# ON THE USE OF METABOLIC RATE MEASUREMENTS TO ASSESS THE STRESS RESPONSE IN JUVENILE SPOTTED GRUNTER, *Pomadasys commersonnii* (Haemulidae, Pisces)

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By

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To my wife Pamela, and the kids…

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# ABSTRACT

Quantification of stress requires the use of a stress indicator that is easy to measure, and which can be readily interpreted in terms of the potential long-term effects to an organism. This study evaluates the suitability of metabolic rate as an indicator of the stress response in fish. By comparing the metabolic with the cortisol stress response, the most commonly used indicator of stress in fish, it was possible to assess the suitability of metabolic rate as a stress indicator. Changes in metabolic rate were used to predict the long-term effects of transport-related stressors. This study also determined the baseline metabolic rates of the fish.

The standard and the active metabolic rates of juvenile *P. commersonnii* were $0.16 \pm 0.02$ (mean ± S.D, $n = 6$) mg $O_2$ $g^{-1}h^{-1}$, and $0.56 \pm 0.04$ mg $O_2$ $g^{-1}h^{-1}$, respectively, whereas the routine metabolic rate for the fish was $0.25 \pm 0.03$ mg $O_2$ $g^{-1}h^{-1}$. The relationship between metabolic rate and body weight was described by the equation $mo_2 = 0.64\ W^{-0.38}$. 24-h oxygen consumption measurements showed that juvenile *P. commersonnii* exhibited diel rhythmicity in oxygen consumption rate, the higher rates occurring at night and the lower rates during the daytime. The higher nocturnal metabolic activity may have been due to increased activity induced by an endogenous rhythm related to feeding. Diel rhythmicity has direct implications for the measurement of baseline metabolic rates since it could result in overestimation or underestimation of these rates. 24-h continuous oxygen consumption measurements enabled the detection of the rhythmicity in oxygen consumption rate, and thereby ensured a greater degree of accuracy in the estimation of these parameters.

The metabolic stress response in juvenile *P. commersonnii* was best described by the equation, $y = -0.0013 x^2 + 0.0364 x + 0.3052$, where x = time after application of stressor, and y = oxygen consumption rate. Using the derivative of this equation, the metabolic stress response was estimated to peak approximately 14 min after application of a simulated capture and handling stressor. Oxygen consumption increased by about 300% as a result of the stress. Approximately 15 min after application of a similar stressor, plasma cortisol levels in stressed fish was 200% higher than baseline levels. However, cortisol levels in fish sampled 30 min after the disturbance was similar to the baseline cortisol levels, indicating that full recovery had occurred. Although the patterns in the metabolic and cortisol stress responses were similar, metabolic rate could be measured continuously, thereby ensuring accurate interpretation of the data. Furthermore, increases in metabolic rate during the stress response are a culmination of physiological events from the primary to the tertiary levels of biological organization and are, therefore, easier to interpret in terms of long-term effects on the fish.

Different transportation procedures elicited variable degrees of stress in juvenile *P. commersonnii*. The cost of metabolism attributed to the effects of capture and handling was twice as much as that attributed to acute temperature elevation. Acute temperature decrease resulted in a significant reduction in the oxygen consumption rate (ANOVA, $p < 0.05$). Oxygen consumption by the fish was not affected by fish density (ANOVA: $F = 2.002$, $p = 0.5$), or by oxygen depletion at dissolved oxygen concentrations above the critical level. Below this level, however, oxygen consumption decreased linearly with further decrease in dissolved oxygen concentration. These results showed that the highest energetic cost to juvenile *P. commersonnii* was incurred as a result of capture and handling. The results also showed that by subjecting fish to different stressors, it was possible to categorize them according to their relative metabolic costs to the fish.

At 25 °C, the effective concentration of 2-phenoxyethanol to fully anaesthetize (Stage IV, McFarland 1960) juvenile *P. commersonnii* was 0.4 ml $l^{-1}$ and the most appropriate concentration for deep sedation (Stage II, McFarland 1960) of the fish for at least 24 h was 0.2 ml $l^{-1}$. A maximum of 3 minutes was required by the fish to recover from the effects of the anaesthetic. There was no correlation between fish weight and the rate of induction of anaesthesia ($r^2 = 0.001$, $p = 0.3$). At the peak of the metabolic stress response, oxygen consumption was twice as high in the un-anaesthetized fish compared to the fish anaesthetized after the application of the simulated capture and handling stressor, suggesting that anaesthetization with 2-phenoxyethanol may have reduced the effect of the disturbance on the fish. Similar oxygen consumption rates for the fish anaesthetized prior to capture and the non-stressed fish suggested that the increases in metabolic rate could be linked to the struggling associated with attempts by fish to escape from the perceived stressor. Anaesthetization of juvenile *P. commersonnii* with 0.3 ml $l^{-1}$ 2-phenoxyethanol resulted in a more than 200% increase in plasma cortisol concentration. The elevated levels of plasma cortisol in the anaesthetized fish suggested a manifestation of 2-phenoxyethanol as a stressor. At the time of capture, cortisol levels in fish that were anaesthetized prior to capture were the same as those measured in the disturbed fish at the peak of the stress response (ANOVA, $p = 0.95$), suggesting that the anaesthetized fish were already experiencing considerable stress at the time they were captured. Undisturbed juvenile *P. commersonnii* that were anaesthetized for 1 h also had cortisol levels that were five times higher than those measured in undisturbed-un-anaesthetized fish, indicating that the duration of exposure to the anaesthetic had a significant effect on plasma cortisol levels.

The results presented in this study demonstrate the usefulness of metabolic rate as an indicator of acute stress in fish. This was achieved by comparing the metabolic and the cortisol stress responses. The ease and accuracy with which oxygen consumption of fish could be measured made it possible to measure the stress response more accurately than by plasma cortisol concentration. It was also possible to monitor metabolic rate continuously over a long duration using polarographic oxygen sensors, thus enabling a better evaluation of the stress response. These results, thus, suggest that metabolic rate measurements could be a more practical way to quantify the effects of acute stressors on juvenile fishes. By detailing the profile of the metabolic stress response in *P. commersonnii*, this study makes a contribution towards understanding the physiological effects of stress in fishes. The study also contributes towards the quantification of baseline metabolic rates of this species under captivity.

This study also contributes towards understanding the effects of 2-phenoxyethanol on the stress physiology of fish. By anaesthetizing fish under different conditions of stress, it was possible to evaluate the effect of 2-phenoxyethanol on the metabolic stress response. The ability of 2-phenoxyethanol to reduce physical activity of the fish, and thereby reduce the impact of acute stress on the metabolic stress response, makes it a good agent for the mitigation of stress during the capture and handling of fish. However, the increase in plasma cortisol concentration during prolonged anaesthetization using this drug suggests that the anaesthetic might be a stressor to fish and may, therefore, not be suitable for long-term sedation.

# CHAPTER 1

## Introduction: Bioenergetics, stress, and the use of anaesthetics in fish transport

### Introduction

The dynamic chemical and physical conditions under which fishes live continuously impact on their homeostatic control systems. Superimposed on this may be the effects of adverse environmental conditions including pollutants and anthropogenic disturbances such as siltation. For fish in artificial conditions, additional stresses may be imposed during handling, transportation, and disease treatment. These disturbances may evoke a variety of changes in the biological condition of the fish that challenge homeostasis and present a threat to fish health. These challenges or stresses can be within the physiological control capacities of the fish and be sub-lethal, or they may require a physiological response in excess of the ability of the fish to accommodate and be lethal or result in disease. Sub-lethal stressors elicit a complex suite of physiological, behavioural, immunological, and pathological changes in fish that can impair vital functions like respiration, blood circulation, osmoregulation and the immune system (Adams 1990). Stress may also alter the metabolic scope of fish (Barton and Schreck 1987) and thereby affect swimming performance, growth and reproductive functions (Barton and Iwama 1997).

In the natural environment the physiological systems of fish must be capable of adjusting to these stressors otherwise the chances of survival would be decreased (Wedemeyer 1996). If the altered physiological state can compensate for the stressor in question, the probability of survival is increased, but often at the expense of non-basal activities like growth and reproduction (Schreck 1981; Davis and Schreck 1997). Interventions are often necessary to reduce the impact of procedures that are linked to aquaculture and fish stock enhancement practices. For example, anaesthetics (Ross and Geddes 1979) and water additives like zeolite (Teo and Chen 1993) and sodium chloride (Forteath 1993) have been used to reduce stress in fish during the performance of many of these procedures. Summerfelt and Smith (1990), however, indicate that anaesthetics may cause considerable stress in fish. It is imperative, therefore, that the effect of anaesthetics on fish physiology is known before they are adopted for use.

Transportation of live fish involves multiple acute stressors that may differ in severity and duration. For example, it has been suggested that the highest levels of stress in fish transport occur during capture and packaging (Robertson *et al.* 1988; Mazur and Iwama 1993). The tolerance of fish to transport conditions might, therefore, depend on the severity of the pre-transport stress. Deterioration of water quality, the level of which might depend on fish density during transport, may also result in considerable stress in the fish. Quantification of the effects of stress is, therefore, desirable in order to rank different transport-related stressors by their importance, and to develop an effective strategy for intervention in the management of stress during fish transport. Physiological responses such as changes in metabolic rate, that can be readily interpreted, should be tested for their potential to measure stress. Furthermore, changes in the metabolic rate of anaesthetized fish might give some indication of the effects of anaesthetics on the physiology of fish.

This chapter provides an overview of the existing literature on the stress response of fish and the various methods used to measure stress in fish. An outline of the role of the stress response in fish physiology, especially as it relates to the bioenergetics of fish, is provided. A review of the use of anaesthetics in stress management during fish transport is presented.

## Stress in fish

Stress has been defined in various ways depending, to a large extent, on the context of the investigation (see review by Adams 1990). Definitions of stress include: the non-specific response of the body to any demand made upon it (Selye 1973); a diversion of metabolic energy from an animal's normal activities (Barton and Schreck 1987); the sum of all the physiological responses by which an organism tries to maintain or re-establish normal metabolism in the face of physical or chemical changes (Wedemeyer and McLeay 1981); alteration of one or more physiological variables to the point that long-term survival may be impaired (Bayne 1985); and the effect of any environmental alteration that extends homeostatic processes beyond their limits (Esch and Hazen 1980). The definitions of Barton and Schreck (1987) and Selye (1973) are of particular significance to this study.

According to the concept of stress proposed by Selye (1973), a stressed organism passes through three distinct response phases viz. alarm, resistance and exhaustion, which he termed the General Adaptation Syndrome (GAS). The alarm reaction is characterized by a rapid physiological response that prepares the organism to fight or flee from the threat. If the organism is not able to escape from the threat, a phase of resistance ensues. During the resistance phase the organism adapts to or compensates for the altered conditions in order

to regain its homeostatic state. This adaptation may entail a reduction in the capacity of the fish to perform normal physiological functions. Where compensation is not possible, a stage of exhaustion is reached that may eventually result in the death of the organism. The importance of the generalized stress response, therefore, depends on the initial stages of the response that acts to promote the best chance of survival in the face of a stressor.

Stress may be acute or chronic. Acute stress responses result from single or several rapid exposures to a stressor, and elicit responses that are akin to the general adaptation syndrome (Schreck 1990). Examples of acute stressors are dip netting, transportation and exposure to toxicants for periods of short duration (Schreck 1990). Chronic stress on the other hand results from either continuous or periodic exposure to low levels of stressors over periods of weeks to years (Schreck 1990). Such exposures include crowded conditions as may be experienced in fish culture, and continuous exposure to toxicants (Heath 1995). A stressor or stress factor is an environmental variable, the challenge of which is sufficiently severe to elicit a compensatory physiological response (Wedemeyer and McLeay 1981). Stressors can be classified into environmental, physical and biological stressors although some stressors may originate from more than one category. Environmental stressors include changes in water quality, whereas physical stressors are those that involve handling, crowding, confinement, transport, or any other form of physical disturbance. Biological stressors can be manifested in dominance hierarchies that develop between individuals within confined spaces such as experimental tanks or in the natural environment (Wedemeyer 1996; Schreck *et al.* 1997). Disease-causing pathogens can also be considered as biological stressors of fish (Iwama *et al.* 1999) although they are generally a manifestation of stress due to increased susceptibility of stressed fish to infectious diseases (Ellis 1981; Angelidis *et al.* 1987; Maule 1989).

The stress response can be classified as primary, secondary or tertiary (Adams 1990), depending on the level of biological organization of the response. The primary or 'alarm' response is an initial response that represents the perception of an altered state and initiates a hormonal response, which forms part of the generalized stress response in fish (Gamperl *et al.* 1994). Cortisol is an essential component of this response (Sumpter 1997; Milligan 1997). The secondary stress response comprises the biochemical and physiological effects that are associated with stress and mediated to a large extent by the 'stress hormones', cortisol and catecholamine. Tertiary or 'whole organism' responses on the other hand, are those responses that are manifest at the level of the whole organism. The stress response begins when stressor stimuli induce an endocrine response in the fish that leads to the GAS response. The GAS response is characterized by rapid changes in the plasma concentrations of catecholamines, primarily adrenaline and noradrenaline, and cortisol (Pankhurst *et al.* 1992; Sumpter 1997). The catecholamines originate from the chromaffin cells scattered throughout the kidneys and the walls of the posterior cardinal veins (Wendelaar Bonga 1993; Sumpter 1997). Various physiological stimuli including acetylcholine, serotonin and altered blood chemistry have been shown to trigger the mobilization of catecholamines (Sumpter 1997). Simultaneous hypothalamo-pituitary-interrenal (HPI) response results in an increase in the levels of circulating plasma cortisol concentration (see review by Sumpter 1997).

The release of the 'stress hormones' initiates a series of responses, the effect of which is the mobilization of energy reserves that are needed for the increased metabolic requirements associated with stress (Jobling 1984). The release of catecholamine results in the optimization of cardiovascular and respiratory functions through changes in blood flow pattern, increased gill perfusion and the oxygen carrying capacity of the blood (Wedemeyer

*et al.* 1990; Thomas 1990; Pickering 1992; Randall and Perry 1992; Jobling 1994; McDonald and Milligan 1997). An increase in the plasma glucose concentration is initiated by adrenaline and maintained by cortisol through the stimulation of gluconeogenesis (Jobling 1994).

Biochemical responses are the earliest indicators of exposure to a stressor (Thomas 1990), and are commonly used to measure the primary and secondary stress response in fish. There are several advantages in using biochemical responses as indicators of stress. Biochemical indicators elicit a rapid response to stimuli and are highly sensitive to sub-lethal stressors (Schreck 1990). The magnitude of the biochemical changes is often related to the severity of the stressor (Barton and Iwama 1991). Furthermore, many biochemical variables show specific responses to certain kinds of stressors, for example heavy metals (Thomas 1990). Most biochemical indices are also easy to measure with standard laboratory equipment (Wedemeyer *et al.*1990), and they can provide both qualitative and quantitative data on sub-cellular and extra-cellular changes in fish. However, the use of biochemical changes to measure stress is limited by the fact that the precise biological significance of the changes for the functional integrity of the organism is often not known. The normal resting values of biochemical parameters for particular fish, and the factors influencing them are also in most cases not known, thus making it difficult to interpret the effect of stress on the fish (Schreck 1990). Nevertheless, biochemical variables whose alterations can be directly related to a physiological function have been used to determine stress effects at the level of the organism. For example, measurement of gill $Na^+$ and $K^+$-ATPase activity has successfully been used to detect osmoregulatory dysfunction of fishes exposed to a variety of physical and chemical stressors (McDonald and Milligan 1997).

Among the biochemical indicators, plasma cortisol is the most commonly used measure of the stress response in fish (Wendelaar Bonga 1997; Mommsen *et al.* 1999). However, not all stressors can be detected by cortisol measurement since only stimuli that cause fright, discomfort, or pain are known to induce the cortisol response (Adams 1990). The complexity of the cortisol response with regard to chronic stressors further complicates the use, and interpretation of cortisol measurements to assess exposure to adverse conditions. For example, Vijayan and Leatherland (1988) observed that under chronic stress plasma cortisol falls back to resting levels even though the stressor is still present.

Other biochemical indicators that are commonly used to measure stress in fish include plasma glucose (Wedemeyer *et al.* 1990) and lactic acid concentration (Reubush and Heath 1996), and liver and muscle glycogen levels (Kindle and Whitmore 1986). Haematological parameters such as the haematocrit and leucocrit values and haemoglobin counts can also be used to measure stress in fish (Murad *et al.* 1990; Barton and Iwama 1991). Plasma glucose concentration is one of the commonly used measurements of the secondary stress effects in fish (Wedemeyer *et al.* 1990). It has been observed that plasma glucose concentration increases after the initiation of stress, but the increase is transient and glucose levels may, or may not, remain elevated in the continued presence of the stressor (Vijayan and Moon 1992, 1994). This is because, like plasma cortisol, the concentration of plasma glucose in circulation is dependent upon the rate of its production and clearance from circulation (Iwama *et al.* 1999). Thus, changes in the rate of clearance of glucose associated with long-term stress can affect plasma glucose concentration. Likewise, the complexity of the glucose response with regard to long-term stressors complicates the use and interpretation of glucose measurements to assess the effects of stress on fish.

