation of the baseline predictions. If these data had been included into the analyses, the resultant regression equations might well have provided more representative predictions. However, inclusion of these data would have involved relatively subjective judgements regarding the true activity level of the fish during these periods. Further, the potential contribution of any "unpaid" oxygen debt to these  $VO_2$  data precluded their inclusion into the regression analyses.

In the light of the preceding discussion, some other estimate of the baseline  $VO_2$  had to be established in order to calculate the EPOC of each trial. The only values which could conceivably be considered as valid estimates of the required baseline values, were the minimum recovery  $VO_2$  values. Where the minimum observed post-exercise  $VO_2$  during the recovery phase of any one of the trials of an experiment fell below the predicted standard  $VO_2$ , this minimum value was employed as the baseline estimate for EPOC calculation in all the trials of that particular experiment. The only exception to this procedure was in the case of the experiment illustrated in Figure 13 C, where the minimum observed post-exercise  $VO_2$  never fell below the predicted standard rate. In this particular case, the predicted standard rate was applied as the baseline for the EPOC calculation.

Post-exercise  $VO_2$  measurements fulfilling the criteria for EPOC calculation specified above are indicated in Figure 13 by shaded bars. In several of the trials, specifically Figure 13 B(iii) & D(ii), sharp elevations in  $VO_2$  were recorded, but no excess activity was observed to account for these isolated observations. These elevations were considered to reflect the occurrence of excitement of the fish, and could not be considered representative of the true course of oxygen debt repayment. However, as mentioned previously, it was extremely difficult to characterize

excitement on either a physiological or behavioural basis, and no objective criterion could be specified justifying the exclusion of these data from the EPOC calculations.

The results of the EPOC calculations are presented in Figure 14 as values of weight-specific oxygen debt accumulation per hour of exercise. Data presented in Figures 14 A to E correspond to the results illustrated in Figure 13 A to E respectively. Considering these results in the light of the fundamental assumption inherent in this series of experiments (that oxygen debt accumulation estimated through measurement of EPOC can be equated with level of anaerobic metabolism occurring during the exercise), and accepting the premise that the higher the level of activity, the greater the contribution of anaerobic metabolism to overall energy expenditure, it is immediately apparent that the results do not exhibit the expected trend. In only two of the five experiments (Figure 14 B & D) was an increase in oxygen debt accumulation evident with increasing swimming speed. The remaining three experiments indicated the reverse.

A possible explanation for this inconsistency may be found in the behaviour of the fishes during the trials, the key factor being the occurrence of excess activity and/or apparent excitement during the trials, both of which were manifested in the data by sharp elevations in  $\dot{V}O_2$ . For the purposes of this discussion, these two factors will be collectively referred to as "excess activity". Excess activity was observed at various stages of virtually all of the trials, the only exceptions being the trials illustrated in Figure 13 B(i), C(iii) & D(i). The remaining trials were all characterized by periods of excess activity during the exercise phases, and in the case of the trials illustrated in Figure 13 A(i), B(iii), D(ii), D(iii) & E(i), during the recovery phase. The occurrence of excess activity appears to be reflected in the magnitude of the oxygen debt accumulation results presented in Figure 14 (trials characterized by excess activity resulted in relatively higher oxygen debt accumulation values than trials where no excess activity was observed). The influence of excess activity appeared to be particularly pronounced during the recovery phases (resulting oxygen debt accumulation estimates were relatively higher than those resulting from the trials where excess activity was only observed during the exercise phases).

The influence of excess activity on estimates of oxygen debt accumulation is more than likely related to the levels of anaerobic metabolism associated with its occurrence. Where excess activity was observed during the trials, it generally took the form of a few intense bursts of swimming activity, lasting for periods of only a few seconds. Activity of this nature is largely supported by anaerobic energy generation (Webb, 1975; Bennett, 1978), resulting in further accumulation of oxygen debt (with resulting elevated EPOC through a prolonged recovery period). The occurrence of such activity would result in significant over-estimates of the level of anaerobic metabolism corresponding to the imposed level of exercise. This effect would be particularly pronounced during periods of low imposed velocities (such as during the recovery phases), where the contribution of such anaerobic energy production to the overall energy expenditure would constitute a much larger component compared to periods of higher imposed exercise.

An additional factor contributing to the apparent inconsistencies evident in the results, relates to observations regarding the minimum observed post-exercise  $\dot{V}O_2$ . These minima were, without exception, observed to occur during the recovery phase of the final trial of each experiment, subsequent to the highest level of imposed exercise (see Figure 13). Further, these minima were generally coupled to the shortest recovery periods of each experiment, the only exceptions being where excess activity was observed during the recovery phase (Figures 13 B & D). Although the reasons for these features are not clear, their cumulative effect was to lower the measured EPOC, contributing to the decreasing trend in the oxygen debt accumulation - swimming speed relationship evident in several of the experiments. A tentative explanation for this apparent paradox is that higher intensity exercise might result in a more "intense" recovery period, during which the fish exhibits a minimum of post-exercise activity and apparent excitement. Another explanation can possibly be found in the results of Scarabello *et al.* (1992). Juvenile rainbow trout subjected to two consecutive bouts of exercise (separated by a 6 hour recovery period) showed improved rates of metabolic recovery following the second bout. Scarabello *et al.* (1992) suggested that this could possibly be attributed to "learning" on the part of the fishes (i.e. the fishes had become familiar with the exercise routine), with a reduction in the psychological influence on the stress-response after the second bout of exercise.

Two key points can therefore be made regarding the results presented in Figure 14. Firstly, the oxygen debt accumulation estimates are almost certainly over-estimates, and are not representative of the actual oxygen debts accumulated at the indicated swimming speeds (with the possible exception of the values obtained from the trials where no excess activity or apparent excitement was observed). Secondly, given that these data are over-estimates, the magnitude of the levels of anaerobic metabolism estimated by these data are extremely low in comparison to the corresponding aerobic energy expenditures illustrated in Figure 9. If the oxygen debt accumulation data are pooled with the aerobic estimates for the corresponding swimming speeds, the anaerobic contribution to the total overall energy expenditure at these swimming speeds is less than 10% in virtually all of the cases. The exceptions are the data from the first two trials in Figure 14 A, which indicated an anaerobic contribution of closer to 20% of the total. Both of these estimates, however, involved high levels of excess activity, and are probably gross over-estimates. Only three of the trials provided what can be considered to be valid estimates of the anaerobic contribution, namely those presented in Figures 14 B(i), C(iii) & D(i). No excess activity or apparent excitement was associated with any of these trials. The results of these particular trials provided estimates of the anaerobic contribution that were of the order of 3% of the total energy expenditure at the corresponding swimming speeds.

On the basis of these results, it was concluded that anaerobic metabolism constitutes a relatively unimportant component of the overall energy production supporting swimming of juvenile rainbow trout at the sustained speeds imposed in this study. This component was consequently ignored in further analyses of the swimming energetics of juvenile trout at sustained levels of activity.

## CHAPTER 7

### LACTATE ACCUMULATION TRIALS

#### Experimental Procedure:

The experimental procedure involved in these trials was identical to that of the oxygen debt experiments, with the exception that immediately after the exercise phase, each fish was removed from the flow chamber, stunned with a blow to the head, and the entire fish then freeze-clamped between aluminium tongs pre-cooled in liquid nitrogen. Each fish was stored at  $-80^{\circ}\text{C}$  for later analysis of lactate content. The lactate analyses were carried out following the methodology of Noll (1984) and Gäde & Grieshaber (1989), a detailed description of which can be found in Appendix A.

Lactate analyses were carried out on the entire fish (i.e. determination of whole body lactate content), in order to avoid the problem of the compartmentalised nature of the distribution of lactate in the tissues (Bennett and Licht, 1972). Since the determination of whole body lactate levels is a destructive technique, involving killing the fish, it is clear that each fish can only be tested at one specific test velocity. This is in contrast to the oxygen debt trials discussed in the preceding chapter, where an individual fish could be tested at several different levels of activity. Four groups of fish were used in these trials (see below). All trials were conducted at  $15^{\circ}\text{C}$ . No simultaneous measurements of oxygen consumption were recorded during these trials.