The commonly used haematological measure of stress, the haematocrit value, might be considered one of the simplest blood variables that can be measured in fish. However, there is a high variability in its measurements mainly as a result of factors related to the sampling method, which produces a variable amount of stress depending on the degree of handling and exposure of fish to air (Houston 1990; Gallaugher and Farrel 1998). The potential for a large margin of error exists, especially when using the acute sampling method, which is likely to be used when sampling blood from small fish. Other factors which influence haematocrit value and haemoglobin count include short-term physiological influences such as aerobic and burst swimming, exhaustive exercise, hypoxia and exposure to toxicants (Gallaugher and Farrel 1998).

Tertiary or 'whole animal' responses that are commonly used to measure stress in fish include metabolic rate, life-cycle tests such as growth rate, reproductive capacity (Wedemeyer and McLeay 1981), condition indices (Goede and Barton 1990), and disease resistance. Life-cycle tests involve long-term exposure of fish to a stressor to assess its effect on the particular variable. Although regarded as the most sensitive and informative for evaluation of stress in fish (Adams 1990), these tests are not only time consuming but also expensive to conduct. Furthermore, only a limited number of stressors can be assessed using these methods (Heath 1987). Stress is known to increase the susceptibility of fish to diseases (Snieszko 1974) due to the suppression of both the numbers and function of circulating lymphocytes (Ellis 1981). Consequently, lymphocyte counts and lowered disease resistance have been used successfully as indicators of stress in fish (Snieszko 1974; Wedemeyer and McLeay 1981; Angelidis *et al.* 1987). Metabolic rate is probably the least studied of the indicators of stress in fish.

## Fish bioenergetics

Physiological energetics or animal bioenergetics is concerned with the rate of energy intake and transformations within an organism (Brett and Groves 1979; Adams and Breck 1990; Cho and Bureau 1995). It provides the framework for the study of the relationships between physiological processes of the organism and the environment. Metabolism (more specifically catabolism) has been likened to the physiological engine that powers activities such as swimming, growth, reproduction and excretion in which the energy is invested (Brett and Groves 1979; Neill and Bryan 1991). Thus, whereas the influence of the environment is on metabolism, the effects of that influence are displayed through the various activities exhibited by the organism. Metabolic rate is, therefore, a fundamental aspect of 'whole-organism' performance capacity that can be affected by stress.

A stress-induced increase in metabolic rate consumes energy available within the metabolic scope of a fish (Fry 1971; Brett and Groves 1979; Priede 1985); metabolic scope is the power available for activity by fish above the standard metabolic rate (Fry 1971). Priede (1985) and Barton and Schreck (1987) reported that as much as 25-100% of the energy available for activity might be required to cope with stress. Since metabolic rate can be affected by stress, its measurement might provide a way to evaluate the effects of a stressor on the bioenergetic capacity of fish.

The selection of an appropriate indicator to measure stress depends on the kind of stressor to be evaluated. The qualities to be considered when selecting an appropriate stress indicator must, however, include sensitivity to the stressor, ease and accuracy of measurement, and the biological significance of the changes (Thomas 1990). An indicator

that incorporates several levels of biological organization will be better placed than one that is based on a single level of organization as the appropriate measure of stress. Changes in metabolic rate are a culmination of many processes involving several levels of biological organization. Metabolic rate changes can also be readily interpreted in terms of the potential long-term effects on the organism (Barton and Iwama 1991). Homeostatic costs are assumed to be higher in stressed than non-stressed fish (Schreck 1990). Consequently, the data on the metabolic stress response can be related to the severity of the stressful situation, thereby enabling the quantification of the effects of the stressor. Metabolic rate, thus, presents an opportunity to measure the stress response in fish.

Although attempts have been made to quantify the effects of stress using metabolic rate (Barton and Schreck 1987; Davis and Schreck 1997), the usefulness of metabolic rate as a measure of the stress response has not been evaluated. A comparison of the metabolic stress response with the cortisol stress response could enable the validation of the use of metabolic rate to measure stress in fish. The changes in the metabolic rate of fish during the stress response might help predict the effect of a stressor on the physiological state of fish. A comparison of the changes in metabolic rate during the stress response might also enable the quantification of the effects of different stressors on fish bioenergetics.

## The use of anaesthetics in fish transport

The growing demand for fishery products has placed pressures on natural aquatic resources (Bartley 1999). Consequently, aquaculture and fish stock enhancement practices are increasingly being adopted as a means to restore, or increase aquatic production (Bartley 1999; Kitada 1999; Leber 1999). Transportation of fish is a pivotal aspect of

these practices since the juvenile fish required for stocking purposes are usually produced in hatcheries, which are often located at considerable distances from the stocking sites. Recent increases in the ornamental fish trade (Wood 1995; Lim and Chua 1993; Bassleer 1994; Chapman *et al.* 1997), and in the trade in live food fish and crustaceans (Forteath 1993; Bradley 1998) are further indicators of the importance of fish transportation. Although deterioration of water quality may result in stress in fish, capture and handling, and crowding during transportation are probably the most severe factors in terms of stress (Robertson *et al.* 1988; Mazur and Iwama 1993). The tolerance of fish to transport conditions, therefore, depends on these factors (Barton *et al.*, 1980; Schreck 1981). Alleviation of the effects of capture and handling, and crowding might result in better post-stress survival rates.

Different strategies have been used to alleviate the effects of stress in fish during transport (Amend *et al.* 1982; Lim *et al.*1993; Teo and Chen 1993; Chow *et al.*1994). These include the fasting of fish prior to transportation, application of anaesthetics, and use of water additives, e.g., zeolite for freshwater fishes, and the reduction of water temperature during transport. Starvation of fish is primarily intended to reduce fouling of water from faecal waste and metabolic end products such as ammonia. The reduction of water temperature, or hypothermia, reduces fish activity and, thereby the rate of oxygen consumption and accumulation of ammonia (Forteath 1993). In addition, low temperature favours higher concentration of oxygen in the water (Jobling 1994). Whereas starvation of fish prior to transportation is an established practice, reduction of temperature may not be beneficial in transporting warm water fish. Mild anaesthetization is commonly used to minimize injury to fish during capture and handling and to reduce stress in the fish during transport.

Anaesthetics are also reported to reduce the rate of fish metabolism and thereby reducing metabolic waste production (Ross and Ross 1984; Wedemeyer 1996).

A wide range of anaesthetics is used in fisheries and aquaculture. These include Tricaine methanesulphate (MS-222), Quinaldine, Benzocaine, Etomidate, Metomidate, 2-phenoxyethanol and clove oil (Ross and Ross 1984; Marking and Meyer 1985; Gilderhus and Marking 1987; Iwama *et al.* 1989; Summerfelt and Smith 1990; Malmstrom *et al.* 1993; Cho and Heath 2000). However, anaesthetic procedures are suspected of causing undesirable side effects as well as stress in fish (Summerfelt and Smith 1990). The efficacy and safety of various drugs used to sedate fish also varies between species (Ross and Ross 1984; Summerfelt and Smith 1990). Knowledge of the efficacy of a given anaesthetic and its ability to reduce oxygen consumption and stress during fish transport is, therefore, a prerequisite to its application. 2-phenoxyethanol has lately come into much use in fish transport. However, information on its efficacy and safety is inconclusive due to the differences reported concerning its effective dose (Weyl *et al.* 1996; Deacon *et al.* 1997) and safety margin (Summerfelt *et al.* 1990). A study of the effects of this anaesthetic might shed some light on the effects of anaesthetics on fish. Knowledge of how metabolic rate and cortisol levels are affected by the application of 2-phenoxyethanol might enable the assessment of the effects of the anaesthetic on the stress response.

Based on the foregoing observations from the available literature, the following hypotheses were proposed:

1. That changes in metabolic rate can indicate the existence and magnitude of stress in fish,

2. That 2-phenoxyethanol can reduce the effects of stress on fish.

Juvenile spotted grunter, *Pomadasys commersonnii*, a sub-tropical marine fish was used as the model species to test these hypotheses. *P. commersonnii* is a shallow water Indo-Pacific fish (Van der Elst 1981) of economic importance in Southern Africa, and is also a candidate aquaculture species (Deacon and Hecht 1995).

## Aims and objectives

The objectives of this study were:

- To investigate the suitability of metabolic rate measurements to assess the stress response of juvenile *P. commersonnii*,
- To evaluate the effects of selected transport-related stressors on the stress levels in this species using metabolic rate as the stress indicator, and
- To investigate the effects of 2-phenoxyethanol on the stress response and to evaluate its suitability for the management of transport-related stressors.

Essential to this study was the estimation of the metabolic and corticosteroid stress response of juvenile *P. commersonnii*. As a prerequisite to the evaluation of the stress response, the primary task in this study was to determine baseline metabolic rates and plasma cortisol levels in the species. These baseline measurements would enable the evaluation of the magnitude of the stress response in the fish. A comparison of the magnitude of the metabolic stress response was expected to provide a way to evaluate the effects and relative importance of different transport-related stressors. Finally, the study aimed at making a

contribution towards understanding the physiological effects of 2-phenoxyethanol on juvenile marine fish.

## Thesis outline

This study was premised on the hypotheses that changes in metabolic rate could indicate the existence and the magnitude of stress in fish, and that appropriate application of 2-phenoxyethanol could alleviate the effects of the stress. A description and critical evaluation of the methods used in the determination of metabolic rate and plasma cortisol concentration is given in Chapter 2. The intermittent respirometry method and the procedures used to measure oxygen consumption, as well as the validation of the respirometer performance, are described. The experimental fish species is also described in this chapter. Before measuring the stress response, it was necessary to establish baseline metabolic rates to enable the assessment of the magnitude of the response. The baseline metabolic rates, and the effects of diel rhythmicity on their determination, are evaluated and discussed in chapter 3.

A primary objective of this study was to evaluate the suitability of metabolic rate to assess the stress response in fish. Essential to this evaluation was the generation of a metabolic and a cortisol response profile. Chapter 4 compares the metabolic stress response and the corticosteroid stress response in juvenile *P. commersonnii*, as a means to validate the former as an indicator of stress in fish. The evaluation of metabolic rate as a stress indicator also required the practical demonstration of its usefulness in measuring stress. This necessitated that metabolic rate be applied in an attempt to evaluate the effects of selected transport-related stressors. Chapter 5 demonstrates the use of metabolic rate in the

quantification of the stress response in fish, and in the evaluation of the effects of different stressors on stress levels in the fish. Using metabolic rate measurements it was possible to distinguish between different acute stressors, and to measure the relative metabolic costs of the stressors to the fish.

The other objective of the study was to investigate the effect of 2-phenoxyethanol on the stress response, and to evaluate the suitability of the anaesthetic for the management of stress in fish. By subjecting juvenile *P. commersonnii* to capture and handling stress and subsequently administering 2-phenoxyethanol it was possible to assess the effect of the anaesthetic on the metabolic stress response of the fish. The results obtained in this study are presented and discussed in Chapter 6. In the concluding chapter (Chapter 7), a comparison is made between the metabolic and the corticosteroid stress responses. The overall effectiveness of metabolic rate as an indicator of stress in fish is presented and discussed. A historical overview of the different methods used to measure stress in fish is presented and discussed to justify the choice of metabolic rate as a measure of stress in juvenile fish.

# CHAPTER 2

## Materials and methods, and a critical assessment of methods used to estimate metabolic rates in fishes

### Introduction

Metabolic rate of fish is usually determined by measuring oxygen consumption. Oxygen consumption in aquatic organisms, including fish, is commonly measured indirectly by respirometry, which quantifies how rapidly oxygen, and by extension, energy is used by the fish. The design of a particular respirometer involves a compromise between sensitivity, time resolution of oxygen uptake, level of external interference, and ease of construction and operation (Gnaiger 1989). The sensitivity of a respirometer and its response time depends mainly on the measuring equipment such as electrodes, pumps and thermostats (Kaufmann *et al.*1989). It is known that the reaction of a fish to respiratory chamber geometry and size and to small variations in the experimental set-up can result in widely differing rates of oxygen consumption under otherwise identical conditions (Fry 1971). For instance, Cech (1990) observed that a large chamber volume to fish size ratio results in a rate of change of oxygen concentration too low for accurate measurement, while too small a ratio results in an elevated oxygen consumption rate due to stress. Consequently, experimental chambers are tailored to suit not only each specific problem, but also different sizes and species of fish, taking into consideration their behaviour and general biology. Thus, most investigators construct their own respirometers depending on the problem under investigation, the level of accuracy desired and the cost of construction.

When long periods are taken to measure oxygen consumption, it is likely that some spontaneous activity might occur, thereby causing fluctuations in the oxygen consumption rate. These fluctuations in measurements might complicate the analysis of the results. Continuous recording of oxygen depletion using Polarographic Oxygen Sensors (POS) helps in interpreting the results obtained from such experiments and are thus an essential component of the respirometer. An ideal POS is a compromise between the speed of response and high sensitivity on the one hand, and stability and low stirring requirement on the other (Gnaiger 1983). Because all applications of POS require stability, calibrations are often necessary to ensure the required precision. The drift of steady-state sensitivity (see Gnaiger 1989) as a result of short-term transient destabilization following any major change in operating conditions, such as change of membrane or temperature, can be easily controlled by waiting for a certain predetermined time before resuming measurements (Hale 1983). Once stabilized, however, a POS settles to a level, which often appears to be constant, but which can drift noticeably over time (Hale 1983). This drift must of necessity be minimized for greater accuracy of the measurements. This may be done through calibrations made at the beginning and at the end of a series of measurements (Gnaiger 1983).

## Respirometer design

Aquatic respirometers are characterized by a delayed response time (Kaufmann *et al.* 1989). Specific delays of the recorded signals are due to the sensor's inertia and to mixing effects within the experimental chamber (Gnaiger 1983). As a result, a respirometer takes some time to equilibrate following introduction of fish or changes in operation. Consequently, an effective mixing of the medium must be facilitated to enable a

homogenous distribution of dissolved oxygen within the measuring chamber and to counter the delays in recorded signals as a result of mixing effects.

Respirometers are classified as either closed system respirometers, in which the time course of oxygen concentration in the water is measured in a closed chamber, or flow-through system respirometers, in which water flows continuously through the respiratory chamber (Gnaiger 1983; Kaufman *et al.* 1989; Steffensen 1989; Wedemeyer *et al.* 1990). In the flow-through system, oxygen consumption is calculated according to the Fick principle (see Steffensen 1989), from the difference in oxygen content between the inflow and outflow and the flow rate. The advantage of this system is the maintenance of stable chemical conditions within the system (Kaufman *et al.* 1989; Steffensen 1989). Flow-through systems can also make it easier to detect and monitor phasic responses of fish to short-term manipulation of input variables. Factors that complicate oxygen consumption measurement using this system are the accumulation of baseline errors that necessitate frequent calibrations, and the effects of dilution rate.

In closed systems, the rate of change in the oxygen content of the water is measured in a closed chamber containing the experimental fish. The changes in the dissolved oxygen concentration in the water, therefore, reflect the rate of oxygen consumption of the fish. Oxygen consumption is calculated according to the formula:

$$mo_2 = V_r * \delta Cwo_2 / \delta t * W$$ (Steffensen 1989), where:

- $mo_2$ is the oxygen consumption rate in mg of oxygen per gram wet weight of fish per hour (mg $O_2$ $g^{-1}h^{-1}$)

- $V_r$ is the volume of the respiratory chamber in litres (L)
- C is the dissolved oxygen concentration in the water in mg per litre (mg $l^{-1}$)
- $\delta Cwo_2$ is the difference in the oxygen concentration of the water at the beginning and end of a measurement
- $\delta t$ is the duration of the measurement
- W is the wet weight of fish (g).

The major disadvantage of closed system respirometers is the reduction in levels of dissolved oxygen and the accumulation of waste products that limit its application for long-term studies. However, since the decrease in oxygen concentration represents the actual amount of oxygen consumed, this disadvantage may be turned into an advantage in experiments where the reduction in oxygen concentration and the accumulation of metabolic waste products are part of the problem to be studied. The reduction in dissolved oxygen concentration with time is suitable for the study of metabolic rate in juvenile fish since it involves the measurement of oxygen uptake by the fish, with the concomitant decrease in dissolved oxygen concentration in the water. The system can also allow introduction of anaesthetics when required.

Closed system respirometers can further be classified as static, closed recirculating or swimming respirometers. Static respirometers contain a noncirculating, fixed volume of water, whereas swimming respirometers contain a closed volume of water that is circulated past the test fish inducing it to swim. The objective behind the swimming respirometer design is that the experimental fish maintains its position in the flow chamber by swimming against the induced water currents at a speed equivalent to the velocity of the water. By forcing fish to swim at different velocities and subsequently extrapolating to zero velocity,

it is possible to estimate the standard metabolic rate of the fish (Brett 1964). A fundamental requirement of the swimming respirometer is that the water current that is induced in the chamber must be accurately measured, regulated and maintained throughout the measurement period (Steffensen 1989). This is disadvantageous considering the requirement for regular calibration of the flow meter (Bell, unpublished) and or regulation of the water pump for optimal operation and accuracy (Gnaiger 1983). Additionally, the pump itself may impart stress on the fish by the pressure pulses and noise it generates. Because of the large volume of water associated with swimming respirometers, they are mainly used to measure the standard and active metabolic rates of large fish.