**Group 1 (control group):** 6 individuals, mean length and wet weight of 8.08 cm ( $SD = 1.036$ ) and 6.76 g ( $SD = 2.825$ ) respectively. These fishes were sampled immediately subsequent to the overnight acclimation phase (a minimum of 15 hours at an imposed flow velocity of about  $0.5 \text{ bl}\cdot\text{s}^{-1}$ ), in order to estimate lactate contents under conditions as close to resting as possible. This assumes that the overnight acclimation phase is of sufficient duration to permit recovery from the effects of handling stress associated with introduction into the respirometer.

**Group 2:** 6 individuals, mean length and wet weight of 8.52 cm ( $SD = 0.504$ ) and 7.09 g ( $SD = 1.445$ ) respectively. After the overnight acclimation phase, the animals were subjected to an exercise phase of 4 hours at an imposed velocity of  $2 \text{ bl}\cdot\text{s}^{-1}$ , prior to analysis.

**Group 3:** 5 individuals, mean length and wet weight of 8.38 cm ( $SD = 0.816$ ) and 6.99 g ( $SD = 1.925$ ) respectively. Identical procedure as Group 2, but with an exercise phase of 4 hours at  $3 \text{ bl}\cdot\text{s}^{-1}$  prior to analysis.

**Group 4:** 5 individuals, mean length and wet weight of 8.48 cm ( $SD = 1.137$ ) and 8.30 g ( $SD = 2.578$ ) respectively. This group were used to determine lactate contents at levels of activity approaching the maximum. The animals were removed from the holding tank, weighed, measured, and placed into separate buckets filled with continuously aerated water. The fishes were left undisturbed in the buckets for a period of about 2 hours in an attempt to minimise the presence of any hypoxia-related metabolites which may have accumulated as a result of handling. Each fish was then subjected to 2 minutes of forced activity (manual chasing of the fish in the bucket), sufficient to induce a state of almost complete fatigue. The fishes were then removed for analysis.

#### **Data Analysis:**

Estimates of whole-body lactate content ( $\mu\text{mol lactate}\cdot\text{g wet weight}^{-1}$ ) for the four groups of fishes were subjected to a simple one-way ANOVA to test for any significant differences between the groups. A second ANOVA was carried out on data from Groups 1 to 3, in order to establish whether any significant differences in lactate content were evident at sustained swimming speeds. To establish whether a relationship could be detected between lactate content and swimming speed over the range of velocities investigated, the data for Groups 1 to 3 were subjected to a simple least-squares linear regression analysis. Data from Group 4 were excluded from this analysis, since no measure of swimming speed was recorded for this group. Further, the exercise phase imposed on Group 4 was of considerably shorter duration than those of the other groups, and the data were consequently not statistically comparable. The data were tested against linear,

exponential and logarithmic models through the transformation procedures described previously, the regression resulting in the highest  $F$  statistic then being selected as the line of "best fit".

### **Results and Discussion:**

The results of the lactate accumulation trials are illustrated in Figure 15. The ANOVA procedure conducted on the data indicated a significant difference between the four groups ( $P < 0.01$ ). The ANOVA conducted on the data from Groups 1 to 3 also resulted in a significant difference between the groups ( $P < 0.05$ ), justifying the regression analyses carried out on these data. The regression analyses resulted in the "best-fit" curve ( $P < 0.01$ ) illustrated in Figure 15. The logarithmic equation describing the curve is presented in the diagram. For comparative purposes, the data from Group 4 are also included in the graph.

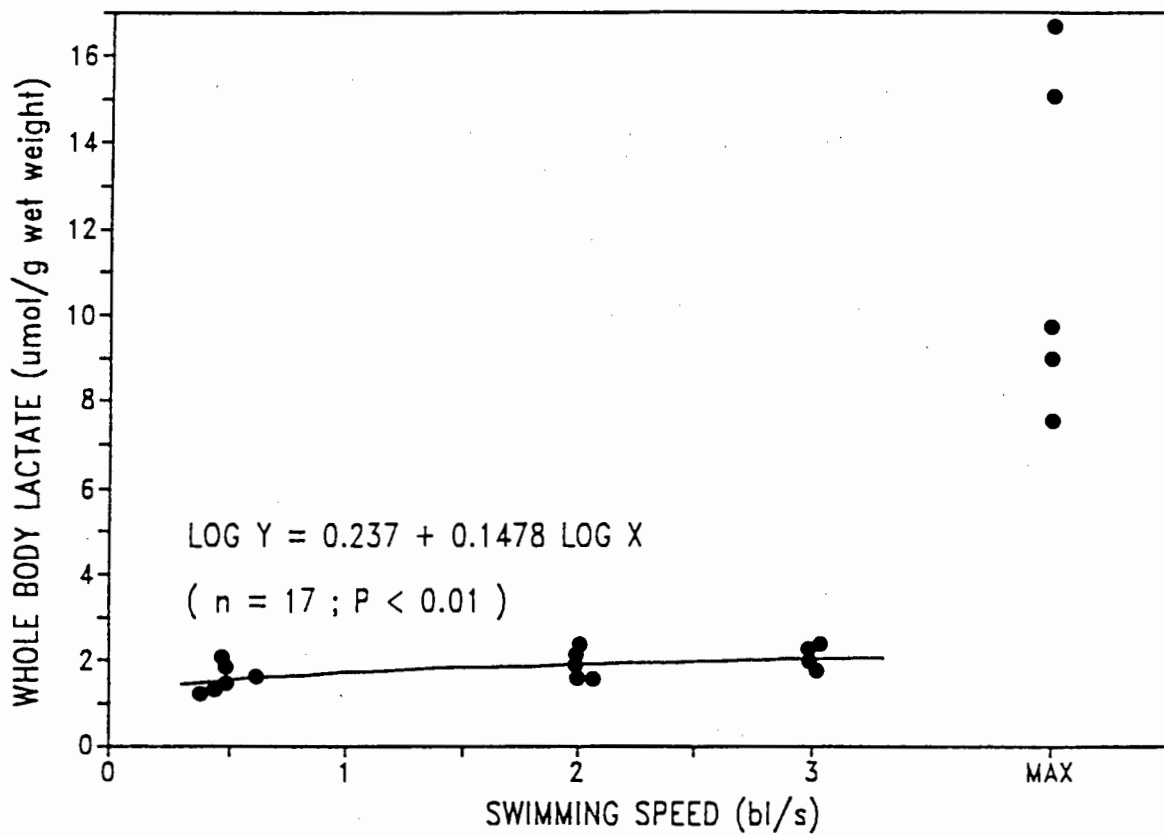
As can be seen from Figure 15, the lactate contents were extremely low and demonstrated a gradual increase with increasing swimming speed. In comparison, body lactate contents at maximum activity levels were an order of magnitude higher. The large degree of scatter in the latter data set are to be expected, considering that the levels of activity imposed on this group were not controlled to the extent of those on the other three groups. It could also be argued that the effects of size and physical "fitness" become more pronounced at these higher levels of activity, although this aspect was not investigated.

In an attempt to assess the contribution of anaerobic metabolism to overall energy expenditure, the values of lactate contents of the trout were converted to units of ATP production, using the conversion values proposed by Bennett and Licht (1972):

1 mg lactate produced = an anaerobic energy production of 0.0167 mmol ATP (assuming that glycogen is the only substrate).

1 ml oxygen consumed = 0.290 mmol ATP generated.

From the regression line illustrated in Figure 15, predicted values of whole body lactate content for a fish of about 7 g wet weight, were calculated for swimming speeds of



**FIGURE 15:** Whole-body lactate contents of juvenile rainbow trout subjected to various levels of activity. Solid dots are lactate contents of individual fishes. The solid line is the regression line resulting from the analysis of the data from groups 1 to 3 (see text).

0.5 bl.s<sup>-1</sup> and 3 bl.s<sup>-1</sup>, and converted to units of ATP production. These were found to be of the order of 0.102  $\mu\text{mol ATP.g wet weight}^{-1}.\text{h}^{-1}$  at 0.5 bl.s<sup>-1</sup>, and 0.764  $\mu\text{mol ATP.g wet weight}^{-1}.\text{h}^{-1}$  at 3 bl.s<sup>-1</sup>.

For comparison, the aerobic energy expenditure of a similar size trout (6.33 g) at the same swimming speeds was calculated from the regression equation illustrated in Figure 9 M, and again expressed in units of ATP production. These values were found to be 42.87  $\mu\text{mol ATP.g}^{-1}.\text{h}^{-1}$  at 0.5 bl.s<sup>-1</sup>, and 65.81  $\mu\text{mol ATP.g}^{-1}.\text{h}^{-1}$  at 3 bl.s<sup>-1</sup>. From these values, it would appear that the anaerobic contribution to total energy expenditure ranged from approximately 0.2% at a swimming speed of 0.5 bl.s<sup>-1</sup>, to about 1% at 3 bl.s<sup>-1</sup>. These results provide further support for the conclusion discussed in the previous chapter, i.e. that the contribution of anaerobic metabolism to overall energy expenditure at sustained swimming speeds is negligible. Driedzic & Kiceniuk (1976) reported no increase in the blood lactate concentrations of rainbow trout at swimming speeds of up to 93% of the critical swimming speed, a result which further supported the above conclusion.

The principle of these lactate accumulation tests rests on the assumption discussed in Chapter 1, i.e. at the low swimming speeds (and levels of stress) involved with these trials, the level of lactate accumulation can be considered to be an indication of the anaerobic metabolism which had occurred during the exercise phase. Although this assumption is open to question, the results presented in this chapter, as well as those of the oxygen debt trials presented in Chapter 6, were consistent with the general consensus expressed in the literature (e.g. Brett, 1964, 1972; Webb, 1975), i.e. the levels of anaerobic metabolism occurring at sustained swimming speeds are minimal, and can most probably be ignored.

## COST OF TRANSPORT AND TOTAL OXYGEN CONSUMPTION

**Cost of Transport:**

Once the aerobic and anaerobic components of the overall energy expenditure at various swimming speeds had been estimated, the cost of transport for fishes of various sizes over a range of swimming speeds could be investigated. This variable was expressed in terms of the amount of oxygen required to support the movement of the entire fish over a set distance at any given swimming speed. In Chapters 6 and 7, it was concluded that the anaerobic contributions to overall energy expenditure at sustained swimming speeds were sufficiently low to justify their exclusion from further analyses. Consequently, only the aerobic component of overall energy expenditure was considered in the cost of transport analyses described below.

To evaluate the energy costs to a juvenile trout moving through a set distance, the regression equations presented in Figure 9 were employed to predict the absolute  $VO_2$  over a range of swimming speeds. Only data from those experiments involving imposed swimming speeds of up to  $3 \text{ bl.s}^{-1}$  and above (the experiments illustrated in Figure 9 G, H, J, K & M) were used. The predictions were never extrapolated beyond the maximum swimming speeds for which valid data had been recorded. The  $VO_2$  values were divided by the corresponding swimming speed (expressed in  $\text{km.h}^{-1}$ ), yielding a value of total cost of transport ( $\text{mg O}_2.\text{km}^{-1}$ ). These values are plotted against swimming speed in Figure 16 A. Net cost of transport values for the same individuals were calculated by subtracting the standard  $VO_2$  from the absolute  $VO_2$  data, before dividing by the swimming speed. These data are illustrated in Figure 16 B. For comparison, the same procedure was carried out on data reported in the literature for sockeye salmon (Brett, 1964) and rainbow trout (Webb, 1975; Facey & Grossman, 1990), and are shown in Figures 16 C & D. In the following discussion, all references to cost of transport refer to values for the entire fish, that is, an absolute measure. The data were not converted into units of weight-specific cost of transport.





Webb (1975) has discussed the use of optimum swimming speed as an approach to the question of swimming efficiency. The relationship between total cost per unit distance and swimming speed is typically described by a U-shape curve (see Figure 16 C). The lowest point of the curve indicates the optimum swimming speed, where the total cost per unit distance is at a minimum. The initial shape of the curve is attributed to the inclusion of standard metabolism into total costs, combined with low muscle and propellor efficiencies at low speeds (Webb, 1975). As swimming speed increases above the optimum, locomotory costs increase, progressively exceeding the standard metabolic rate.

The results illustrated in Figures 16 A & B are comparable to those reported in the literature, specifically the data of Facey & Grossman (1990) for rainbow trout of similar size (see Figures 16 C & D). The curves of total cost of transport calculated for juvenile rainbow trout in the present study do not, however, exhibit the U-shape discussed above, a result similar to that of Duthie (1982). This is most probably due to the fact that the swimming speeds imposed during these experiments were not extended to levels where the curve would begin to show the upward trend apparent in Figure 16 C. In other words, the trout used in this study were not subjected to velocities above their optimum swimming speed.

Another factor to consider is the size effect. The larger the fish, the greater the amount of oxygen required to move through a set distance. This is apparent in Figures 16 C & D, and to a lesser extent in Figures 16 A & B, where both total and net cost of transport are higher for the larger fishes. The effect of size on the optimum swimming speed would appear to follow the reverse trend, where larger fishes exhibit relatively lower optimum swimming speeds. A similar trend was found for sockeye salmon by Brett (1965), where the equation relating the maximum 60-minute sustained swimming speeds to length was found to have a slope of 0.5, demonstrating a rapid decrease in relative performance with increasing size.

This decrease in performance with increasing size is reflected in the slopes of the net cost of transport curves (Figures 16 B & D). The implication of this observation is that an increase in size reflects a more rapid increase in locomotory costs with increasing swimming speed. Evident

in Figure 16 B is that the net cost of transport curves are almost linear, compared to the exponential nature of the curves resulting from the data of Brett (1964) and Webb (1975) (see Figure 16 D). This could be explained by the range of swimming speeds for which data was available in the present study, coupled with the size effect discussed above. The data of Facey & Grossman (1990) (Figure 16 D) for trout of similar size showed an almost linear increase in the net cost of transport curve up to approximately  $8 \text{ bl}\cdot\text{s}^{-1}$ , supporting the above explanation.

### **Total Oxygen Consumption:**

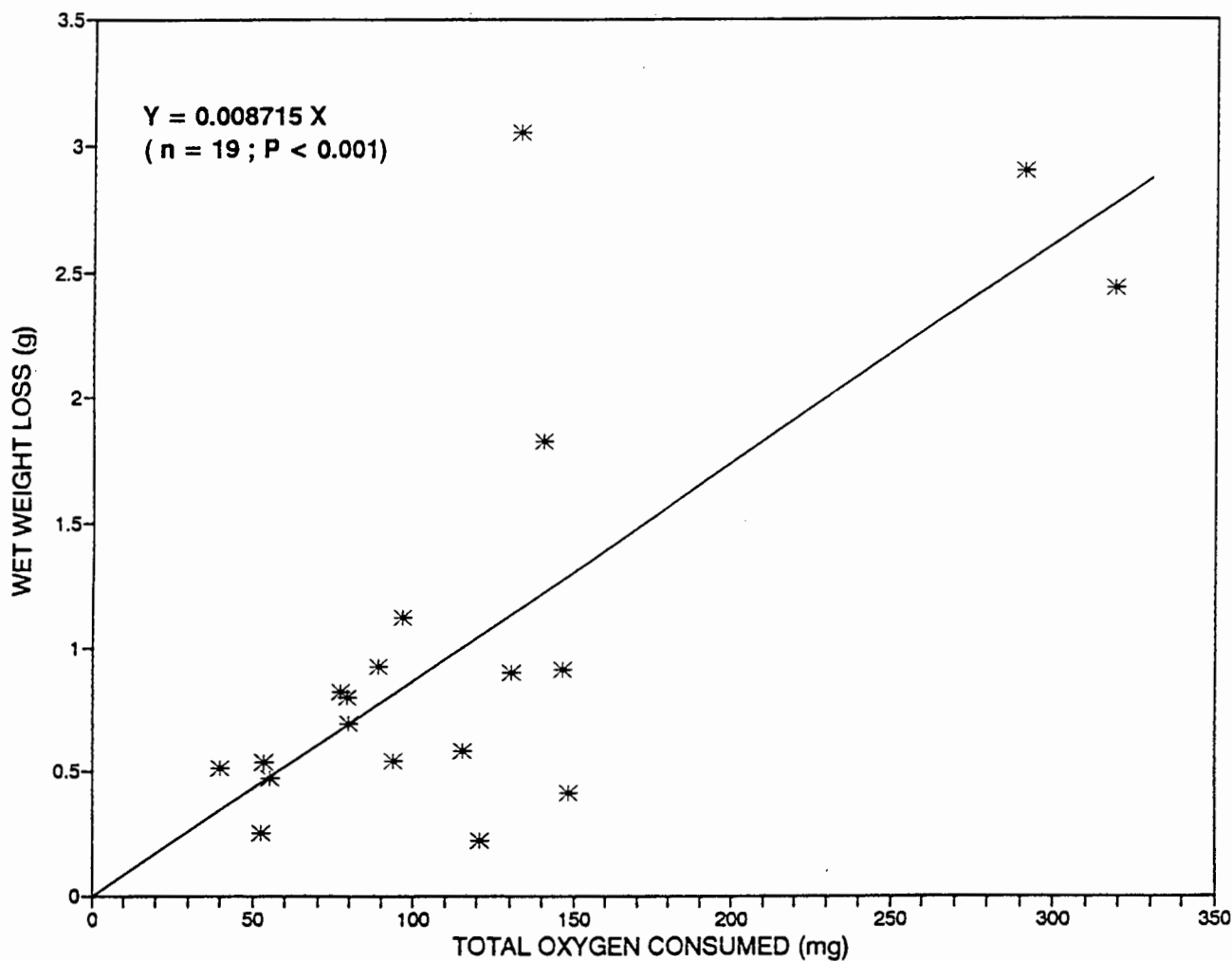
Measurements of body wet weight before and after each of the experiments described in Chapters 4, 5 and 6 yielded direct estimates of body weight loss during each experiment. Concomitant  $\text{VO}_2$  measurements permitted an examination of the relationship between the total amount of oxygen consumed, and the decrease in body weight which occurred. If a significant relationship exists between these two variables, this could be used to estimate energy expenditure under conditions where measurement of oxygen consumption and/or body fuel depletion are impractical or impossible.