A third type of respirometer, the intermittent flow respirometer is a compromise between continuous flow respirometers and closed system respirometers. Such a system retains some of the simplicity and low cost of closed system respirometers, without limiting the duration of the experiment due to depletion of oxygen (Quetin 1983). The objective behind the design of intermittent systems is that the respiratory chamber is flushed periodically between oxygen measurements and fresh oxygenated water introduced into the chamber before repeating the measurements. By flushing the chamber at defined intervals the concentration of dissolved oxygen is maintained at non-stressful levels, and accumulation of waste products is reduced (Kaufmann *et al.* 1989). Experimental procedures that were used during this study, such as the transfer of fish into, and their confinement in the respirometer can result in a transient increase in oxygen consumption rate (Schurmann and Steffensen 1997). It was expected, therefore, that the fish would require adequate time to acclimate to the respirometer before oxygen consumption could be measured. Metabolic rate measurements would also be made over a prolonged period of time during which it would be necessary to maintain dissolved oxygen concentration above a certain level. A

supply of oxygenated water would be required during this period. Using the intermittent respirometer, it would be possible to conduct prolonged metabolic rate measurements while maintaining the dissolved oxygen concentration above a designated level. Because of these advantages and potential, a partially closed recirculation system with an intermittent respirometer was used to measure oxygen consumption rate in this study.

The primary objective of this chapter is to present a critical evaluation of the different methods that are commonly used to measure oxygen consumption in fishes. This chapter also aims at describing the experimental system and the methods that were used to estimate metabolic rate, and to describe the fish species used in this study. An overview of the closed system and the flow-through system respirometers and their advantages and disadvantages is presented. A description of the intermittent system respirometer that was used in this study is also given. The general methods that are applied in the measurement of oxygen consumption are described in this chapter. However, specific methods that were used to estimate the changes in metabolic rate resulting from specific experimental conditions are described in the relevant chapters.

## The experimental system

The partially closed recirculating respirometer system used in this study (Fig. 2.1), consisted of a reservoir tank, an overhead tank and a water bath containing a respiratory chamber and was situated in a controlled temperature room. A submersible water pump (Nocchi Vipvort 180/6) pumped seawater from the reservoir tank to an overhead tank from where it flowed by gravity to the water bath. Water was recirculated through an overflow outlet near the top of the water bath, which also ensured a constant water level in the tank.

The respiratory chamber (Fig 2.2) was connected to the recirculation system through a 5 mm diameter plastic tube. A clamp attached to the tube was used to control the water flow. Closing down this clamp converted the respirometer into a closed system. Submersible heaters located in each tank maintained a constant temperature.

One of the conditions for determination of metabolic rate is that the experimental fish should be able to swim freely in the respiratory chamber and be protected from external disturbance. It should also be free of the effects arising from the transfer into the respiratory chamber. Consequently, the tank containing the respiratory chamber was shaded with dark polythene paper to protect the fish from visual disturbances. Only the side of the tank from which the experimenter operated was shaded, thus minimising changes in the lighting conditions. Different sizes of volumetric flasks that allowed free movement of the experimental fish were used to measure oxygen consumption of different sizes of fish. A 350-ml flask was used to measure oxygen consumption of fish weighing less than 6 g whereas a 690-ml flask was used to measure oxygen consumption of larger (6 – 30 g) fish. A rubber stopper was used to seal the respirometer. Vaseline jelly was used to make the chamber airtight. A small diameter inlet and outlet hole in the rubber stopper enabled the flow of water in and out of the chamber. By boring through the rubber stopper with a hypodermic needle a syringe access was created to facilitate the addition of anaesthetic into the respiratory chamber. An Oxyguard Gamma oxygen meter (Oxyguard International A/S, Denmark) was used to measure dissolved oxygen concentration. An electric mechanical stirring device was incorporated in the chamber through the stopper. The mechanical stirrer ensured homogenous distribution of dissolved oxygen within the chamber and maximum sensor activity. Although the stirrer could generate some level of stress, this was minimal and constant for all the test fish.

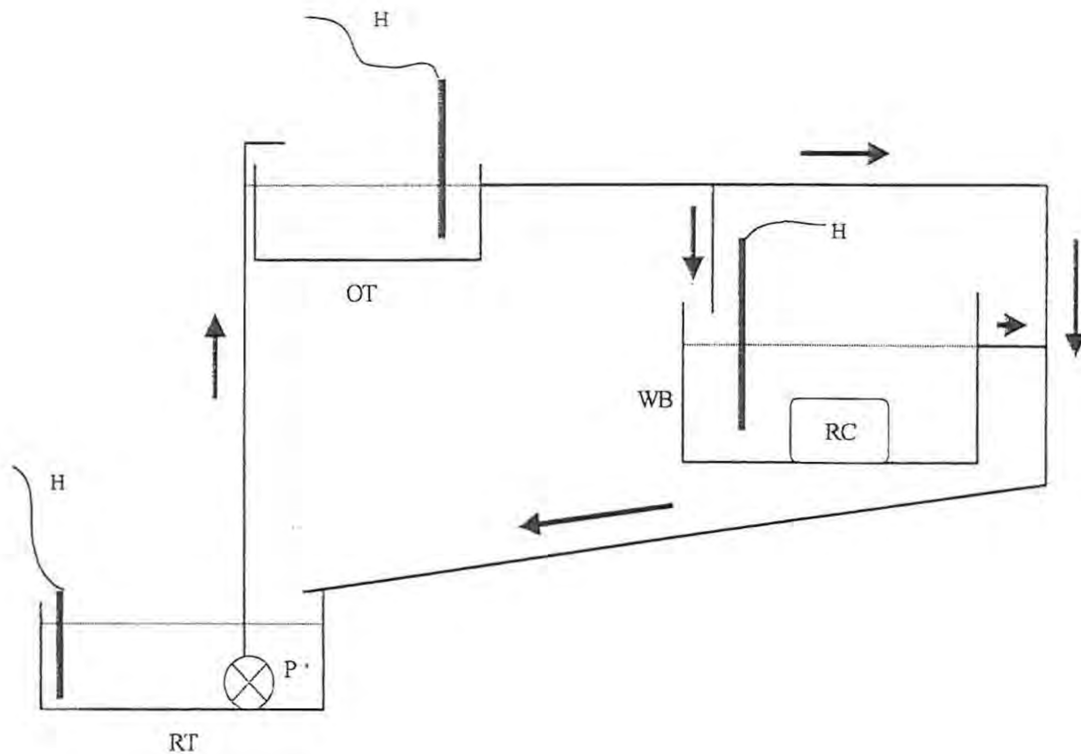


Figure 2.1. The partially closed recirculation system used to measure oxygen consumption of juvenile *P. commersonnii*. The system consisted of a reservoir tank (RT), from which water was pumped by a submersible pump (P) to an overhead tank (OT). Water from the overhead tank flowed to a water bath (WB) housing the measuring chamber. An outlet near the top of the water bath allowed the recirculated water back to the reservoir tank. A thermostatic heater (H) in each tank controlled the water temperature.

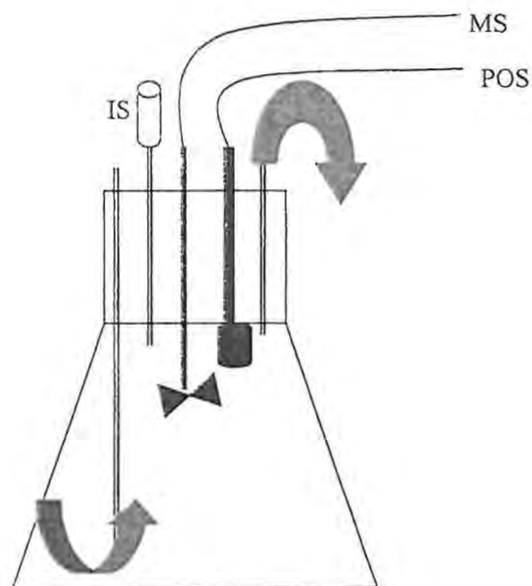


Figure 2.2. The respirometer used for measuring oxygen consumption. The respirometer consisted of a glass flask with a mechanical stirrer (MS), a Polaroid oxygen sensor (POS) and an injection syringe (IS). The arrows indicate the direction of water flow through the measuring chamber.

## Experimental fish

| | |
|---|---|
| Order: | Perciformes |
| Family: | Haemulidae |
| Genus: | Pomadasys |
| Species: | *Pomadasys commersonnii* (Lacepede, 1802) |
| Common names: | Spotted grunter |

All experimental work was undertaken on the spotted grunter, *Pomadasys commersonnii* (Haemulidae), a candidate species for aquaculture in South Africa. *P. commersonnii* is a shallow water (< 30 m) Indo-Pacific fish, which inhabits the warmer waters of temperate and tropical coasts (Van der Elst 1981). It is considered a potential aquaculture species in South Africa (Deacon and Hecht 1995). Along the East Coast of southern Africa it is most commonly found off sandy beaches and in estuaries (Wallace *et al.* 1984). The spotted grunter spawns at sea during late winter and the juveniles recruit into estuarine nursery areas at less than 50-mm total length during summer (Wallace 1975). Newly recruited juvenile spotted grunter are pelagic and feed mainly on copepods and mysids, whereas more mature stages feed on benthic organisms (Whitfield 1998; Hecht and Van der Lingen 1992). The fish migrate back to the sea after about one year where they mature at approximately 30-40 cm total length. However, fish of up to 87 cm have been recorded (Van der Elst 1981). *P. commersonnii* is euryhaline and can be found at salinities ranging from 0 to 90 ppt (Whitfield 1998). The thermal preferendum of the fish lies between 24 and 25 $^{\circ}$ C (Deacon and Hecht 1995). The spotted grunter has also been shown to cope with high water turbidity (Hecht and Van der Lingen 1992).

Juvenile *P. commersonnii* were obtained by beach seine netting from the Great Fish River estuary (33$^{\circ}$ 30'S; 27$^{\circ}$07'E) and acclimated to captive conditions for a period of 30 days at

the field station of the Department of Ichthyology and Fisheries Science (DIFS) in Port Alfred. At the field station the fish were kept in 300-l rectangular tanks linked to a 4000-l partially recirculated seawater system under natural light and temperature conditions. They were fed to satiation once a day on a diet of fresh chopped squid. After the period of acclimation, experimental fish were divided into two groups. One group of fish was used to measure oxygen consumption rate, whilst the other group was stocked in grow- out cages in a sheltered part of the Kowie estuary in Port Alfred for later experimentation. When required, fish were moved to the Grahamstown laboratory where all experiments were carried out. At the Grahamstown laboratory, the fish were sub-divided into different groups and were kept in separate 300-L holding tanks within a 4000-L recirculating system. Seven days before experimenting fish from a selected holding tank were moved into the controlled-temperature room where all oxygen consumption measurements were conducted. A 12L: 12D photoperiod was maintained throughout the acclimation period and during the experiments. Table 2.1 summarises the procedures for the capture, transportation and preparation of experimental fish. For the duration of the experiment the fish were kept in 90-L shaded glass aquarium tanks (10 fish / tank) with *in situ* box-type gravel filters. Water temperature was kept at $25 \pm 0.7$ ° C and the fish were fed on fresh chopped squid throughout the test period. Ammonia and nitrite levels were kept below 0.02 mg $l^{-1}$ by exchanging 20 % of the water daily, and removing uneaten food immediately after feeding the fish.

Table 2.1 Summary of the procedures and duration for the capture, transportation and preparation of experimental fish.

| Process | Procedure | Duration |
|---|---|---|
| Fish capture | Beach seine | Various intervals during a 30 day period (March - April 2000) |
| Transportation to Port Alfred | Road transport | < 30 min |
| Holding in Port Alfred | - Tank acclimation<br>- Cage culture | 30 days<br>6 - 12 months |
| Transportation to Grahamstown | Road transport | < 60 min |
| Holding in tanks | - | > 7 days |
| Acclimation in temperature controlled room | - | 7 days |
| Acclimation in respirometer | - | > 12 h |

## Measurement of oxygen consumption rate

To measure oxygen consumption rate individual *P. commersonnii* were weighed before introduction into the measuring chamber where they were acclimated overnight. Experimental fish were not fed for 24 h prior to the introduction. After introducing the fish into the respirometer chamber, the chamber was sealed under water with a rubber stopper. Vaseline jelly was spread around the chamber opening as an extra measure against uncontrolled gaseous exchange during the measurements. The recirculation system was left in the flow mode at a flow rate of 0.1 l $min^{-1}$, the theoretical water exchange rate, throughout the night. After the overnight acclimation water flow through the unit was

turned off and dissolved oxygen concentration was measured for the period of time specific to the respective experiments.

Oxygen consumption of *P. commersonnii* was measured during a series of experimental trials. An experimental trial was designed to obtain oxygen consumption measurements from either a single fish (Chapters 3 – 6) to estimate the metabolic rate of each fish, or a group of fish (Chapter 5) to study the effect of fish density on oxygen consumption rate. A measurement period was designated as the duration of one trial, and lasted between 1 h and 24 hours. The measurement period was further divided into measurement intervals, which defined the frequency of oxygen consumption measurement. Two measurement intervals were adopted. They were half-hourly intervals for measurement periods lasting up to 6 h and hourly intervals for measurement periods lasting for 24 hours. At each interval, oxygen measurements were recorded every 15 seconds for up to 20 minutes. The high frequency of data collection enabled detection of any abrupt increases in oxygen consumption rate, such as may occur during sporadic movement that could affect the interpretation of the results. One experimental trial was considered a replicate within one experiment. For each experiment five trials, representing five replicates were conducted with five different fish.

For purposes of analysis, the data obtained during the 20-min measurement duration was divided into five-minute intervals consisting of 20 measurements each. Only the data collected during the last five minutes were used to determine the oxygen consumption rate for each fish. This was done to pre-empt any variations in oxygen consumption rate that might occur as a result of the interruption of water flow while flushing the respirometer chamber. Oxygen consumption rate was calculated according to the formula:

$$\dot{m}o_2 = V_r * \delta Cwo_2 / \delta t * W$$ (details given above)

The overall oxygen consumption rate of a fish was calculated using the mean (per interval) oxygen consumption measurements for the five replicates. A summary of the procedure used to estimate the metabolic rate is given in Fig. 2.3. Unless otherwise specified, dissolved oxygen levels in the water were kept above 4 mg $O_2$ $l^{-1}$ during all experiments. Details of each experiment are described in the relevant chapters. Also unless otherwise stated, all variations reported in this thesis are standard deviations from the mean.

## Validation of respirometer performance

An Oxyguard Handy Gamma (Oxyguard International A/S, Denmark) oxygen meter was used to measure dissolved oxygen concentration. This oxygen meter has a response time of less than 10 seconds, an error of less than 1% of reading ± 1 digit, depending on calibration, and a repeatability of ± 0.5% of the measured value within a range of 0 - 50 mg $l^{-1}$ dissolved oxygen concentration and 0 - 600% saturation. After constructing the respirometer, five experimental trials were conducted using a blank chamber to test its stability and to estimate the amount of oxygen that could be lost from the system due to bacterial metabolism. To accomplish this, the same procedures as outlined above were followed during the blank trials. The measuring chamber without fish was connected to the recirculating system the evening before the experiment. The chamber was sealed under water and Vaseline jelly spread around the chamber opening to prevent infusion of oxygen during the measurements. The recirculating system was left in the flow mode throughout the night. At the beginning of the experimental trial water flow through the chamber was turned off and dissolved oxygen measurements were recorded after every five minutes for a

period of 1 h, a duration equivalent to three times the 20-min measurement duration required for determining oxygen consumption. An equation to calculate the system drift was developed as follows:

$$\text{Drift} = \delta Cwo_2 \, / \, Cwo_2 \; * \, 100; \text{ where:}$$

- Drift is the amount of oxygen lost from the system due to systematic (POS) and non-systematic (biological) reasons
- $Cwo_2$ is the dissolved oxygen concentration in the respiratory chamber at the beginning of the measurement
- $\delta Cwo_2$ is the difference between the initial dissolved oxygen concentration and the concentration at the end of the measurement period. The drift is expressed as a percent of the mean of five trials.

At 25 $^{o}$ C, the average dissolved oxygen concentration in the water was 6.2 mg $l^{-1}$. Drift accounted for 0.1 mg $O_2$ over a period of six hours. Using the above formula, the 'oxygen loss' as a result of the effects of drift, bacterial activity, or other system artefacts was less than 1.6 %. This loss was, however, compensated for by the short experimental run-time (max. = 20 min). In addition, fish were placed into the respiratory chamber at least 12 h before the experiment to allow enough time for respirometer stabilization.

It was therefore concluded that the effects of drift, bacterial activity, and other system artefacts on the sensitivity and stability of the respirometer were negligible. Any delays in temporal resolution were overridden by a protracted measurement period, which allowed any changes in signal transmission to be picked up as increases or decreases in dissolved

oxygen measurements over the measurement interval. The fish were allowed to acclimate in the respirometer for at least 12 h before the measurements, and thereby minimizing the effects of random movements of the fish. The fast response time of the oxygen meter and the good repeatability of measurements assured acceptable levels of accuracy in the measurements of dissolved oxygen concentration.