Implementation of such an approach requires a detailed analysis of body composition and energy content, the relative contributions of various metabolic pathways, and proportions of body fuels being catabolized during various activities and feeding regimes. Although such an analysis is beyond the scope of the present study, it was nonetheless felt that the results discussed below could be of use in further research of this nature. Only two investigations of this nature appear to have been reported to date. Brett (1973) conducted a comparative study on salmon, assessing the validity of the application of generally accepted oxycalorific equivalents (see Chapter 1). Boggs & Kitchell (1991) estimated the metabolic costs of three species of tuna from measurements of energy losses during starvation. Estimates of metabolic costs calculated from measurements of mass losses and changes in energy content were consistent with previously reported results obtained through respirometry. It would therefore appear that valid data could be generated by this technique.

It should be emphasised that due to a lack of data in certain key areas, a number of unsubstantiated assumptions had to be made in the course of the analysis. Several potential sources of error introduce further complications, resulting in a high degree of uncertainty in the results (see Brett, 1973 for detailed discussion). Nevertheless, it is felt that such analyses could be of use in highlighting areas where further research is required if this relationship is to be established as a valid technique for estimating energy expenditure.

Measurements of  $VO_2$  from the experiments described in previous chapters were integrated over time to provide a single value of total oxygen consumed ( $mg O_2$ ) by each fish. Where gaps in the data series occurred (for example during the flushing periods and the overnight acclimation phases), an average value of oxygen consumption corresponding to the imposed velocity was calculated, this value then being applied to the calculation of total oxygen consumption. The corresponding body weight loss data (the dependent variable), are plotted against total oxygen consumed in Figure 17. A simple least-squares regression analysis was carried out on the data, forcing the intercept through the origin. This constraint was imposed on the analysis, since the underlying assumption was that all loss of body substances could be attributed to aerobic metabolic pathways. The data were tested against linear, exponential and logarithmic models through the transformation procedures described previously. The line of "best fit" is shown in Figure 17.

At this point, two major sources of error become apparent. Firstly, the oxygen consumption records used all had major gaps (particularly during the overnight periods where no measurements were recorded). Mean values corresponding to the swimming speeds imposed during these periods were consequently applied. These averaged values do not necessarily reflect the actual oxygen consumption rates, and hence incorporate an unknown degree of error. Secondly, this analysis assumes that all weight loss observed during the course of the experiments is a result of aerobic catabolic processes. Any weight losses which may have occurred through unrelated processes which were not accounted for (e.g. anaerobic metabolism, excretory and faecal losses), would introduce a further error component, the net result of which would be an overestimate of total energy expenditure.



**FIGURE 17:** Wet weight loss (g) corresponding to estimated total oxygen consumption (mg O<sub>2</sub>). The solid line is the best-fit regression line obtained by forcing the intercept through the origin (sse text).

The slope of the regression line illustrated in Figure 17 indicated a wet weight loss of 8.715 mg for every mg O<sub>2</sub> consumed. Application of the oxycalorific equivalent of 4.63 kcal.l O<sub>2</sub><sup>-1</sup> (Brett & Groves, 1979) yielded a value of energy expenditure equivalent to 0.3718 kcal.g wet weight<sup>-1</sup>. Since no analyses of the body energy contents of the trout used in this study were conducted, data reported for rainbow trout (with a mean wet weight of 3 g) by Staples & Nomura (1976), were used to estimate this quantity in an attempt to establish how the above value compared to generally accepted physiological equivalents. The fishes used by Staples & Nomura (1976) were fed a diet comprising 45.2% protein, 7.4% fat, 0.8% fibre and 10% moisture, similar to that of the feed used in the present study (see Chapter 3).

The data presented by Staples & Nomura (1976) indicated a mean caloric content of 5.7 kcal.g dry weight<sup>-1</sup>, and 1.17 kcal.g wet weight<sup>-1</sup>. Assuming that 80% of the energy present in body fuels is physiologically available to the fish, the balance being lost in excretion (Brett, 1973), the physiologically available energy content of juvenile trout body tissue was estimated to be in the region of 0.936 kcal.g wet weight<sup>-1</sup>. This value is 2.5 times higher than the value of 0.3718 kcal.g wet weight<sup>-1</sup> indicated in Figure 17. If the use of the data of Staples & Nomura (1976) is considered to be valid, then the measurements of weight loss illustrated in Figure 17 grossly underestimate actual energy expenditure. Two potential sources of error contribute to this:

(a) Weight losses through processes not attributable to aerobic catabolism. In other words, an unknown proportion of the measured weight loss was not attributable to the measured oxygen consumed, the net result of which would be an underestimate of actual energy expenditure through measurements of weight loss.

(b) The averaged oxygen consumption rates applied to the gaps in the data record were not representative of the true oxygen consumption rates. If these average values were underestimates (i.e. more oxygen was consumed than estimated), the net result would again be an underestimate of actual energy expenditure through measurement of weight loss. If, on the other hand, the average values were overestimates (i.e. less oxygen consumed than estimated), corresponding

measurements of weight loss would overestimate the energy expenditure of the fish. It is considered unlikely that error from this latter source has any major influence on the results.

In view of the above, it can be concluded that the error inherent in the weight loss versus total oxygen consumed relationship illustrated in Figure 17 can be attributed to the combined effect of excretory and faecal losses, and the underestimation of the total oxygen consumed over the course of the experiment. It is felt that while some anaerobic metabolism may have occurred at various stages, the error due to this factor is more than likely insignificant (see Chapters 6 & 7). As has been described previously, the fishes used in these experiments were all starved for long periods prior to each experiment, and while it is possible that the results have been influenced by faecal losses (although these were never observed), this is not considered to be a major source of error. The underestimation of the total oxygen consumed over the course of each experiment is felt to be primary factor contributing to the apparent error in the weight loss - total oxygen relationship. The averaged values applied for the overnight periods were those corresponding to the swimming speeds imposed over these periods. Since no observations of the swimming behaviour of the fishes over these periods were recorded, it is likely that excess activity on the part of the fishes may have occurred, and the resulting elevations in oxygen consumption rates were not accounted for in the estimate of total oxygen consumption.