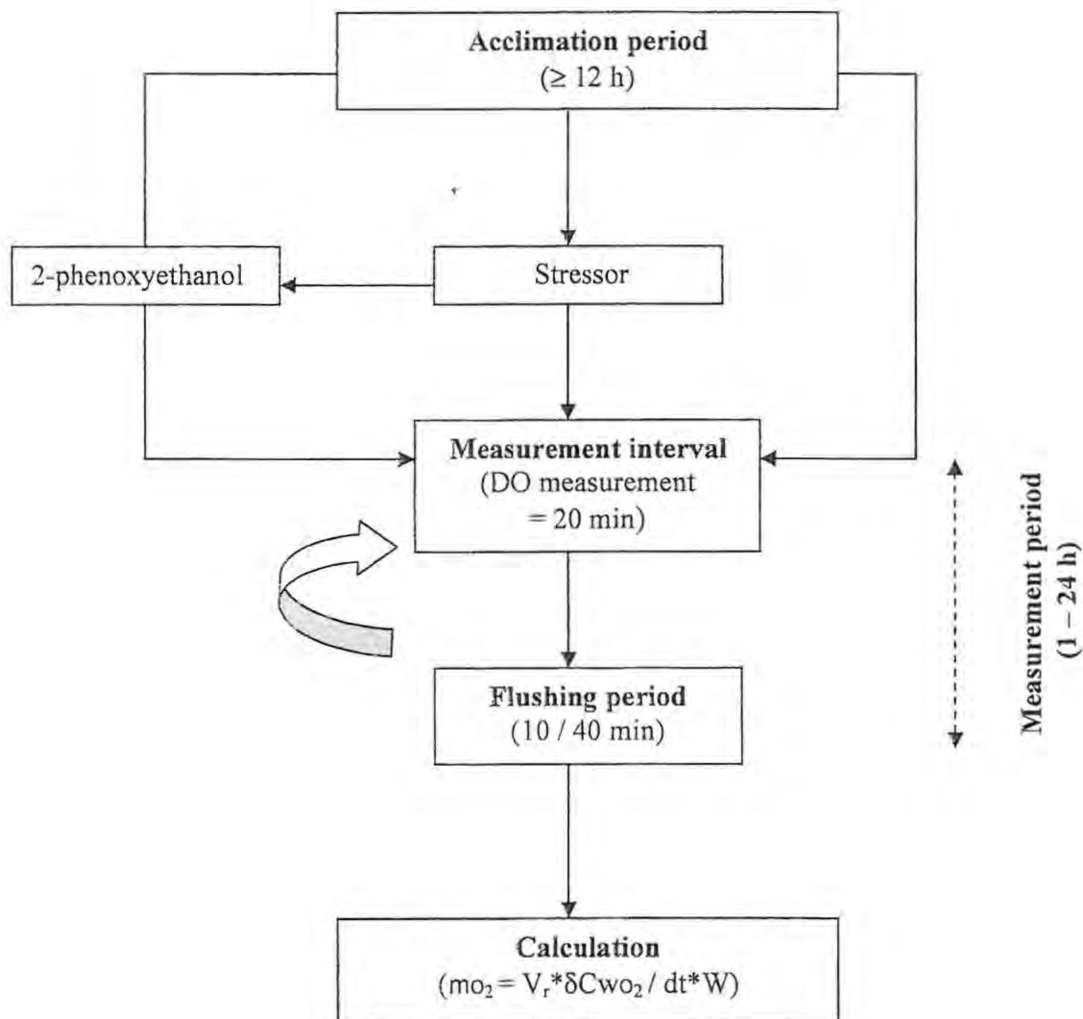


Figure 2.3. Summary of the procedures used to measure oxygen consumption rate of juvenile *Pomadasys commersonnii*. Oxygen consumption was calculated using the measurements recorded during the last five minutes of the 20-min measurement interval.

# CHAPTER 3

## Baseline metabolic rates of juvenile *P. commersonnii* and the effect of diel rhythmicity on oxygen consumption rate

### Introduction

Measurement of metabolic rate is a direct way to evaluate the effects of stressors on the bioenergetic capacity of fish. The establishment of baseline metabolic rates of a species is, therefore, a prerequisite to evaluating the effects of any stressor. The metabolic rate of fish is commonly measured by determining oxygen consumption rate. However, wide variations in oxygen consumption may be obtained for fish in the same state of physical activity (Fry 1971). For example, Fry (1971) observed that the metabolic rate of a resting fish ranged between its standard metabolic rate to over a half of its maximum metabolic rate. Metabolic rate is also sensitive to factors such as excitement, fish size, and temperature (Brett and Groves 1979). Although the tunnel respirometer has enabled the estimation of the standard and active metabolic rates in large fish (Brett 1964), variations in oxygen consumption by individual fish may constitute a major problem in the estimation of baseline metabolic rates of juvenile fishes. Another problem in measuring oxygen consumption in fish is related to the phenomenon of circadian rhythmicity. Most fish show a cyclic pattern in daily activity levels that are related to a periodically changing environment. Du Preez *et al.* (1986) and Deacon and Hecht (1995) observed rhythmic patterns in routine oxygen consumption and physical activity of *P. commersonnii*. Earlier work by Brett (1964) demonstrated a good correlation of oxygen consumption with the level of physical activity of young sockeye salmon. A review of biological rhythms in fish

by Schwassmann (1978) demonstrated a daily periodicity in oxygen consumption of largemouth bass (*Micropterus salmoides*). Because of the diel fluctuations in activity levels, measurements of metabolic rate may vary for the same fish depending on the time of day at which the measurements are taken. Oxygen consumption measurements taken during the low activity period might reflect lower metabolic rates, whereas measurements taken during the peak activity period might reflect relatively higher metabolic rates for the same fish. Establishing a suitable sampling time that takes into consideration the circadian rhythm in oxygen consumption is, therefore, essential in order to maintain uniformity in the measurement of oxygen consumption rates.

The term metabolism refers to all processes by which food energy is converted to become available for activity (Fry 1971). Such activities include locomotion, growth, reproduction and excretion. Metabolism can be separated into standard, routine and active metabolism depending on the level of activity of a fish. Standard metabolic rate (SMR) is the minimum level of energy required to meet the maintenance needs of a fish (Brett and Groves 1979). Standard metabolic rate is regarded as the minimal maintenance or resting metabolic rate of a fish below, which physiological functions are impaired (Priede 1985). Fry (1971) equates standard metabolic rate to an approximation of the minimum rate of metabolism, as measured in a temperature-acclimated undisturbed fish in a post-absorptive state, and at complete rest. Active metabolic rate (AMR) refers to the maximum rate of metabolism of a fish swimming steadily at a maximum sustained speed, whereas routine metabolic rate (RMR) is the mean rate of metabolism of fish exhibiting random movements (Fry 1971; Brett and Groves 1979). Routine metabolic rate is a reflection of the degree to which the fish responds to the directive effects of the environment. A directive factor exerts its effect on the organism by stimulating a

transductive response (Fry 1971). For example, signals such as photoperiod elicit a hormonal change that might prepare the organism for imminent events related to day length (Liley 1971). Routine metabolism reflects the energy requirement of a fish in an environment with a minimum of stressors. A fish exhibiting routine metabolism may, therefore, be considered to be in a non-stressed state. Consequently, RMR was used to assess the magnitude of the stress response in juvenile *P. commersonnii*.

Fish metabolism is primarily a function of water temperature and body size (Fry 1971; Brett and Groves 1979), but it can also be affected by water quality variables such as dissolved oxygen (Fry 1971) and ammonia levels (Neill and Bryan 1991). Generally, large fish consume more oxygen than small fish (Joling 1994). However, per unit body weight, small fish consume more oxygen than larger ones. The relationship between metabolism and fish weight can be described by the allometric equation, $mo_2 = aW^b$ (see Fry 1971), where $mo_2$ is the rate of metabolism (expressed as oxygen consumption rate), W is the body weight, and a, b are constants. According to this equation metabolic rate per unit body mass decreases with increasing fish weight. The decline in oxygen consumption rate with increasing fish weight has been attributed, in part, to changes in the relative sizes of different organs (Oikawa *et al.* 1991; Jobling 1994). The decline might also result from a reduction in the metabolic intensities of different body organs with age (Jobling 1994). The magnitude of the decline has been attributed to interspecific differences (Fry 1971). Thus, fish size must be taken into consideration when comparing metabolic rate within, and between species.

The first objective of this experiment was to study the patterns in oxygen consumption rate of juvenile *P. commersonnii* and to establish a suitable time for taking oxygen

consumption measurements. The second objective was to establish baseline metabolic rates including the standard, active and routine metabolic rate of this species, that are necessary for assessing the stress response. The effect of fish size on the metabolic rate of juvenile *P. commersonnii* is also examined, with the aim of presenting species-specific coefficients for the metabolic rate equation.

## Materials and methods

### Patterns in diel oxygen consumption rate

Continuous oxygen consumption measurements were performed over a 24-h period to search for patterns in diel oxygen consumption rate and to determine the most suitable time for measuring oxygen consumption in *P. commersonnii*. Juvenile *P. commersonnii* were obtained from the Great Fish River estuary and acclimated in Port Alfred for 30 days before experimentation (see Chapter 2). After acclimation to captive conditions, experimental fish were transported in 300-L cylindrical tanks to the laboratory in Grahamstown where they were acclimated in an indoor water recirculating system for at least 7 days. Ten fish (wt = 14.5 ± 1.5 g) from this batch were used to determine the patterns in diel oxygen consumption rate. Oxygen consumption was measured separately for each fish following the protocols outlined in Chapter 2. Individual fish were selected at random and weighed before introduction into the measuring chamber where they were acclimated for at least 12 h. The fish had not been fed for 24 h prior to the introduction. After the 12 h overnight acclimation period, dissolved oxygen concentration was measured at hourly intervals for 24 hours. At each interval, oxygen measurements were recorded every 15 seconds for 20 minutes. To determine the hourly oxygen consumption

rate, the measurement period was further divided into five-minute intervals consisting of 20 measurements each (see Chapter 2, Fig 2.3). Oxygen consumption rate was determined using the measurements recorded during the last five minutes of the measurement interval, and was calculated using the formula:

$$mo_2 = Vr * \delta Cwo_2 / \delta t \text{ (see Steffensen 1989), where:}$$

$Mo_2$ is oxygen consumption rate, $V_r$ is volume of water in the respiratory chamber, $\delta Cwo_2$ is the difference in the oxygen concentration of the water at the beginning and at the end of the measurement period, and $\delta t$ is duration of the measurement.

The mean 24-h oxygen consumption data for six fish (four data sets having been discarded for lack of consistency) were pooled together to obtain the diel oxygen consumption pattern for juvenile *P. commersonnii*. The pattern in the diel oxygen consumption rate was used to investigate differences in the nocturnal and diurnal oxygen consumption rates, and to determine the standard and active metabolic rate of the fish. The pattern in oxygen consumption was also used to determine a suitable time for oxygen consumption measurement. Sampling time for subsequent oxygen consumption experiments was established to coincide with the period during which the fish elicited the lowest observed spontaneous activity.

**Determination of SMR, AMR, and metabolic scope**

Standard metabolic rate is an approximation of the minimum rate of metabolism of an organism and is usually determined as the value found at zero activity by relating

metabolic rate to controlled swimming activity (Brett and Groves 1979). Active metabolic rate on the other hand is the maximum rate of metabolism of a fish swimming steadily at a maximum sustained speed. The most ideal method to measure standard and active metabolic rate of fish is by using the tunnel respirometer (Brett and Groves 1979). In this study, however, a method based on intermittent respirometry was adapted to determine these rates in juvenile *P. commersonnii*. It is known that after transfer into a respirometer fish experience an elevation in oxygen consumption rate (Schurmann and Steffensen 1997). This may last for several hours during which period a gradual decrease in oxygen consumption rate can be observed. Following this period, oxygen consumption rate stabilizes at a distinctive lower level. In this study, the lower level delineated the standard metabolic rate while higher measurements were attributed to random activity. On the other hand, the elevated oxygen consumption rate that coincided with sustained nocturnal activity in juvenile *P. commersonnii* was taken as an estimation of the active metabolic rate of the fish.

Data obtained from the 24-h oxygen consumption measurements were used to estimate the standard and active metabolic rate of juvenile *P. commersonnii*. The data had been obtained by measuring oxygen consumption of individual fish that were acclimated in the respirometer overnight and had recovered from the effects of handling. The SMR was estimated as the Y-intercept of the line fitted through the five lowest (mean hourly / per interval) oxygen consumption measurements by least-square linear regression. The AMR was similarly estimated using the five highest oxygen consumption measurements from the pooled 24-h oxygen consumption data.

The routine metabolic rate was regarded as the rate of oxygen consumption by fish that had been acclimated to the respirometer for at least 12 h and was determined during a series of experimental trials conducted at 25 $^{0}$ C. To determine the RMR, single fish were introduced into the measuring chamber and acclimated overnight before measuring oxygen consumption. Oxygen consumption was subsequently measured at half-hourly intervals for a period of 3 h, resulting in six data points. The procedure used to measure oxygen consumption was identical to that used in the other trials (see Chapter 2). Oxygen consumption was calculated using the formula: $mo_2 = Vr * \delta Cwo_2 / \delta t$ (details given above). Five replicate trials were conducted using different fish. Routine metabolic rate was calculated as the mean of the pooled ($n$ = 30) oxygen consumption measurements.

**Effect of fish size on metabolic rate**

In poikilothermic organisms, it is difficult to define a normal resting oxygen consumption rate, or to even define the relation of oxygen consumption to body weight because of the many factors that affect their metabolic rate. To determine the relationship between metabolic rate and fish weight for a given species it is necessary to specify the environmental and experimental conditions under which the measurements are done. These conditions are described in Chapter 2. Oxygen consumption of 32 fish ranging in weight between 2 – 18 g was measured to study the relationship between fish weight and metabolic rate. The oxygen consumption measurements were conducted at 25 ± 0.7 $^{0}$ C, which was close to the temperature preferendum of *P. commersonnii* (24 – 25 $^{0}$ C) reported by Deacon and Hecht (1995). The fish used for this experiment were obtained from the acclimation facilities at the Port Alfred field station (see Chapter 2) and transported to the Grahamstown laboratory for experimentation. Oxygen consumption

was measured following the methods outlined in Chapter 2. Experimental fish were introduced into the respirometer and acclimated overnight. After the overnight acclimation, oxygen consumption was measured at half-hourly intervals for 3 hours. At each measurement interval dissolved oxygen concentration was recorded every 15 seconds for 20 minutes. Oxygen consumption for a single fish was calculated as the average of the six oxygen consumption measurements obtained during the 3-h period. The oxygen consumption measurements were calculated using the formula provided in the previous section (see also chapter 2). The oxygen consumption measurements for all the fish were pooled together to determine the effect of weight on metabolic rate. Regression analysis was used to model the effect of fish weight on metabolic rate using the equation, $mo_2 = aW^b$.

## Results

### Effect of diel rhythms on oxygen consumption rate

Juvenile *P. commersonnii* consumed significantly more oxygen at night than during daytime (t-test: $t = 3.08$, $p < 0.0001$). Oxygen consumption increased from $0.18 \pm 0.06$ mg $O_2\ g^{-1}h^{-1}$ during the daytime to $0.56 \pm 0.04$ mg $O_2\ g^{-1}h^{-1}$ at midnight, before declining in the early morning hours (Fig. 3.1). The elevated oxygen consumption rates coincided with an increase in random movements by the fish.

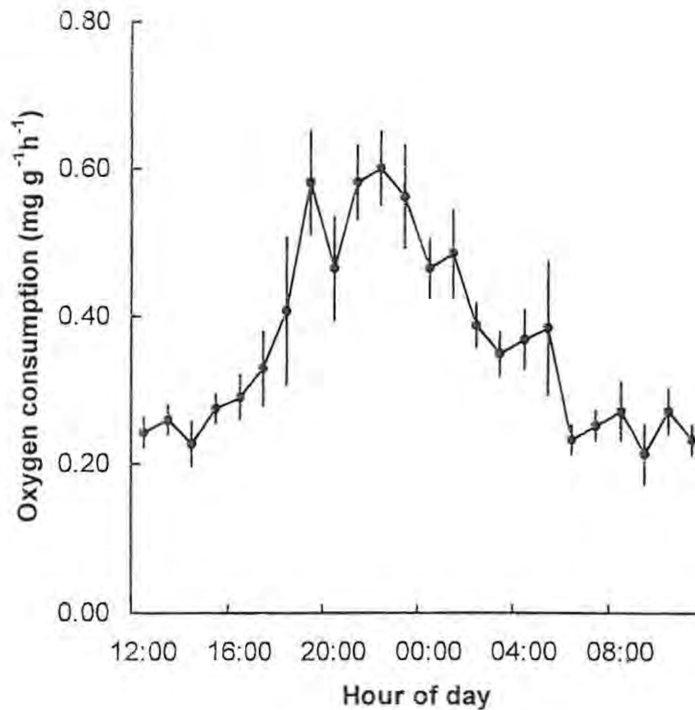


Fig: 3.1. The diel oxygen consumption patterns of juvenile *Pomadasys commersonnii.* Data points are mean (± S.D., $n = 6$) hourly oxygen consumption rates measured during a 24-h period.