Use of the weight loss - total oxygen consumption relationship to estimate energy expenditure through measurement of weight loss relies on an accurate and precise measure of total oxygen consumed. This can be achieved through continuous monitoring of oxygen consumption rates over the period for which the measured weight loss occurred. The relative proportions of body fuels being catabolized would also have to be accounted for (as well as the factors discussed in Chapter 1, regarding the application of oxycalorific and physiological equivalents). With the establishment of an adequate database, this method could be a valuable tool in the estimation of energy expenditure under circumstances precluding the use of measurement of gaseous exchange. Field studies of fish energetics are one area where the application of this technique could be extremely beneficial.

## GENERAL DISCUSSION

In Chapter 1 of this document, it was emphasised that the primary objective of this study was the development of an apparatus suitable for investigations into the swimming energetics of small aquatic organisms (i.e. estimating the energy expenditure associated with various levels of activity). As has been described in Chapters 1 and 2, the design of the apparatus was based on an indirect calorimetric approach, where energy expenditure at set levels of imposed activity was estimated through measurement of oxygen consumption. It is evident from the references cited throughout this thesis that this approach is by no means a novel one, and has been employed by several researchers in the field. Many of the features of the respirometers described in the literature have been incorporated into the apparatus described in Chapter 2.

The performance of the apparatus developed in the present study was tested during the course of the experiments conducted on juvenile rainbow trout. Since the results of these experiments are of fundamental importance in establishing whether the respirometer was capable of generating data comparable to that reported in the literature, this aspect of the study will be discussed prior to the final evaluation of the apparatus in the closing sections of this chapter.

### **1. Diurnal Variation in the Metabolic Rate of Juvenile Rainbow Trout.**

While seasonal differences in the metabolic rates of fishes have been demonstrated (e.g. Beamish, 1964b; Evans, 1984; Facey & Grossman, 1990), there does not appear to be any general agreement concerning the existence of any diurnal differences in metabolic rate. Evans, Purdie & Hickman (1962) reviewed evidence suggesting either a direct or indirect influence of photoperiod on metabolic rate, but presented data for rainbow trout which demonstrated that the photoperiod did not significantly affect this variable. Degani *et al.* (1989) mentioned that time of day has an influence on metabolism, but provided no information substantiating this statement. Brett & Zala (1975) investigated diurnal variation in the  $VO_2$  of sockeye salmon which were fed

at the same time every morning. The results demonstrated an anticipatory elevation in  $VO_2$  prior to feeding and the light phase of the applied photoperiod. This elevation was related to the activity levels of the fishes, and was observed to decline when the fishes were starved.

Fry (1971) has stated that most fishes display a daily cycle of activity which may influence measurements of metabolic rate, in spite of precautions to maintain constant conditions during experiments (Van den Thillart & Verbeek, 1991, however, were able to depress such spontaneous activity in goldfish by keeping them in complete darkness). Evidence suggesting endogenous diel rhythms in  $VO_2$  has been reported for three species of marine teleost, *Lithognathus mormyrus* and *Lithognathus lithognathus* (Du Preez, Strydom & Winter, 1986), and *Lichia amia* (Du Preez, 1987), and for the marine elasmobranchs *Rhinobatos annulatus* and *Myliobatos aquila* (Du Preez, McLachlan & Marais, 1988), but no indication was provided as to whether these rhythms were activity related. In contrast, experiments on rainbow trout reported by Dickson & Kramer (1971) and Evans *et al.* (1962) indicated no differences in respiratory metabolism during the daylight hours.

The data presented in Chapter 4 provided no evidence supporting the presence of an such underlying rhythmic fluctuation in metabolic rate. Although significant elevations in  $VO_2$  were observed in several cases after the switching phases of the applied photoperiod, these could not be ascribed to anything other than a purely behavioural response to the visual stimuli of the relatively rapid changes in light intensity. These  $VO_2$  elevations were always associated with the incidence of excess activity on the part of the fish. The influence of these elevations were generally restricted to the short term, lasting for a maximum of 2 hours after the switch in the photoperiod. Considering that all  $VO_2$  measurements recorded during the incremental velocity and oxygen debt trials were collected during the daylight hours, and usually after 9 am, it is unlikely that these data incorporated a significant degree of error from this source.

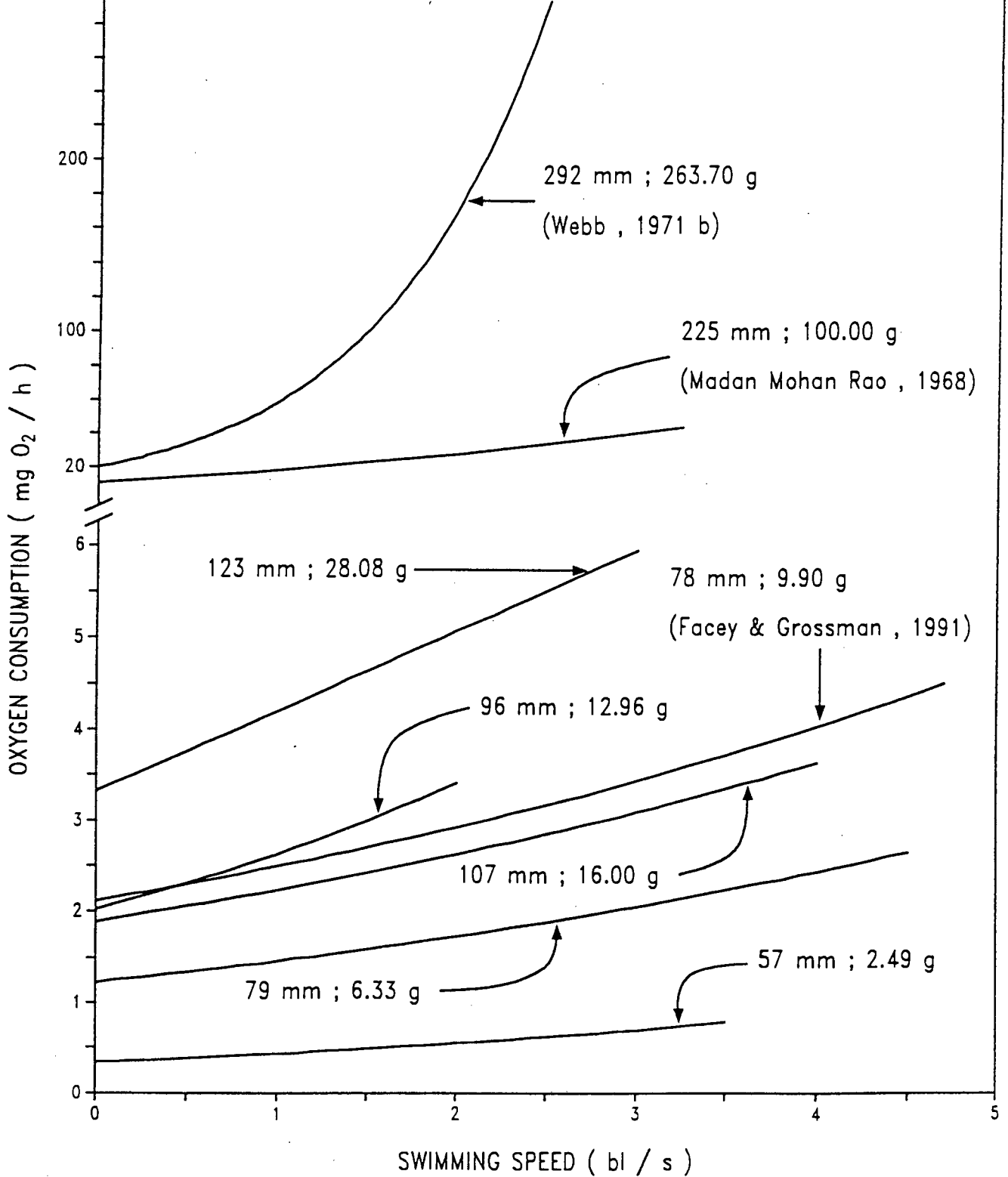
On the basis of the results obtained from this series of experiments, it was concluded that diurnal variation in the metabolic rates of juvenile rainbow trout would not introduce a major source of error into measurements of oxygen consumption recorded during experiments where this variable

was used as an estimate of energy expenditure. These data were consequently considered to be robust, provided that the complicating factors of excess activity and inadequate starvation periods were accounted for.

## **2. The Oxygen Consumption - Swimming Speed Relationship at Sustained Swimming Speeds.**

The results of the incremental velocity experiments presented in Chapter 5 supported the generally accepted exponential nature of this relationship (e.g. Brett, 1964; Webb, 1971b and 1975; Goldspink, 1977b; Facey & Grossman, 1990). Although data recorded during several of the experiments indicated a linear increase in oxygen consumption with increasing swimming speed, this has been explained by the range of swimming speeds over which the data were recorded.

To establish how the data recorded in the present study compare with those reported in the literature, the results of several selected incremental velocity trials are plotted in Figure 18. For comparative purposes, the data reported by Madan Mohan Rao (1968), Webb (1971b) and Facey & Grossman (1990), are included in the plot. While the data from the present study are for individual fishes, this was not the case for the curves obtained from the literature. All of these curves represent mean values of  $VO_2$  versus swimming speed for a number of individuals. In most cases, the literature data sets were obtained from a relatively wide size range of fishes, compared to those used in the present study. This is particularly evident in the data of Madan Mohan Rao (1968), where regression equations relating oxygen consumption to wet weight at four set swimming speeds ( $0 \text{ cm.s}^{-1}$ ;  $18.5 \text{ cm.s}^{-1}$ ;  $45.1 \text{ cm.s}^{-1}$  and  $72.7 \text{ cm.s}^{-1}$ ) were presented for a wet weight range of 54 to 135 g. To convert these swimming speeds into relative units ( $\text{bl.s}^{-1}$ ), the length - weight regression equation presented in Madan Mohan Rao (1971) for the same group of fishes, was used to estimate the length of a 100 g trout, and the corresponding oxygen consumption rates then calculated from the  $VO_2$  - weight regressions mentioned above.



**FIGURE 18:** Absolute oxygen consumption rate ( $\text{mg O}_2 \cdot \text{h}^{-1}$ ) - swimming speed ( $\text{bl} \cdot \text{s}^{-1}$ ) relationships in rainbow trout of indicated length and wet weights. All at  $15^\circ\text{C}$ . Data from Madan Mohan Rao (1968); Webb (1971b); Facey & Grossman (1991) and the present study (unreferenced curves).

Data relating  $VO_2$  and activity for rainbow trout of the same size as those investigated in the present study appear to be in short supply in the literature. Only the data from Facey & Grossman (1990) illustrated in Figure 18 can be considered representative of fishes of similar size. Although appearing to be of slightly lower magnitude, the data from the present study compare favourably with those of Facey & Grossman (1990). The data do not, however, conclusively support the contention stated in Chapter 5 that the "settling down" period of approximately 60 minutes employed by several researchers in the field, was of insufficient duration to obtain valid results, and that the data reported in these publications would more than likely be over-estimates. Although the curves resulting from the present experiments generally seem to be slightly lower than that of Facey & Grossman (1990) for trout of similar size, the difference is not convincing, and does not support the prolonged stabilization periods employed in the experimental protocol described in Chapter 5.

An interesting observation in this regard is reported by Wokoma & Johnston (1981), who monitored changes in whole body lactate contents of rainbow trout over time, subsequent to commencement of a high level of sustained exercise (a swimming speed of  $3.5 \text{ bl.s}^{-1}$ ). Whole body lactate contents increased by approximately 16% during the 25 second acceleration period, and then exhibited a substantial linear increase for the next 8 minutes. Following this peak, the lactate contents then gradually decreased, reaching levels not significantly different from the "resting" condition. Wokoma & Johnston (1981) concluded that the initial anaerobic contribution at this level of swimming activity was likely of the order of 38%. After the 8 minute peak, there appeared to be decreased reliance on anaerobic metabolism, as the fishes settled down to a more economical mode of swimming (after commencement of the exercise, the fishes were observed to swim unsteadily in a series of flick-and-glide manoeuvres, an observation similar to that of Webb, 1971b), permitting significant catabolism of the lactate. The data of Wokoma & Johnston (1981) indicated that 60 minutes after the increase in velocity, the whole body lactate contents had decreased considerably, and were only slightly higher than the "resting" levels. These results support the contention expressed in the literature that a stabilization period of about 1 hour is usually sufficient for valid determinations of oxygen consumption rates.

A further aspect to be considered in the  $VO_2$  - swimming speed relationships established in this study, is that of physical training of the fishes. Various training protocols have been shown to improve endurance (Johnston & Moon, 1980a; Lackner, Wieser, Huber & Dalla Via, 1988), reduce resting and swimming oxygen consumption rates (Nahhas, Jones & Goldspink, 1982; Woodward & Smith, 1985), increase the maximum sustainable swimming speed (Hammond and Hickman, 1966), increase heart size and blood haemoglobin levels (Hochachka, 1961), increase growth rate (Lackner *et al.*, 1988), reduce endogenous fuel depletion during swimming (Pearson *et al.*, 1990) and result in more rapid recovery of muscle and liver glycogen (Hochachka, 1961). Further, such training improves the response to stress (Woodward & Smith, 1985; Lackner *et al.*, 1988; Pearson, Spriet & Stevens, 1990) enhances the capacity of the red muscle fibres to perform anaerobic work (Johnston & Moon, 1980a & 1980b) and induces improved rates of metabolic recovery following exhaustive exercise (Pearson *et al.*, 1990; Scarabello *et al.*, 1992). Hochachka (1961) reported that trained rainbow trout could acquire an oxygen debt three times that of untrained fishes.

In view of the fact that the trout used in the present study were not subjected to any training protocol at all, it is possible that the data have been influenced by this factor, perhaps resulting in  $VO_2$  - activity relationship slightly higher than what would be the case if the fishes had been trained prior to the experiments. The general consensus, however, appears to be that the influence of training is most profound in terms of increasing the anaerobic capacity of the red muscle fibres, and in enhancing recovery from strenuous exercise (see previous paragraph). It is felt to be unlikely that these effects would be a significant influence at the levels of activity imposed in the present study.

The influence of size on the  $VO_2$  - swimming speed relationship has been discussed in detail in Chapter 5. In general, the illustrated relationships (see Figures 10, 11 & 12) compare favourably with those reported in the literature, with several inconsistencies. Most notably, the decrease in the slope of the  $VO_2$  - weight relationships at swimming speeds above  $1 \text{ bl.s}^{-1}$ , whereas results documented in the literature (see Chapter 5) indicated an increase in the slope of this relationship with increasing activity levels. The inconsistencies evident in the data from the present study

were attributed to a combination of the lack of data for larger fish at the higher swimming speeds, together with the statistical procedures employed during the analysis of the data.

### **3. The Anaerobic Component of Energy Expenditure at Sustained Swimming Speeds.**

The results of the oxygen debt trials and lactate accumulation experiments presented in Chapters 6 & 7, supported the general consensus expressed in the literature, in that it was concluded that the anaerobic contribution to overall energy expenditure at sustained swimming speeds in juvenile rainbow trout could be considered negligible. Estimates of the magnitude of anaerobic metabolism arrived at through measurement of EPOC indicated an anaerobic contribution ranging from approximately 3% of the overall energy expenditure, to a maximum of about 20%. However, as was described in Chapter 6, this latter estimate was almost definitely a gross over-estimate, since the trials yielding this result were characterized by a high incidence of excess activity by the fish during the exercise and recovery phases, with associated elevated  $VO_2$  and EPOC. It was concluded that a figure of approximately 3% of the total energy expenditure was a more reliable estimate of the anaerobic contribution at the swimming speeds involved (up to about  $3 \text{ bl}\cdot\text{s}^{-1}$ ). As stated previously, this result rests on the assumption that the concept of EPOC as a measure of oxygen debt accumulation is a valid indicator of the level of anaerobic metabolism which had occurred during the exercise.