### Standard metabolic rate, active metabolic rate and metabolic scope

Figure 3.2 shows the 24-h oxygen consumption data that were used to calculate the standard and active metabolic rates of juvenile *P. commersonnii*. The standard metabolic rate was 0.16 ± 0.02 (mean ± S.D, $n = 6$) mg $O_2$ $g^{-1}h^{-1}$, whereas the active metabolic rate was 0.56 ± 0.04 mg $O_2$ $g^{-1}h^{-1}$. The routine metabolic rate was 0.25 ± 0.03 mg $O_2$ $g^{-1}h^{-1}$.

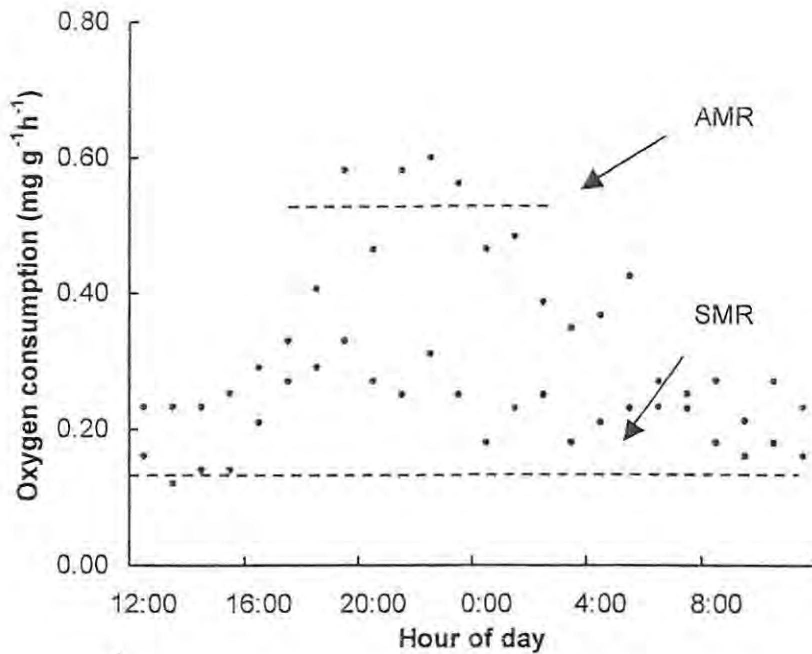


Fig: 3.2. The standard metabolic rate (SMR) and active metabolic rate (AMR) of juvenile *Pomadasys commersonnii*. Data points are mean oxygen consumption rates of six fish measured during a 24-h period.

### Effect of fish size on oxygen consumption rate

Figure 3.3 shows the changes in the mass-specific rate of oxygen consumption by juvenile *P. commersonnii.* Oxygen consumption ranged between 0.5 mg $O_2\ g^{-1}h^{-1}$ for fish weighing less than 3 g to 0.25 mg $O_2\ g^{-1}h^{-1}$ for fish weighing up to 18 g. There was an inverse relationship between oxygen consumption rate ($Mo_2$) and fish weight, which was described by the allometric equation, $mo_2 = 0.64\ W^{-0.38}$ ($r^2 = 0.82$).

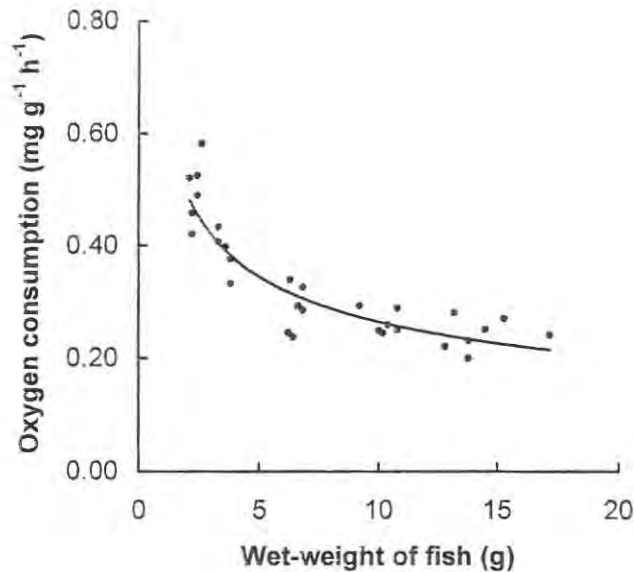


Fig: 3.3. Changes in mass-specific oxygen consumption rate of *Pomadasys commersonnii*. The inverse relationship between weight and oxygen consumption was described by the equation, $mo_2 = 0.64\ W^{-0.38}$ ($r^2$=0.82).

## Discussion

Oxygen consumption of juvenile *P. commersonnii* was significantly higher at night than during daytime, indicating the presence of a diel rhythm in the oxygen consumption pattern. The high nocturnal oxygen consumption rate coincided with increased levels of physical activity observed in this study, and by Du Preez *et al.* (1986). It has been suggested that the high nocturnal activity levels in *P. commersonnii* may be related to feeding. Lasiak (1984), for example, observed that juvenile *P. commersonnii* were mainly caught at twighlight when the fish moved closer inshore to feed and argued that the high nocturnal metabolic rate of this species may be due to increased feeding activity during these periods. Du Preez *et al.* (1986) also recorded up to 100 % higher nocturnal oxygen consumption rates in *P. commersonnii* weighing between 50 - 2390 g wet weight. In this study, a 12L: 12D photoperiod was maintained throughout the 7 days acclimation period

and during the experiments (see also Chapter 2). In addition, the precautions taken during the experiments such as shading of the measuring chamber, the observation of an acclimation period after introduction of fish into the chamber and the use of a polarographic oxygen sensor to continuously monitor dissolved oxygen concentration in the chamber, ensured the accuracy of the measurements. Prior to the experiments, fish were fed only once during the day. The high nocturnal metabolic rates were, therefore, not likely to be associated with an anticipation of feeding, but perhaps with an endogenous rhythm.

Diel rhythms are primarily endogenous (Muller 1978) and show an ecological usefulness the adaptive value of which may be a restriction of certain activities to the most favourable time of the day. The restrictions of activities are determined by abiotic factors such as photoperiod and temperature, or biotic factors such as prey availability. The perception of changes in these variables may initiate a train of physiological events that serve to prepare the fish to meet the biological consequences induced by the perceived factor (Schwassman 1978; Boujard and Leatherland 1992). *P. commersonnii* exhibits increased activity at the onset of darkness (Du Preez *et al.* 1986) that has been associated with the availability of prey. The increase in nocturnal oxygen consumption of juvenile *P. commersonnii* may have resulted from an increase in activity.

The higher nocturnal oxygen consumption exhibited by juvenile *P. commersonnii* indicates that rhythmic fluctuations in metabolic rate could have direct implications on the determination of baseline metabolic rates of fish. Fluctuations in metabolic rate might result in overestimation or underestimation of the standard and active metabolic rate of the fish, depending on the time of day at which the measurements are taken. However, the

24-h continuous measurements enabled the detection of these fluctuations, and thereby ensured greater accuracy in the estimation of these parameters.

Standard metabolic rate is an approximation of the minimum rate of metabolism as measured in a fish at complete rest (Fry 1971). Active metabolic rate on the other hand, is measured in fish swimming steadily at a maximum sustained speed (Brett 1964). SMR and AMR are, therefore, basically measurements of the minimal and maximal rates of metabolism of the fish. Oxygen consumption rates measured in resting fish during the day, and that measured during sustained nocturnal activity, as was exhibited by juvenile *P. commersonnii* would, therefore, give a fairly reasonable approximation of these rates. The estimated AMR of juvenile *P. commersonnii* was the same as the maximum oxygen consumption rate of the fish estimated from data recorded after a capture and handling stressor (see Chapter 4). This agrees with Bradley (1998), who argues that the highest oxygen consumption rates of a fish are likely to be similar to the rates measured in the fish during oxygen-debt repayment following near-exhaustive activity levels, such as can occur during capture and handling. Following these observations, it is suggested that the use of 24-h oxygen consumption measurements could provide a useful way to estimate the metabolic rate of fishes that exhibit diel fluctuations in activity. Du Preez *et al.* (1986) and Schurmann and Steffensen (1997) used similar methods to estimate the metabolic rate of juvenile *P. commersonnii* and Atlantic cod (*Gadus morhua*), respectively.

Oxygen consumption of juvenile *P. commersonnii* decreased with increasing weight following the equation, $mo_2 = 0.64\ W^{-0.38}$. The exponent determined for juvenile *P. commersonnii* in this experiment was the same as that obtained by Du Preez *et al.* (1986) for *P. commersonnii* (b-value = 0.4, wt: 30 - 3000 g), but was lower than the published

b-values (0.4-1.0) reported for many species (Schmidt-Nielsen 1984). The size range of fishes used by Schmidt-Nielsen (1984) to determine the b-values were, however, of the order of several magnitudes larger than that used to describe the size-dependent oxygen consumption of juvenile *P. commersonnii*. Other authors have used similarly wide size ranges to determine the b-values for fish. For example, Mitz and Newman (1989) used a range of sizes of approximately two orders of magnitude to determine the b-value for mosquito fish, *Gambusia affinis*. Oikawa *et al.* (1992) used fish ranging in size between 0.0002 g and 2.9 g to study the relationship between metabolic rate in vitro and body mass in porgy, *Pagrus major*.

The patterns from the 24-h oxygen consumption measurements enabled the sampling time for oxygen consumption measurements to be established between 10 h 00 and 16 h 00 to coincide with the period of least activity in the fish. It is suggested that patterns in oxygen consumption rate might provide a way of establishing a suitable sampling time, and thereby avoid the pitfalls in experimental design that may result in erroneous estimations of baseline metabolic rates of fishes that exhibit cyclic fluctuations in metabolic rate. Since stress is only manifest in fish that has been subjected to an environmental change in excess of the normal (Wedemeyer and McLeay 1981), the use of standard metabolic rate as the baseline rate to assess the effect of stressors on fish metabolism may be misleading. Consequently, routine metabolic rate was chosen as the baseline rate for assessing the effect of stressors on the metabolic stress response of juvenile *P. commersonnii*. For purposes of uniformity in the oxygen consumption measurements, all experiments performed during the rest of this study, including both oxygen consumption and plasma cortisol measurements, were carried out between 10 h 00 and 16 h 00 in conformity with the sampling time established earlier on.

# CHAPTER 4

## Metabolic and plasma cortisol stress responses in juvenile *P. commersonnii*

### Introduction

Stress in fish is a 'state' caused by a factor, or stressor, that deviates from a normal resting or homeostatic state (Barton and Iwama 1997). Stress itself cannot be measured and only the responses to stressor stimuli can be quantitatively determined to reflect the severity of the stress experienced. Stress may, therefore, be considered as a change in biological condition beyond the normal resting state, which challenges homeostasis. As such it represents a possible threat to the fish. The stress response in fish can be classified as primary, secondary or tertiary depending on the level of organization of the response (Adams 1990). The primary response represents the perception of an altered state and initiates the secretion of neuroendocrine stress hormones that are an essential component of the generalized stress response in fish (Gamperl *et al.* 1994). The primary stress response is commonly measured by the concentration of plasma cortisol (Thomas 1990; Mommsen *et al.* 1999). Plasma cortisol is used to assess the primary stress response mainly because of its responsiveness to acute stressors. During the stress response, the plasma concentration of cortisol increases proportionately to the intensity and duration of the stress (Barton *et al.* 1980) thus enabling the estimation of the effects of the stressor. Plasma cortisol can also be measured easily and accurately using commercially available radioimmunoassay (RIA) or Enzyme-Linked Immunosorbent Assay (ELISA) kits (Barry

*et al.* 1993; Nash *et al.* 2000). Furthermore, it has been suggested that non-stress levels of plasma cortisol may be obtained by proper sampling procedure, including sampling under anaesthesia (Iwama *et al.* 1989; Cho & Heath 2000). Cortisol is also used to measure stress because of its functional significance in physiological processes affecting fish health (Barton and Iwama 1991; Barry *et al.* 1993).

Cortisol is the principal corticosteroid in teleost fishes (Wendelaar Bonga 1997; Mommsen *et al.* 1999). It is always present in measurable quantities in vertebrates even under non-stress conditions but its effects only become apparent under abnormal circumstances (Mommsen *et al.*1999). It is also noteworthy that under chronic stress such as prolonged crowding, plasma cortisol may fall back to resting levels even though the fish may still be exposed to the stressful situation (Vijayan and Leatherland 1988). Cortisol is a lipid soluble hormone, but because of the presence of binding proteins in plasma, its physiologically significant concentrations may differ considerably from what chemical analysis might reveal. Plasma cortisol concentrations reflect the net effect of production and the plasma clearance of the hormone (Mommsen *et al.*1999). The clearance of cortisol from the plasma is dependent on binding proteins, target tissue receptors, tissue uptake and catabolism of cortisol (Mommsen *et al.* 1999). Consequently, these processes may modulate the animal's physiological response to the hormone. Factors affecting any of these parameters will in turn modify the cortisol response (Mommsen *et al.* 1999). Considerable variation has, therefore, been reported in the corticosteroid response of different fish species, and also between different individuals of the same species (Barton 1980; Schreck 1981; Donaldson 1981; Pankhurst *et al.* 1992; Mazur and Iwama 1993; Caldwell and Hinshaw 1994; and Staurnes *et al.* 1994). Recorded levels of cortisol in unstressed fish range from less than 2 ng $ml^{-1}$ in rainbow trout,

*Oncorhynchus mykiss*, to 250 ng $ml^{-1}$ in striped bass, *Morone saxatilis* (see review by Barton and Iwama 1991). Cortisol levels have been reported to rise to 213 ng $ml^{-1}$ in rainbow trout, and to 2 000 ng $ml^{-1}$ in striped bass, in response to acute physical stress. The variability in cortisol levels may also be attributed to the diversity of approaches in manipulating cortisol levels during tests and to differences in sampling techniques (Wedemeyer *et al.* 1990). Moreover, plasma cortisol levels can also be affected by the nutritional condition of the fish, and photoperiod and sexual maturity (Mommsen *et al.* 1999), in addition to environmental factors such as prior exposure to prophylactics (Pickering and Pottinger 1989) and water quality (Pickering and Pottinger 1987). Mommsen *et al.* (1999) reviewed the dynamics, mechanisms of action, and metabolic regulation of cortisol.

Although plasma cortisol is the most commonly measured indicator of stress in fish, not all kinds of environmental stressors can be detected by its measurement alone (Adams 1990). For example, exposure to certain types of toxicants are obviously detrimental to fish in that they cause direct mortality or impair fish health, but do not necessarily evoke the characteristic increases in cortisol normally associated with the stress response (Barton and Iwama 1997). Because the stress response comprises a chain of interconnected events that occurs from one level of biological organization to another (Iwama *et al.* 1999), the effects of stress could be best evaluated by measuring indicators such as metabolic rate, that are a culmination of a chain of responses at different levels of biological organization.

Stress is an energy-demanding process (Davis and Schreck 1997). It is, therefore, hypothesized that changes in metabolic rate during the stress response are a fundamental response to the stressor. A key element in the provision of the energy required during the stress response is a change from storage to a catabolic state, in which cortisol plays an

adaptive role (Pickering 1992; Morgan and Iwama 1996; Milligan 1997). Cortisol also plays a role in ionic and osmotic regulation following the stress response (Iwama *et al.* 1999; Mommsen *et al.* 1999). The stress response in fish is accompanied by rapid changes in the plasma concentrations of catecholamines, primarily adrenaline and noradrenaline (Pankhurst *et al.* 1992; Sumpter 1997). The catecholamines originate from the chromaffin cells scattered throughout the kidneys and the walls of the posterior cardinal veins (Wendelaar Bonga 1993; Sumpter 1997). Various physiological stimuli including acetylcholine, serotonin and altered blood chemistry have been shown to trigger the mobilization of catecholamines from chromaffin cells (Sumpter 1997). A simultaneous hypothalamo-pituitary-interrenal (HPI) response results in increased plasma cortisol concentrations (see review by Sumpter 1997) that maintain hyperglycaemia during the stress response. Because of the linkage between cortisol and energy-mobilization during the stress response, metabolic rate could be used to complement cortisol measurements as the principal indicators of stress in fish. Wedemeyer *et al.* (1990) also suggested the use of metabolic rate as a possible method for evaluating stress in fish. Although changes in metabolic rate have been used to quantify the effects of stress on several fish species (Robertson *et al.* 1988, Iwama and Mazur 1993, Pankhurst *et al.* 1992), a comparison of metabolic rate and the commonly used indicators of stress in fish such as plasma cortisol, has not been done. The usefulness of metabolic rate as a stress indicator, therefore, remains untested.