The lactate accumulation trials discussed in Chapter 7 yielded data which led to the same conclusion as stated above. Estimates of the anaerobic component arrived at through analysis of the changes in whole body lactate (ranging from 0.2% at  $0.5 \text{ bl}\cdot\text{s}^{-1}$ , to about 1% at  $3 \text{ bl}\cdot\text{s}^{-1}$ ) were considerably lower than those calculated from the oxygen consumption trials. This could be taken as further support for the criticism of the use of lactate metabolism as a measure of anaerobiosis documented by Scarabello *et al.* (1991) and Wood (1991), both of which studies indicated that lactate metabolism seriously under-estimated the anaerobic component as measured by EPOC. Another possibility is that since the fishes were sampled immediately subsequent to the exercise phase for analysis of lactate content, these data were not subject to the error

associated with excess activity during the recovery phase, which the oxygen debt data doubtless incorporated.

A source of error which could potentially influence the lactate accumulation data is the duration of the sampling period, in other words, the time which elapsed between the removal of the fish from the apparatus and subsequent freeze-clamping, effectively arresting all metabolic activity. During this period, changes in lactate content could occur, either through ongoing lactate generation through the stress involved with the sampling procedure, or catabolism of the lactate prior to freeze-clamping. During the experiments described in Chapter 7, the fishes were always freeze-clamped within 60 seconds of removal from the chamber. Since the process of lactate catabolism appears to be a relatively slow one, it is felt that the error from this source is probably negligible. On the other hand, lactate generation can occur very rapidly. Wokoma & Johnston (1981) document a 16% increase in whole body lactate within 25 seconds of an increase in flow velocity. It is therefore conceivable that a significant amount of lactate could have been produced anaerobically during the sampling period. Error from this source, however, would over-estimate the actual lactate accumulation resulting from the imposed exercise. Since the figures of lactate contents mentioned above are already extremely low, error from this source would not influence the final conclusion arrived at from this series of experiments.

The results of Wokoma & Johnston (1981), regarding the time-course of lactate production in rainbow trout swimming at  $3.5 \text{ bl.s}^{-1}$  discussed previously, demonstrated the generally accepted progression of metabolism with the onset of activity. Initially, the energy demand associated with an increase in activity cannot be met aerobically, and consequently has to be supported by anaerobic pathways (Jones, 1982; Bennett, 1983), with resulting production of lactate. A brief period of unsteady swimming behaviour adds to this production. Once the cardiovascular and ventilatory support systems have been mobilized, and the fish has settled into a more efficient mode of swimming, the energy requirements are met through aerobic pathways, with a concomitant decrease in lactate production. Since available oxygen is presumably in excess, the lactate which has been accumulated is also slowly catabolized (in a similar manner to the EPOC concept). However, since a certain proportion of anaerobic metabolism is still occurring, there is

an ongoing dynamic process of lactate production and catabolism. Eventually, the influence of the increase in swimming speed is no longer apparent, and the system settles into a "steady-state", where a certain amount of lactate is being continually produced and catabolized (Thomas *et al.*, 1987). From the data presented by Wokoma & Johnston (1981), this state is almost attained after about 60 minutes of exercise at  $3.5 \text{ bl}\cdot\text{s}^{-1}$ . The swimming speeds employed by Wokoma & Johnston (1981) were considered to be sustainable, and hence are comparable to those employed in the present study.

The lactate accumulation trials conducted in the present study employed a 4 hour stabilization period subsequent to the increase in velocity prior to sampling. It was assumed that by this time, the system had achieved steady-state status, and that the levels of lactate present in the tissues of the fishes were representative of the level of anaerobic metabolism associated with the imposed swimming speed. As has been discussed previously, the principle of the lactate accumulation trials rests on the assumption that this variable can be regarded as an index of the level of anaerobic metabolism at sustained swimming speeds. Although the validity of this assumption is open to question, the results of the oxygen debt experiments substantiated the final conclusion. Further research aimed at anaerobiosis in fishes at sustained activity levels would perhaps shed more light on this question.

#### **4. Overall Energy Expenditure at Sustained Swimming Speeds.**

Given that the anaerobic component of total energy expenditure at sustained swimming speeds was concluded to be negligible (with the exception of a limited contribution associated with rapid changes in swimming speed - Jones, 1982), the aerobic fraction (measured by rates of oxygen consumption) can be considered to be representative of the overall energy expenditure at the swimming speeds imposed in the present study. The results of the cost of transport calculations discussed in Chapter 8 indicated that the fishes were subjected to levels of activity well below their optimum swimming speeds. By general consensus (Jones, 1982; Randall & Daxboeck, 1982), activity at these levels is supported virtually entirely by aerobic metabolism (RQ's of less

than unity reported by Kutty, 1968b for rainbow trout at swimming speeds up to 80% of the critical swimming speed, substantiate this assumption).

Quantification of the optimum swimming speeds could be of great interest, particularly with regard to commercial farming of rainbow trout. A continuous water flow through the holding tanks of such farming enterprises has to be induced to ensure a supply of fresh water to the fishes, as well as removing waste products. Information regarding the most efficient swimming speed has profound implications in terms of the flow rates through the tanks which would minimise locomotory costs, and maximise growth rates.

As will be discussed below, the apparatus was somewhat limited in terms of the maximum velocity which could be imposed. The data illustrated in Figure 16 indicated that the optimum swimming speeds were beyond the capabilities of the apparatus. Consequently, it was impossible to gain an idea of this optimum, or to set the limits of sustained swimming in juvenile rainbow trout.

##### **5. Performance of the Apparatus.**

In view of the preceding discussion of the results generated from the apparatus developed for this study, it is felt that the design of the respirometer was satisfactory for the purposes envisaged. The data collected during the various experiments were considered to be comparable to those reported in the literature. Several shortcomings of the apparatus were, however, evident.

Firstly, the respirometer was somewhat limited in terms of the maximum flow velocity which could be generated by the main circulating centrifugal pump. In several of the experiments, particularly those using the larger fishes, the maximum flow velocity did not allow for the procurement of satisfactory data at higher swimming speeds. Clearly, this problem could easily be rectified by incorporating a more powerful pump into the system. Whether this would influence the temperature regulating capabilities of the system would have to be established and accounted for.

The second major shortcoming of the system involved the sensitivity of the oxygen sensor. This problem was particularly apparent in experiments involving the smaller fishes, where oxygen consumption rates were so low that prolonged periods were required for the dissolved oxygen content of the water to decrease sufficiently to be detected by the sensor. The net result of this was a loss of resolution in the data. The oxygen debt trials were especially effected by this problem. With current advances in technology, increasingly precise equipment is becoming available. Alternative sensors providing improved levels of precision could be applied to an apparatus such as this, greatly enhancing the quality of the data. Although the concept of a flow-through system was rejected in Chapter 2, for certain applications such a system is undoubtedly superior. An oxygen sensor incorporating a higher level of precision would perhaps allow the application of a flow-through system to be a viable proposition in a study of this nature.

Further modifications which would improve the overall performance of the system include a more sophisticated flow meter, some form of data logging device (e.g. the apparatus described by Steffensen *et al.*, 1984), as well as techniques for measuring dissolved CO<sub>2</sub> and ammonia. Incorporation of additional flow chambers into the apparatus would permit experiments to be run on several fishes simultaneously (e.g. Degani *et al.*, 1989), considerably reducing the time factor in obtaining a representative data set.

An interesting observation made in Chapter 6 has bearing on the design of the experimental protocol of the incremental velocity trials. A major problem of these experiments was the occurrence of excess activity on the part of the fishes, with resulting VO<sub>2</sub> elevations. This feature can introduce a substantial source of error into such an investigation, particularly at low imposed velocities. During the oxygen debt experiments, excess activity was observed to be minimal during the recovery phases following exercise (particularly the higher levels of exercise). This observation raises the possibility of using decreasing velocity tests to simultaneously investigate the VO<sub>2</sub> - swimming speed relationship, and EPOC. Initially, high velocities could be imposed, gradually decreasing in set increments. Following each velocity decrease, continuous monitoring of VO<sub>2</sub> would provide an estimate of EPOC, and once recovery is complete and the oxygen debt repaid, an estimate of the oxygen consumption corresponding to the level of activity imposed.

This procedure could possibly minimise the influence of excess activity on measurements of  $\dot{V}O_2$ , providing more robust estimates of this variable.