In Chapter 1, it was hypothesized that changes in metabolic rate could indicate the existence and magnitude of stress in fish. To prove this hypothesis, it was necessary to establish a protocol for measuring metabolic rate that could be used without causing stress to the fish unless required. The methods and protocols for the determination of metabolic

rate were outlined in Chapter 2, whereas the baseline metabolic rates for the test species were established in Chapter 3. In this chapter, and in subsequent chapters (5 and 6), the suitability of metabolic rate as a measure of stress in fish will be demonstrated. It will be shown that for juvenile fish, metabolic rate may be a better indicator of acute stress than the commonly used plasma cortisol measurement.

To facilitate this demonstration, it was necessary to generate a profile of the metabolic stress response. Consequently, the objective of this series of experiments were:

1. To study the effect of acute stress on the metabolic rate of juvenile *P. commersonnii*,
2. To study the effect of acute stress on the corticosteroid stress response in the fish, and
3. To evaluate the suitability of using metabolic rate in comparison with plasma cortisol concentration as a measure of acute stress

To achieve these objectives two experiments were performed to (1) evaluate the change in the metabolic rate of juvenile *P. commersonnii* and (2) to measure the change in plasma cortisol concentration in response to acute stress. The relationships between metabolic rate and plasma cortisol concentration, and the potential use of metabolic rate change as an indicator of stress are discussed.

The size of an organism often has a direct effect on the rates of change of biochemical and physiological variables in the organism (Schmidt-Nielsen 1984). It is therefore necessary that scale effects be taken into account when measuring such parameters. Whereas, it has been established that size has an effect on the metabolic rate of fish (Fry 1971), it is not known whether fish size might influence the cortisol stress response and thereby affect the

concentration of circulating cortisol in the fish. The effect of fish size on plasma cortisol concentration was also investigated.

## Materials and methods

### 1. Measurement of the metabolic stress response

The metabolic stress response was studied by measuring oxygen consumption of juvenile *P. commersonnii* that were subjected to a well-defined and controlled stressor. Juvenile *P. commersonnii* were obtained by beach seine from the Great Fish River and acclimated to captive conditions for 30 days at the Port Alfred field station (see Chapter 2). The fish were acclimated for a further 7 days at the Grahamstown laboratory before experimenting. All experiments were conducted at 25 ± 0.7 ° C, close to the thermal preferendum of this species (Deacon and Hecht 1995). To study the metabolic stress response replicate trials were conducted using five fish (wt. = 14.7 ± 2.8 g). Oxygen consumption of each fish was measured separately following the protocols outlined in Chapter 2. Single fish were acclimated in the respirometer for at least 12 h before experimenting. Before measuring oxygen consumption, the fish were subjected to a simulated capture and handling disturbance that lasted less than 2 minutes. During the simulation, stirring within the respirometer chamber as well as the water flow through the chamber was stopped and the respirometer chamber was shaken vigorously while under water. Immediately after this, oxygen consumption was measured for 4 h. In an experiment conducted to study the effect of oxygen depletion on oxygen consumption rate of individual *P. commersonnii* (wt = 14.9 ± 2.4 g, mean ± S.D., Chapter 5), it took at least 30 min before dissolved oxygen in the chamber was depleted to limiting levels. Consequently, to measure the metabolic

stress response, oxygen consumption was measured continuously during the first 30 minutes and thereafter at half-hourly intervals. At each interval oxygen measurements were recorded every 15 seconds for 20 minutes. All measurements were conducted between 10 h 00 and 16 h 00.

The dissolved oxygen measurements were divided into five-minute intervals consisting of 20 measurements each. Oxygen consumption was calculated using the measurements recorded during the last five minutes of the measurement interval (see Chapter 2). Oxygen consumption rate was calculated according to the formula: $mo_2 = Vr * \delta Cwo_2 / \delta t$ (see Steffensen 1989), where: $mo_2$ = oxygen consumption rate (mg $O^2$ $g^{-1}l^{-1}$); Vr = volume of water in the respiratory chamber (l); $\delta Cwo_2$ = the difference in the oxygen concentration of the water at the beginning and end of measurement; $\delta t$ = duration of measurement.

**2. Measurement of the corticosteroid stress response**

**Effect of fish size on the cortisol stress response**

Two experiments were conducted to study the effect of fish size on plasma cortisol concentration, and to determine whether size has an effect on the cortisol stress response in the fish. The fish used in the study were obtained from the Great Fish River at the same time as those used to measure the metabolic stress response. At approximately 30 g, the fish intended for cortisol measurements had been transferred to net cages in a sheltered part of the Kowie estuary in Port Alfred. A random sample of 60 fish was taken from this population and moved to the Grahamstown laboratory where they were divided into

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groups of 15 and held in four 300-l holding tanks to facilitate the sampling process. All fish were held for a minimum of 7 days before the experiments began.

Sampling for cortisol measurements was done following the recommendations by Robertson *et al.* (1988) and Pankhurst *et al.* (1992) that blood sampling should be completed within 3 minutes to evade the effects of stress to the fish due to handling. To study the effect of size on plasma cortisol levels fish were captured individually from a previously undisturbed holding tank and blood samples were obtained in less than 90 seconds by severing the caudal peduncle. These samples represented the 'resting' or basal levels of cortisol in the fish. In the second experiment to test whether fish size has an effect on the cortisol stress response, individual fish of indeterminate sex[1] were subjected to a simulated capture and handling disturbance that lasted approximately 2 minutes. The fish were then left undisturbed for 15 min before obtaining a blood sample. Twenty fish ranging in size between 30 and 150 g were measured during each experiment. Regression analysis was used to assess the effect of fish size on the plasma cortisol levels. The estimation of the cortisol stress response and other subsequent cortisol measurements were based on the results from these experiments.

**Estimating the corticosteroid stress response**

The corticosteroid stress response was studied by measuring plasma cortisol concentration in juvenile *P. commersonnii* after subjecting them to a simulated capture and handling stressor that was identical to that used to evaluate the metabolic stress response. The fish used for these experiments were obtained from the same population as that used to

[1] The effect of sexual differences on the stress responses was not considered because the experimental fish were at a sexually inactive stage where such differences might become evident.

measure the metabolic stress response, and were kept under similar conditions in the laboratory, thereby minimizing the effects of culture conditions and genetic variability on the stress response. In the laboratory, experimental fish were divided into separate holding tanks to facilitate the sampling process. To measure the cortisol response, four fish were obtained simultaneously from a previously undisturbed holding tank after the simulated capture and handling procedure. One of the captured fish was immediately sampled for blood, and was subsequently designated as the control fish for this group. The [2]control, thus, represented the [3]pre-stress or 'resting' cortisol levels. The remaining fish were transferred into separate shaded aquaria and were sampled for blood after 15, 30 and 60 minutes, respectively. All blood samples were taken from fish that had been placed into a lethal solution (25 ml $L^{-1}$) of 2-phenoxyethanol. Samples were taken by severing the caudal peduncle and draining the blood into a sterile 5-ml vacuum tube (BD Vacutainer Systems, Preanalytical solutions, Plymouth, UK). The whole process of capture, caudal severance and blood sample collection lasted less than 90 seconds. The blood samples were allowed to clot for 10 minutes, centrifuged to separate serum from blood cells and stored at 4 $^{0}$ C for subsequent analysis. The same sampling procedure was repeated using one holding tank after another until 10 replicate blood samples were obtained for each of the designated sampling intervals. All blood samples were preserved and analyzed at the laboratories of Du Buisson and Partners; Pathologists (Pretoria, South Africa) following the procedures outlined below.

[2] In this study, control measurements represented the levels of the measured variable, i.e. plasma cortisol or oxygen consumption rate, before the application of a stressor. Any prior elevation in the levels of these variables e.g. as a result of transfer from Port Alfred or captive conditions was considered to be the same for all experimental fish.

[3] Pre-stress / resting / basal levels refers to the levels of the measured variable at the time of capture from the holding tanks.

**Determination of plasma cortisol concentration**

Plasma cortisol is usually measured using radioimmunoassay or enzyme-linked immunosorbent assay techniques. Enzyme immunoassays are based on enzyme labelling of either antigen or antibody (Clausen 1988) and are performed in three stages viz. labelling of the antigen or antibody with an enzyme, purification of the conjugates, and measurement of the residual enzyme activity. In this study, plasma cortisol concentration was measured using the competitive enzyme-linked immunosorbent assay (ELISA) test. The competitive assay technique is based on the ability of free antigens to competitively inhibit the deposition of antibodies in the immune complex with corresponding enzyme-labelled antigens. The assay system consists of an antibody in a solid phase, a specific or free antigen, and an enzyme-linked antigen. Because antibodies are able to form covalent bonds through their free terminal $NH_2$-groups with reagents reactive towards such groups, these reagents are attached to a solid phase that makes it possible to prepare insoluble but reactive antibodies. During the assay, the sample containing the antigen in an unknown concentration (free antigen) together with the corresponding specific antibody is incubated in a single step with a certain amount of the enzyme-linked antigen. The two antigenic reactants, thus, compete for the same antibody sites. At equilibrium the measured enzyme activity of the solid phase immunosorbent is inversely proportional to the concentration of the antigen in the sample (Clausen 1988). The incubated sample is purified before measuring residual activity in order to remove non-reacted enzyme. The residual enzyme activity may be determined spectophotometrically or using other chemiluminescence techniques. Typical assays using competitive labelling include total T4 assay, Oestradiol assay, and Cortisol and Digoxin assays.

To measure cortisol concentration in juvenile *P. commersonnii*, 10 µL of the serum sample from the fish was added to 11.5 ml of alkaline phosphate conjugated to cortisol in a buffer solution. The sample was incubated together with polyclonal rabbit anti-cortisol attached to beads in a cortisol bead pack. The whole mixture was incubated for 30 min before the assay. Three ml of processed high-cortisol and low-cortisol concentrations were used as cortisol adjusters. The non-reacted enzyme was washed with distilled water before measurement. Residual enzyme activity was measured using an Immulite 2000 cortisol analyzer (Diagnostic Products Corporation, 5700 West 96$^{th}$ Street, Los Angeles, CA).

**Sensitivity and specificity of the assay**

The assay had a sensitivity of 5.5 nmol $l^{-1}$ and was highly specific to cortisol. Intra-assay variability, measured as a percentage of the coefficient of variation (100 x SD/mean, Heppel *et al.* 1999), was assessed by conducting duplicate assays. The average intra-assay variation was 7.4% ($n$ = 31) and the average inter-assay variation, evaluated in the same manner using the same replicates assays, was 9.4%. Within the cortisol production pathway the antiserum had a cross-reactivity of 0.2% with 17α - hydroxyprogesterone. Progesterone was not detected. Details of the preparation, setup, dilutions, adjustments, assay, and quality control procedures are given in the Immulite 2000 Operator's manual.

**Statistical analyses**

Oxygen consumption rate was modeled as a function of time using polynomial regression to estimate the metabolic response of juvenile *P. commersonnii* after stress application. Data were analyzed using the STATISTICA program (Statsoft Inc., 1984 - 2001). A

regression model was fitted using the forward selection procedure (Zar 1999). Beginning by fitting a linear regression to the data followed by a $2^{nd}$ order quadratic model, the selections were repeated until the best possible fit (the model resulting in the largest coefficient of determination or $r^2$) was obtained. For purposes of interpretation, the metabolic stress response model was divided into a stress effect phase comprising a response phase and a recovery phase, which had a parabola-like shape (Fig. 4.1). The derivative of the $2^{nd}$ order equation that described the stress effect phase was used to estimate the magnitude of the metabolic stress response.

One-way ANOVA was used to test for differences in plasma cortisol concentration measured at the specified measurement intervals. Normality of the data and the homogeneity of the variances were tested using the Shapiro-Wilks and the Levene's test, respectively. A log transformation was performed on the data before analysis. Homologous groups were distinguished by the Schaffé test. For purposes of comparing the metabolic and the corticosteroid stress responses of juvenile *P.commersonnii*, the metabolic stress response was divided into discrete intervals (3, 15, 30 and 60 min) and the oxygen consumption data compared using ANOVA.

## Results

### 1. Metabolic stress response

The metabolic response to capture and handling consisted of a response phase, which was characterized by an increase in oxygen consumption rate and a recovery phase, during which oxygen consumption rate returned to the pre-stress levels. During the response

phase oxygen consumption increased from $0.26 \pm 0.05$ mg $O_2$ $g^{-1}h^{-1}$ before the application of stress to $0.60 \pm 0.06$ mg $O_2$ $g^{-1}h^{-1}$ at the peak of the stress response, reflecting a three-fold increase in oxygen consumption rate. The metabolic stress response peaked approximately 15 minutes after stress application and normal metabolic rate was re-established after approximately 90 minutes. During the early part of the recovery period the fish remained stationary showing little or no visible reaction to mild physical disturbance instituted by tapping the respirometer chamber with the sharp end of a plastic ruler. The return to normality was evidenced by the resumption of random movements.

Regression analysis of the changes in metabolic rate as a function of time after capture and handling showed that the model that best described the metabolic stress response ($y = - 5E - 13 x^6 + 4E - 10 x^5 - 1E - 07 x^4 + 2E - 05 x^3 - 0.0013 x^2 + 0.0364 x + 0.3052$; $E = 10$, $r^2 = 0.89$, Fig. 4.1) could be divided into two parts. The first part of the model described a parabola-like stress effect phase with a maximum value at the peak of the stress response. Using the derivative of the quadratic equation ($y = - 0.0013 x^2 + 0.0364 x + 0.3052$) that described the stress effect phase, the metabolic stress response was estimated to peak 14 minutes after capture and handling, at which time the maximum oxygen consumption rate was estimated to be 0.56 mg $O_2$ $g^{-1}h^{-1}$, a close approximation to the observed maximum rate. The metabolic stress response model also showed that following the initial recovery period (~ 90 min) the fish exhibited random fluctuations in metabolic rate that could probably be attributed to factors such as confinement and random physical activity.

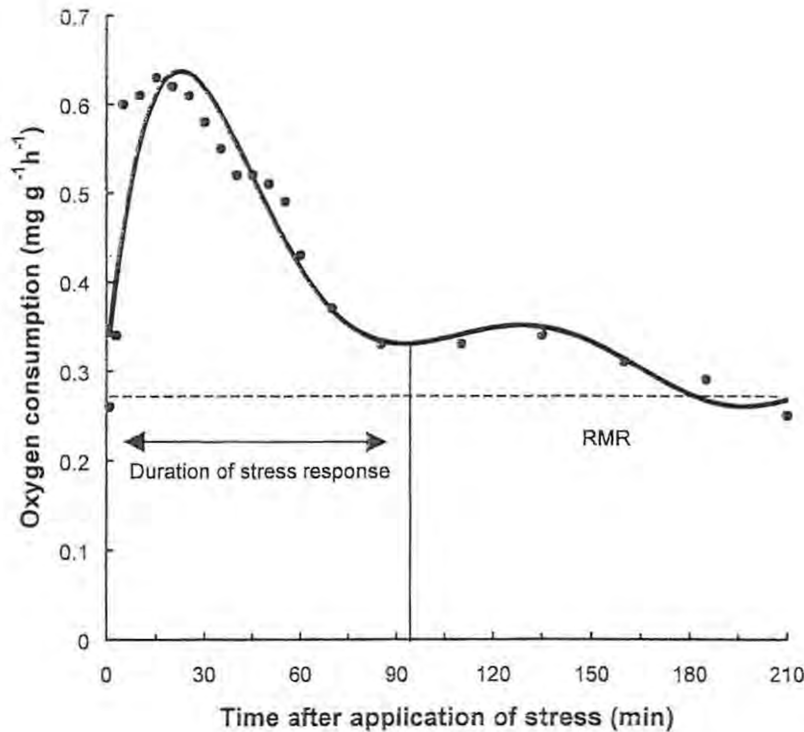



Figure: 4.1. The metabolic stress response profile for juvenile *Pomadasys commersonii* ($n = 5$) following a 2-min capture and handling stress. A sixth order polynomial ($y = -5E - 13\ x^6 + 4E - 10\ x^5 - 1E - 07\ x^4 + 2E - 05\ x^3 - 0.0013\ x^2 + 0.0364\ x + 0.3052$, $r^2 = 0.89$) was fitted to the measurements. The magnitude of the stress response, measured by the maximum oxygen consumption rate at the peak of the stress response, was estimated using the derivative of the quadratic equation ($y = -0.0013\ x^2 + 0.0364\ x + 0.3052$). The area demarcated by the duration of the stress response comprises the response and recovery phases of the metabolic stress response. The dashed line designated RMR indicates the mean (pre-stress) metabolic rate of the fish.

## 2. The cortisol stress response

### Effect of fish size on stress levels

There was no correlation between fish size and plasma cortisol concentrations in juvenile *P. commersonnii* as shown by linear regression analysis of plasma cortisol data obtained from the fish immediately upon capture (Fig 4.2) indicating that plasma cortisol concentration was independent of fish size within the range tested. Similarly, the cortisol stress response was not affected by the size of the fish ($r^2 = 0.06$, $p = 0.5$).

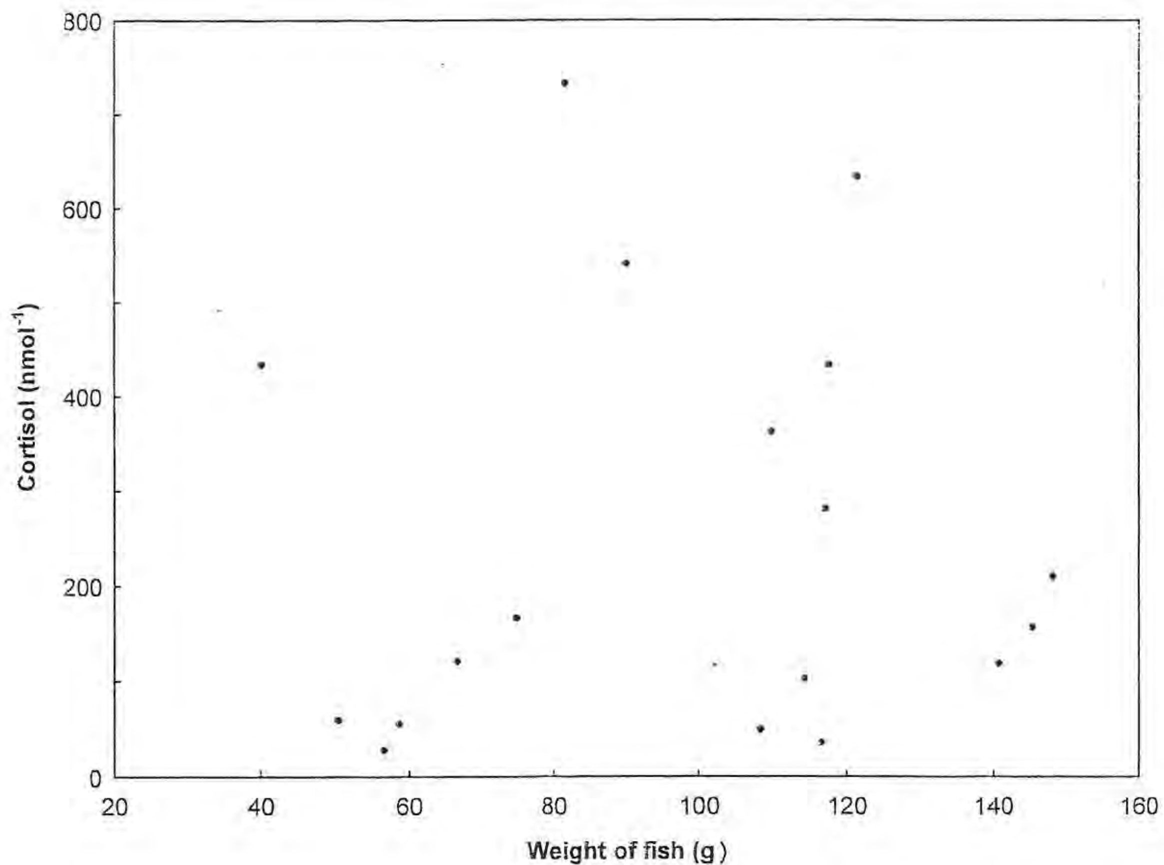



Figure 4.2. The effect of fish size on plasma cortisol concentration: data points are the cortisol concentrations measured in individual unstressed *Pomadasys commersonnii* immediately after capture.

## The corticosteroid stress response

Plasma cortisol concentrations were significantly higher (ANOVA: $F = 6.63$, $p < 0.001$; Fig 4.3) in juvenile *P. commersonnii* that were sampled 15 minutes after capture and handling than in those sampled immediately after capture. However, cortisol concentrations in the fish that were sampled 30 min after the disturbance was similar to those sampled immediately after capture, suggesting a re-establishment of normal cortisol levels after the stress. The corticosteroid response, therefore, consisted of a response phase that was similar to the metabolic response, and which was characterized by an increase in plasma cortisol concentration and a recovery phase during which cortisol

levels declined to the pre-stress levels. After the capture and handling disturbance cortisol concentration increased from 159 ± 85 nmol $l^{-1}$ to 370 ± 165 nmol $l^{-1}$, reflecting a more than two-fold increase in plasma cortisol levels in the fish. As is evident in Fig 4.3, there were wide variations in plasma cortisol concentration both before, and after stressor application. Pre-stress levels of circulating cortisol were re-established after approximately 30 minutes.

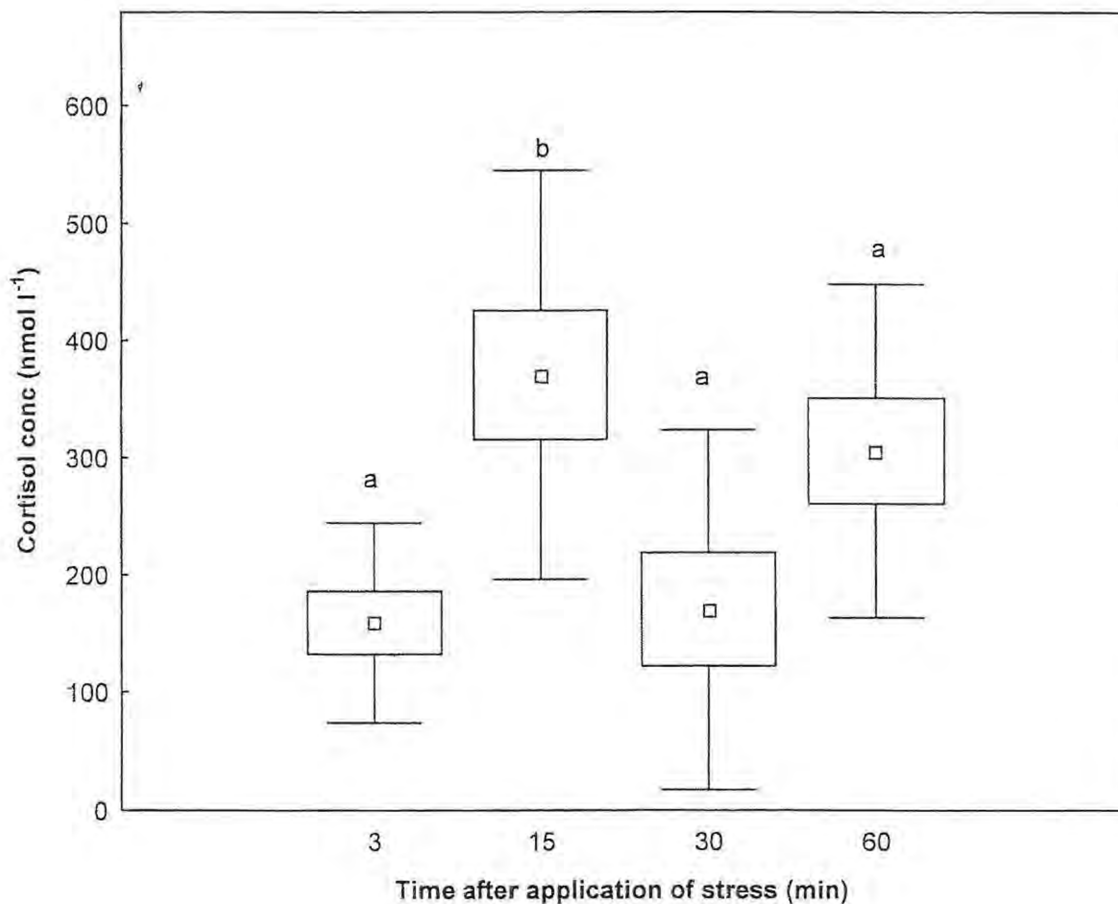



Figure 4.3. Plasma cortisol levels in juvenile *Pomadasys commersonnii* after a capture and handling disturbance. Cortisol measurements were taken immediately after the disturbance and after 15, 30, and 60 minutes. The box plots represent the mean, standard error and standard deviations. Significant differences are denoted by different captions.

## DISCUSSION

The metabolic stress response in juvenile *P. commersonnii* occurred in two phases, a response phase during which oxygen consumption rate increased to a peak after approximately 15 minutes, and a recovery phase when oxygen consumption rate gradually returned to pre-stress levels. This response pattern, which is typical of the response of stenothermic poikilotherms to acute stress (Jobling 1994), has also been reported in other fish species (Robertson *et al.* 1988, Dalla Via *et al.* 1989, 1998, Mazur and Iwama 1993, Pankhurst *et al.* 1992 and Barrionuevo and Fernandes 1998). The increase in metabolic rate is premised on the fact that stress imposes a metabolic load on fish that increases the demand for metabolizable energy. Exposure of fish to a stressor induces a generalized stress response that includes a switch from anabolism to catabolism, and results in the release of the metabolic energy required to cope with the stressor (Barton and Iwama 1991). The energy expended by fish to cope with stress varies with species and level of stress. For example, Brett (1964) found that during short bursts of physical activity the metabolic rate of young sockeye salmon (*Oncorhynchus nerca*) increased by more than 400%. Barton and Schreck (1987) and Davis and Schreck (1997) on the other hand recorded a 200% increase in oxygen consumption of juvenile steelhead (*O. mykiss*) and coho salmon (*O. kisutch*) as a result of stress. Oxygen consumption of juvenile *P. commersonnii* increased by more than 200% after the simulated capture and handling stress which is consistent with the reported increase in post-stress oxygen consumption rates of some fish species. The difference between oxygen consumption rate of juvenile *P. commersonnii*, before and after application of the stressor, thus, reflected the additional energy required by the fish to cope with the simulated capture and handling stressor.

The time at which measurements of an indicator variable are made after the beginning or end of a stressful experience greatly influences the magnitude or extent of the responses observed (Schreck 1990). That is to say it would be of no use measuring a biological variable with a short response time if the measurements were taken at some point after that time; conversely, it would be equally futile to measure a variable that took a relatively long time to respond if the measurement were taken before that variable had a chance to change. Proper timing of measurements requires knowledge of the temporal scaling of the response, i.e., when the response peaks and when baseline levels of the variable are re-established (Schreck 1990). This may be done by developing a response profile for the variable in question. However, developing this profile may not always be possible. For example, whereas it was possible to develop a profile for the metabolic stress response of juvenile *P.commersonii*, it was not possible to do the same for the cortisol response, mainly because the size of the experimental fish did not allow for repeated blood sampling. In conducting the cortisol experiments, therefore, a time frame of 90 minutes equivalent to the observed duration and recovery of the metabolic stress response in juvenile *P. commersonnii* was adopted. Sampling for cortisol was carried out at designated intervals that were similar to those reported in similar experiments with cortisol (Barton *et al.* 1980; Barton and Schreck 1987; Pankhurst *et al.* 1992; Ackerman *et al.* 2000), and in conformity with the hypothetical cortisol stress response profile (see General discussion, Chapter 7).

Fifteen minutes after the capture and handling disturbance, cortisol concentration in juvenile *P. commersonnii* had increased by more than 200%. However, cortisol levels in the fish that were sampled 30 min after the disturbance were similar to those in unstressed fish, suggesting a re-establishment of pre-stress cortisol levels. This also indicated that,

like the metabolic stress response, the corticosteroid response consisted of a response phase and a recovery phase, characterized by an increase in cortisol concentration during the response phase and a decline in the levels of this hormone during the response phase. A corticosteriod stress response time of approximately 15 min has been reported for other fish species (Barton *et al.* 1980; Barton and Schreck 1987; Pankhurst *et al* 1992; Ruane *et al.* 2001).

The magnitude and time course of the corticosteroid stress response usually reflects the severity and duration of the stressor. For example, Barton *et al.* (1980) found that intense handling and severe confinement caused a rapid elevation in plasma cortisol to levels two to four times higher than that found in fingerling rainbow trout *(O. mykiss*) subjected to less vigorous stressors. Gentle agitation and intermittent restraint with a dip net on the other hand resulted in a gradual increase in plasma cortisol concentration. Davis and Schreck (1997) also measured significantly higher cortisol concentrations in juvenile Coho salmon (*O. kisutch*) subjected to severe physical handling compared to those subjected to less severe handling. The 200% increase in plasma cortisol concentration in juvenile *P. commersonnii* after application of the stressor was similar to those reported in other fish species. The two-fold elevation in cortisol levels suggested that capture and handling might have caused severe stress to the fish.

The concentration of cortisol in the blood reflects the net effect of production and plasma clearance of the hormone (Mommsen *et al.* 1999). The rate of plasma clearance of the hormone may, on the other hand, be affected by factors like rate of biosynthesis and secretion, metabolic clearance and uptake and catabolism (Mommsen *et al.* 1999). These factors are known to vary between different species as well as between individuals of the

same species (Wendelaar Bonga 1999). The variation in cortisol levels, irrespective of whether or not juvenile *P. commersonnii* were stressed, could be a reflection of the influence of these factors. These variations also showed how much the cortisol response might vary between individual fish. Barton and Schreck (1987) and Pottinger and Carrick (1999) reported similar variations in plasma cortisol concentrations in rainbow trout.

The similarity of the metabolic and the corticosteroid stress responses in juvenile *P. commersonnii*, which was reflected in the increase in metabolic rate and the levels of plasma cortisol, and in the temporal scaling of both responses, suggests that the two responses occur simultaneously and might, therefore, be related. Reports by Barton and Schreck (1987) and Davis and Schreck (1997) suggested that there was a correlation between plasma cortisol levels and oxygen consumption in fish. However, an examination of their data revealed a number of differences in the reported correlation between oxygen consumption rate and plasma cortisol concentration. For example, Davis and Schreck (1997) found a significant correlation between plasma cortisol and metabolic rate of juvenile coho salmon subjected to a moderate stressor but did not find any correlation between these parameters in fish subjected to more severe stress. Davis and Schreck (1997) also observed that the supposed correlation was only significant immediately after application of stress but not at any other time.

The controversial nature of the relationship between plasma cortisol levels and metabolic rate is also reflected in the different results obtained during controlled cortisol experiments. For example, Chan and Woo (1978) observed an increase in oxygen consumption rate of hypophysectomized Japanese eel (*Anguilla japonica*) after the fish were injected with exogenous cortisol. Davis and Schreck (1997), on the other hand,

observed that juvenile coho salmon given exogenous cortisol did not exhibit an increase in oxygen consumption. The findings of Chan and Woo (1978) implied a functional relationship between cortisol and metabolism, whereas that of Davis and Schreck (1997) indicated that cortisol alone was unlikely to have a major effect on metabolic rate. From different observations (Leatherland 1985; Barton and Schreck 1987; Davis and Schreck 1997), it is evident that the hormonal changes in response to stress, and the subsequent changes in metabolic rate often co-vary, but the available data does not show any causal link between these two responses. The role of cortisol during the stress response has been reviewed by Sumpter (1997) and Mommsen *et al.* (1999).

Cortisol plays a metabolic as well as a physiological role in fishes (Mommsen *et al.* 1999) that makes it an essential component of the stress response. However, it is its metabolic role that relates cortisol concentration to increases in metabolic rate during the stress response. The primary stress response results in the release of cortisol into the blood stream (Gamperl *et al.* 1994; Sumpter 1997). Cortisol activates key enzymes for hepatic intermediary metabolism (Vijayan *et al.* 1991) that stimulates glycolysis and gluconeogenesis from protein and lipid sources (Wendelaar Bonga 1999). The effect of this is the mobilization of energy reserves for the increased metabolic requirements that are associated with stress (Pickering 1992; Randall and Perry 1992). The simultaneous occurrence or co-variation of the metabolic response may be explained by the fact that the initiation and sustenance of the energy generating processes and changes require an energetic input (Jobling 1994; Beyers *et al.* 1999), which results in the increase in metabolic rate. The question, therefore, arises: could metabolic rate be a better indicator of the stress response in fish compared to cortisol?

The selection of an appropriate indicator of stress is dependent on the kind of stressor to be evaluated, i.e., whether it is acute or chronic. The qualities that must be considered when selecting the appropriate indicator include sensitivity to the stressor, a rapid response time, ease and accuracy of measurement, and its biological significance (Schreck 1990). At the beginning of this chapter, it was suggested that metabolic rate might be a better indicator of acute stress in fish than the commonly used plasma cortisol measurement. However, comparison of the metabolic and corticosteroid responses of juvenile *P. commersonnii* revealed a similarity in the response pattern of the two variables that was highlighted by, among others, the fact that both responses were characterized by an elevation in the variable that could be detected within three minutes of stressor application, and that both responses lasted for approximately the same duration. Plasma cortisol concentration and metabolic rate could also be measured easily and accurately using the Enzyme-Linked Immunosorbent Assay (ELISA) test and respirometry, respectively. Biologically, changes in the levels of cortisol have a functional significance in physiological processes affecting fish health (Barton and Iwama 1991), whereas changes in metabolic rate portend a diversion of energy from activities such as growth and reproduction.

Results obtained in the current study suggest that metabolic rate has several advantages over cortisol as a measure of stress in juvenile fish. Using intermittent respirometry, it was possible to monitor metabolic rate continuously by polarographic oxygen sensors installed in the respirometer, enabling a better evaluation of the stress response. It was, however, not possible to obtain repeated cortisol measurements because of the small fish sizes used. The high variability that was evident in the cortisol measurements of individual *P. commersonnii* could, therefore, not be adequately explained. The main benefit of using

cortisol to measure stress, thus, seems to be the relative ease of taking blood samples and measuring cortisol, particularly in larger fish.