The ultimate objective of studies investigating the activity physiology of fishes is generally the quantification of the locomotion term of the equation describing the energy budget of the species concerned (see Chapter 1). Laboratory-derived estimates of the energy costs associated with various levels of activity obtained from such studies, can be applied to field-derived measurements of routine swimming activity (i.e. all the volitional movements of the fishes in the natural environment - Webb, 1991) in order to set up an energy budget which reflects natural conditions (e.g. Feldmeth & Jenkins, 1973; Ware, 1975, 1978; Puckett & Dill, 1984). The fundamental assumption implicit in this approach (i.e. that estimates derived in the laboratory have external validity) has been questioned by Webb (1991). Considering that such laboratory studies subject the experimental animals to conditions far removed from what they would experience in the natural environment (e.g. physical handling, confinement in enclosed spaces and prolonged periods of forced steady swimming), such criticism may have some founding.

Webb (1991) discussed the results of several recent studies which suggested that unsteady motions of fishes were associated with substantial increases in energy costs, compared with the costs of steady swimming at the same average speed. In contrast, results of theoretical analyses suggested that an intermittent swimming mode ("burst-and-coast" swimming) was energetically advantageous over steady swimming at velocities below the maximum aerobic cruising speed (Weihs, 1974), as well as at anaerobic speeds (Videler & Weihs, 1982). These results appeared to be substantiated by observations of the swimming behaviour of fishes. In spite of this disparity of opinion, and in view of the fact that steady swimming at a constant speed is rarely observed in the natural environment (Webb, 1991), the application of estimates of locomotory costs obtained from experiments involving steady swimming to measurements of routine swimming, may well be inappropriate.

If this issue is to be resolved, reliable estimates of the metabolic costs of activity of wild fishes under field conditions have to be obtained. This problem has proved to be a very elusive one for

fisheries biologists (Kaseloo, Weatherley, Lotimer & Farina, 1992). Recent advance in biotelemetry, however, may provide a possible solution. Kaseloo *et al.* (1992) have described a telemetry system capable of obtaining, transmitting and recording electromyograms (EMG's) generated during muscle activity of free-swimming fishes, which can be used as quantitative indicators of overall activity. The system described by Kaseloo *et al.* (1992) was an improved version of the system developed by Weatherley, Rogers, Pincock & Patch (1982) and subsequently applied in the field (Rogers, Church, Weatherley & Pincock, 1984). Weatherley *et al.* (1982) reported a very good correlation between measurements of activity and concomitant  $VO_2$  values, and suggested that the system could be "calibrated" to provide estimates of oxygen consumption. Although attempts to deduce the metabolic costs of activity from the data were prone to error (Rogers *et al.*, 1984), the prospects of this technique are very exciting. It is hoped that further research of this nature may increase the reliability of such systems, the application of which could generate information of great value to many fields of investigation in fish biology.

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## LACTATE DETERMINATION METHODOLOGY

Reference to Noll (1984) and Gäde & Grieshaber (1989) will provide a complete description of the enzymatic assay technique for determination of lactate concentration in biological samples. A synopsis of the techniques employed in this study is presented below for those with no easy access to these references.

### **Sample Collection and Preparation:**

In order to quantify the whole-body lactate content of the trout investigated in the present study, through enzymatic analysis, two pre-requisites had to be fulfilled (Gäde & Grieshaber, 1989):

- the sample to be analysed had to be representative (i.e. a homogeneous tissue sample of the entire fish);
- no decomposition of the lactate should occur prior to the analysis .

The sample collection and treatment procedures recommended by Gäde & Grieshaber (1989) were adopted to fulfill these criteria. Immediately after the fish had been subjected to the specific exercise level of each experiment, it was stunned with a blow to the head, and freeze-clamped between aluminium plates which had been pre-cooled in liquid nitrogen. This technique results in a thin compressed layer of tissue, which is rapidly completely frozen, arresting all metabolic activity which might lead to the decomposition of the metabolite to be analysed. The entire freeze-clamping procedure was always completed within 45 to 60 seconds subsequent to removal of the fish from the chamber.

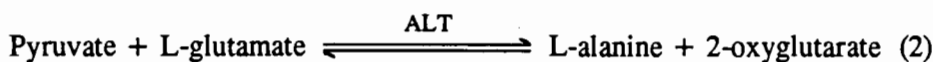
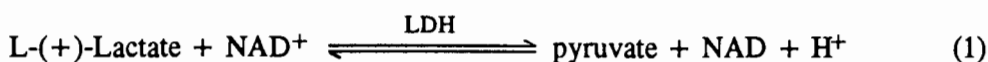
### **Disintegration and Extraction:**

Once the sample had been collected, the entire body of the fish was pulverized into a fine powder with a porcelain mortar and pestle which had been precooled in liquid nitrogen. Frequent addition of liquid nitrogen during the grinding of the tissue prevented any thawing of the sample.

The samples were stored at  $-80^{\circ}\text{C}$  between treatments. Once the samples had all been reduced to powder form, they were deproteinized with 0.6 M perchloric acid ( $\text{HClO}_4$ ). Approximately 1 g of the frozen tissue powder was added to about 5 ml of the ice cold perchloric acid, mixed, homogenized for 20 seconds, and the mixture then centrifuged at 3000 g for 20 minutes at  $2^{\circ}\text{C}$ . The supernatant was subsequently saved, and the pellet again deproteinized as described above. The supernatants were combined, and an aliquot (4ml) of the solution was neutralized by addition of 3 M KOH (the pH of the solution was established with pH paper). The resulting sample solution was stored frozen at  $-20^{\circ}\text{C}$  until the enzymatic analysis was conducted.

### Enzymatic Analysis:

The principle of the assay rests on two reactions:



Where:

LDH = lactate dehydrogenase

ALT = alanine aminotransferase, formerly called GPT (glutamate-pyruvate transaminase).

The equilibrium of reaction 1 is greatly in favour of lactate (i.e. the conversion of pyruvate to lactate occurs more readily than does the reverse). However, lactate can be completely dehydrogenated by LDH if pyruvate is removed from the equilibrium by conversion to alanine (reaction 2). The increase in NADH concentration (measured by the change in absorbance at a wavelength of 340 nm) is directly proportional to the amount of lactate present in the sample.

Prior to the assay, the extracts of the tissue samples were diluted with a 0.52 M glutamate buffer solution, usually by a factor of 100, and up to 1000 in those samples corresponding to maximal activity levels. This ensured the presence of sufficiently low lactate levels to reach the reaction end point in a reasonable period of time.

For the assay, the following solutions were successively pipetted into a cuvette:

Glutamate buffer	0.67 ml
NAD (24mmol.l <sup>-1</sup> )	0.10 ml
Sample	0.20 ml
ALT suspension (4U per assay)	0.05 ml
Water	1.96 ml

The mixture was incubated for 10 minutes at 25°C, and then the absorbance (*A1*) measured in a spectrophotometer. Following this, 0.02 ml of the LDH suspension (11U per assay) was added, and then the absorbance monitored until a constant reading was obtained (*A2*), indicating that the reaction end point had been reached. A blank was also run, since even in the absence of lactate, a slight increase in absorbance occurs. The blank was run with a 1.8 M ammonium sulphate solution instead of the ALT suspension, and with water instead of sample. Absorbance *A1* was subtracted from *A2*, providing a change in absorbance for the assay. This value was then corrected for the blank, yielding a value *A*, the corrected change in absorbance.

The concentration of lactate in the assay sample could then be calculated from the following equation:

$$c \text{ (mmol.l}^{-1}\text{)} = A \times [V \div (\epsilon \times d \times v)]$$

where *V* = assay volume = 3 ml

$\epsilon$  = extinction coefficient at 340 nm =  $6.3 \times 10^2 \text{ l.mmol}^{-1} \text{ .mm}^{-1}$

*d* = light path = 10 mm

*v* = sample volume = 0.20 ml

This value then had to be corrected for the various dilutions of the original sample, including the deproteinization and neutralization dilutions, before a value of lactate content per gram wet weight of tissue could be calculated. In order to check the accuracy of the assays, three standards were run during the course of the analysis. These took the form of solutions containing known amounts of lactate. The concentration of lactate in these solutions were selected so as to represent the extremes and approximate mid-point of the range of expected lactate concentrations in the samples. The level of accuracy was found to be satisfactory for the purposes of the analysis.