To summarize, assessment of the metabolic stress response shows that:

1. Metabolic rate was highly sensitive to acute (capture and handling) stress
2. The metabolic stress response had a rapid response time of less than three minutes
3. Metabolic rate could be monitored throughout the measurement period, and thereby ensuring the accuracy of the measurements
4. The increase in metabolic rate due to stress, being the culmination of. many physiological events from the primary to the tertiary levels of biological organization, is more indicative of the effect of the stressor on the growth and survival of the organism

Based on these observations, it is suggested that metabolic rate could be a more useful indicator of the stress response than cortisol. However, a variety of commonly known stressors should be tested to confirm the usefulness of metabolic rate as an indicator of the stress response. Such tests will show if there are differences in the magnitude of the changes in metabolic rate associated with different stressors, and how accurately this method measures the stress response. Chapter 5 reports on the changes in the metabolic rate of juvenile *P. commersonnii* with respect to handling, acute temperature changes, and crowding.

# CHAPTER 5

## The effects of oxygen depletion, acute temperature changes, and crowding on the metabolic rate of juvenile *P. commersonnii*

### Introduction

In chapter 4, changes in metabolic rate were used to measure the response of juvenile *P. commersonnii* to a simulated capture and handling stressor. Consequently, it was suggested that a variety of commonly known stressors should be tested to confirm the usefulness and accuracy of metabolic rate for measuring the stress response in fish. Transportation of live fish, a pivotal aspect of aquaculture and fish stock enhancement practices (Bartley 1999; Kitada 1999), is an example of a process that involves potential multiple acute stressors. These include capture and handling (Barton *et al.* 1980; Barton and Schreck 1987; Davis and Schreck 1997), temperature fluctuations that might occur during the transportation of fish (Chow *et al.* 1994), dissolved oxygen depletion (Jobling 1994), and high fish density (Staurnes *et al.* 1994). This study presented the opportunity to evaluate the effects of these variables on the stress response in fish. Knowledge of how juvenile *P. commersonnii* responds to these stressors could lead to a better understanding of the effects of transportation on the stress physiology of fish. A brief overview of these factors is necessary to interpret their effects on fish physiology.

Temperature

Fry (1971) classified the effects of the environment on organisms into five categories, viz. lethal, controlling, limiting, masking, and directive factors. Lethal factors restrict the range of the environment within which the organism can exist. Controlling and limiting factors govern metabolic rate, whereas a masking factor modifies the operation of other factors on the organism. A directive factor, or cue, exerts its effect on the organism by stimulating transductive responses. Temperature is the principal factor controlling fish metabolism (Fry 1971). Temperature sets the pace of metabolism through control of cell and tissue molecular dynamics, and through control of biochemical reactions in the cells (Jobling 1994). A given temperature range permits an upper and a lower limit to metabolism, represented by the active metabolic rate and standard metabolic rate, respectively (Fry 1971). Within these limits, metabolic rate increases with increase in temperature, doubling, or in some cases tripling, with every 10 $^{\circ}$ C temperature rise (Neill and Bryan 1991). Outside these limits temperature becomes lethal, destroying the integrity of the organism (Jobling 1994).

The responses of fish to acute temperature changes, or heat *senso stricto*, are described in terms of thermal tolerance, thermal resistance and thermal preference (Fry 1971; Jobling 1994). The zone of thermal tolerance is defined by limits within which 50% of a fish population can survive indefinitely (Fry 1971). The upper limit of the resistance zone is defined as the critical thermal maximum (Jobling 1994), whereas the lower limit is the critical thermal minimum. Outside these limits there is a progressive increase in thermal stress (Elliot 1981; Jobling 1994). Exposure of fishes to acute changes in temperature within the lower and upper temperature limits may effect characteristic stress reactions

(Wedemeyer 1973; Jobling 1994). The indications of thermal stress begin with abnormal behaviour such as cessation of feeding, progressing to debilitation and loss of equilibrium (Jobling 1994). A series of experiments were conducted to study the effects of acute temperature changes on the metabolic rate of juvenile *P. commersonnii* within these thermal limits. Knowledge of the critical temperature tolerance limits of the fish was deemed necessary to avoid unnecessary mortality during experimentation and to delineate the temperatures beyond which the effects of thermal stress are evident.

Thermal tolerances of fish have been quantified in the laboratory by both incipient lethal and critical thermal methods. Using the incipient lethal temperature (ILT) method, groups of fish acclimatized at various constant temperatures are transferred directly into water of different temperatures, and mortality is recorded over time (Fry 1971). The ILT is then estimated as the temperature that is lethal to 50% of the fish. The critical thermal maximum (CT max) and minimum (CT min) are the extreme measures of thermal resistance (Becker and Genoway 1979). The critical thermal temperature is obtained by progressively increasing or decreasing the water temperature at a constant rate until a predefined sublethal endpoint e.g. loss of equilibrium is reached (Jobling 1994; Currie *et al.* 1998). The rate at which the temperature is adjusted is important to this process. Adjustment rates that are too slow may lead to temperature acclimation through concomitant physiological adjustment by the fish, whereas a rate that is too fast may not reflect the physiological effect of the temperature adjustment since the fish's internal body temperature may not be the same as that of the water. In their evaluation of methods for determining CT max, Becker and Genoway (1979) recommended a heating rate of 0.3 $^{o}$C $min^{-1}$, which they considered optimal since the increase in water temperature paralleled the internal temperature of the fish, but at the same time did not allow for compensation

by acclimation. Although both CTM and ILT end points are quantitatively expressed as temperature, they do not quantify the same response (Currie *et al.* 1998). The CTM method involves the interaction of temperature as both a lethal factor and as a factor controlling the metabolic rate of the fish, whereas in the ILT method lethal effects are independent of metabolic rate (Fry 1971). The CTM method thus quantifies the effect of temperature on metabolic rate while the ILT quantifies the mortality of fish at a given temperature without a clear indication of the cause of death (Fry 1971). Furthermore, because the fish need not be handled before the temperature change, the CTM method does not confuse handling stress with thermal stress (Bennett and Judd 1992). For these reasons, the CTM method was used to study the effect of acute temperature changes on the stress response of juvenile *P. commersonnii*.

**Oxygen**

Mobilization of energy reserves requires oxygen, which a fish acquires through respiratory exchange at the gill surface (Houston 1990). The gradient of oxygen tension between fish blood and surrounding water is the driving mechanism for oxygen uptake by the fish (Jobling 1994). In addition, the oxygen concentration in the water indicates how much water must be pumped over the gills in order for the fish to obtain a given amount of oxygen (Fernandes and Rantin 1994). When fish are exposed to reductions in dissolved oxygen concentration a variety of physiological responses that result in increased ventilation rate are initiated. This enables the fish to maintain uniform rates of oxygen uptake independent of dissolved oxygen concentration (Jobling 1994). A resting fish in water with gradually declining oxygen concentration thus maintains a relatively constant rate of oxygen uptake. However, below a species-specific critical oxygen concentration,

oxygen availability becomes a factor that limits the metabolic rate of fish (Fry 1971). A reduction in the supply of oxygen below the critical concentration can therefore reduce the metabolic rate of an organism. Moreover, acute reductions in dissolved oxygen concentration such as might occur as a result of high fish densities or abrupt changes in temperature during fish transport could result in fish mortality (Teo and Chen 1993; Forteath 1993). Knowledge of the critical oxygen concentration of a species is therefore important to the survival of the fish during transportation.

**Handling and packaging**

Fish density and fish-loading capacity are biological criteria for fish culture that are often used in the wrong context due to a lack of proper definition. Fish density, or crowding, describes the behavioural requirements of fish for physical space and is usually expressed in terms of the weight density or weight of fish / unit volume of water, whereas fish-loading capacity defines the maximum number of individuals per unit of space. Both criteria are, however, important in fish transport because economic considerations usually dictate that maximum use is made of both water and space (Wedemeyer 1996). The main objective in the transport of live fish is to maximize the fish-loading capacity of freight packages (Forteath 1993). Exceeding the density tolerance of a given species in the intensive culture systems results in stress in the fish (Wedemeyer 1997). It has also been shown that a high fish density results in stress in fish (Mazur and Iwama 1993; Wedemeyer 1996; Barcellos *et al.* 1999). The effect of density on the stress levels of fish held under optimal water quality conditions is, however, not known. Meanwhile, it has been suggested that the highest levels of stress in fish transport occur during the capture and packing process (Robertson *et al.*, 1988; Mazur and Iwama 1993) but there is little data to support this suggestion. These

experiments presented the opportunity to study the effects of fish density on the stress response in fish. They also enabled the evaluation of the effects of capture and handling on the stress response.

This study was based on the hypothesis that changes in metabolic rate could indicate the existence and the magnitude of stress in fish. Consequently, it was hypothesized that changes in metabolic rate could be used to measure, and subsequently to rank different stressors according to their metabolic costs to fish. In Chapter 4, changes in metabolic rate were used successfully to measure the response of juvenile *P. commersonnii* to a simulated capture and handling stressor. This series of experiments demonstrate the use of metabolic rate measurements to determine the metabolic cost of acute temperature changes, oxygen depletion, and high fish density, and were based on the hypothesis that acute temperature changes, oxygen depletion, and high fish density could lead to an increase in metabolic rate. The objectives of the experiments were, therefore, to study the effect of (1) acute temperature changes, (2) oxygen depletion, and (3) high fish density on the metabolic rates of juvenile *P. commersonnii*. To achieve these objectives, experiments were conducted using juvenile *P. commersonnii* previously kept at a constant temperature of 25 $^{\circ}$ C, which is close to the temperature preferendum of the fish (Deacon and Hecht 1995), for at least 21 days. The standardised experimental procedures for measuring oxygen consumption (see Chapter 2) were followed before subjecting the fish to these stressors.

## Materials and methods

### Determination of the critical thermal maximum (CT max) and minimum (CT min)

The critical thermal maximum and minimum for juvenile *P. commersonnii* was determined following the methods of Currie *et al.* (1998). Sixty experimental fish were obtained from the Port Alfred field station (see Chapter 2), and moved to the Grahamstown laboratory for experimentation. In the laboratory, the fish were separated into two groups of 30 fish each. The first group of fish was used to determine the thermal maximum and minimum, whereas the second group was used to study the effect of acute temperature changes on oxygen consumption rate. The two groups were further subdivided into two groups of 15 fish each to facilitate the sampling process. All fish were acclimated at $25 \pm 1$ ° C for at least 21 days before experimenting. To determine the critical thermal levels, 30 fish (wt = $14.3 \pm 1.3$ g, mean ± S.D.) were netted individually from a holding tank and placed into a 45 cm (L) x 30 cm (W) x 25 cm (D) shaded glass aquarium containing water drawn from the same tank. The fish were allowed approximately 1h 30 min to recover from the effect of handling before determining the critical thermal levels. CT max was determined by increasing water temperature at a constant rate of $0.3\ ^{\circ}C\ min^{-1}$ using thermostatic heaters placed at the centre of the aquarium tank, whereas CT min was determined by reducing water temperature at the same rate by adding chilled water. Aerators placed at the centre and the corners of the tank allowed continuous mixing of the water. Loss of equilibrium (LOE) was chosen as the end point for determining the critical thermal levels. Consequently, during the trials water temperature was increased using thermostatic heaters, or decreased by adding chilled water at a constant rate until the fish lost its equilibrium. Fish were deemed to have lost equilibrium when they were unable to maintain a dorso-

ventral position and kept rolling from side to side. Once a fish reached this endpoint, the temperature was measured to ± 0.1 ° C using an Oxyguard Gamma (Oxyguard International A/S, Denmark) temperature and oxygen meter. These LOE endpoint determinations were repeated 10 times with different fish. The CT max and CT min were calculated as the arithmetic mean of the respective ten endpoints.

**Effect of acute temperature changes on oxygen consumption rate**

Experiments were conducted to study the effect of acute temperature increase (heat stress) and reduction (cold stress) on the oxygen consumption rate of juvenile *P. commersonnii*. Prior to conducting these experiments oxygen consumption of ten fish (wt = 14.9 ± 2.4 g, mean ± S.D.) selected at random from the holding tanks was measured at the acclimation (25 ± 1 ° C) temperature. The ensuing oxygen consumption rate was regarded as the control (pre-stress) oxygen consumption rate. To study the effect of heat stress on metabolic rate, five *P. commersonnii* were introduced individually into the respirometer chamber where they were acclimated at 25 ± 1 ° C for at least 12 h. The fish had not been fed for at least 24 h prior to the introduction. Before measuring oxygen consumption the fish were treated to an acute temperature elevation whereby water temperature was increased from 25 ° C to 32 ° C at a constant rate of 0.3 ° C / min following the procedure used to determine the thermal limits for the fish. The heating process lasted approximately 20 minutes. After the test temperature (32 ° C) was reached, oxygen consumption was measured at half-hourly intervals for 3 hours following the procedures outlined in chapter two. Oxygen measurements were recorded every 15 seconds for 20 minutes, and only the data collected during the last five-minutes were used to determine the oxygen consumption rate for each fish. Oxygen consumption during a given measurement interval

was calculated according to the formula: $\dot{m}o_2 = V_r * \delta Cwo_2 / dt * W$ (Steffensen 1989). The overall oxygen consumption rate for that interval was then calculated as the arithmetic mean of the oxygen consumption measurements for the five fish.

The same procedures used to investigate the effect of heat stress were used to investigate the effect of cold shock on oxygen consumption rate. Experimental fish were acclimated overnight. Before measuring oxygen consumption, water temperature was decreased from 25 $^{o}$ C to 17 $^{o}$ C at a rate of 0.3 $^{o}$ C / min by adding chilled water. The chilling process lasted for 20 minutes. Oxygen consumption was subsequently measured for 3 hours at the same intervals as described above and was repeated for the same number of replicates. Oxygen consumption rate was determined as outlined in the previous section. One-way analysis of variance was used to examine the changes in the metabolic rate as a function of time after application of the stressor. Post-hoc analysis was done using the Schaffé test.

**Effect of oxygen depletion on oxygen consumption rate**

These experiments were conducted using the respirometer as a closed system. Thus, during the experiment the fish were subjected to a continuous depletion in dissolved oxygen concentration in the respirometer chamber. To study the effect of oxygen depletion on oxygen consumption rate, five fish (wt = 14.9 ± 2.4 g, mean ± S.D.) were introduced singly into the measuring chamber where they were acclimated for at least 12 h at 25 ± 1 $^{o}$ C. Before measuring oxygen consumption, water flow through the respirometer was stopped and oxygen concentration in the respirometer was allowed to deplete with time until the fish lost equilibrium. Dissolved oxygen concentration was recorded every 15 seconds for the entire period. The concentration at which the fish

showed the first indications of abnormal behaviour such as debilitation and unbalanced swimming movements was also recorded.

There is no set method for the determination of critical oxygen concentration, partly because the initial depression of metabolic rate is often not clearly delineated (Prosser 1973). Different methods have, therefore, been used to calculate the critical oxygen concentration (Marvin and Heath 1968; Ultsch *et al.* 1978; Schurmann and Steffensen 1997). For purposes of analysis, oxygen consumption rate of juvenile *P. commersonnii* under declining dissolved oxygen concentrations was calculated for every two-minute interval using the formula given in the previous section. The oxygen consumption data were analyzed by breakpoint regression analysis to determine the point at which metabolic rate began to decline. The critical oxygen concentration was demarcated by the point of intersection of the two estimated models.

**Effect of fish density on oxygen consumption rate**

Oxygen consumption measurements were performed on juvenile *P. commersonnii* at three different fish densities (13, 23, and 50 g $l^{-1}$). A 1.5-L bell jar was used as the respiratory chamber. The test fish (wt = 12.4 ± 1.7 g) were acclimated to the respirometer overnight singly or as groups. After the acclimation, oxygen consumption was measured at half-hourly intervals for a period of 3 hours. Oxygen consumption measurements were performed following the procedures outlined in Chapter 2. The experiments were performed in triplicates. One-way analysis of variance was used to examine the differences in oxygen consumption rate between the different fish densities. Normality of the data and the homogeneity of their variances were tested using the Shapiro-Wilks and

the Levene's test, respectively. Post-hoc comparison of the means was done using the Scheffé.

## Results

### Critical thermal maximum and minimum

The CT max for juvenile *P. commersonnii* acclimated at 25 ± 0.7 ° C was 34.5 ± 1.1 ° C (mean ± S.D.), whereas the CT min for the fish was 15.1 ± 2.5 ° C. Loss of equilibrium (LOE) was more evident during the temperature elevation trials in comparison to the temperature reduction trials. As temperature increased, the fish exhibited signs of agitation accompanied by sporadic bursts of activity, which progressed to loss of equilibrium at the critical thermal maximum. During the CT min trials, there was a progressive decrease in fish activity followed by an increase in muscle spasms and irritability. Loss of equilibrium was only evident at near lethal low temperature levels. All experimental fish survived the temperature elevation for at least 24 h after transfer back to fresh seawater of 25 ° C, whereas 12 out of 15 fish died after the cold treatment during the same period.

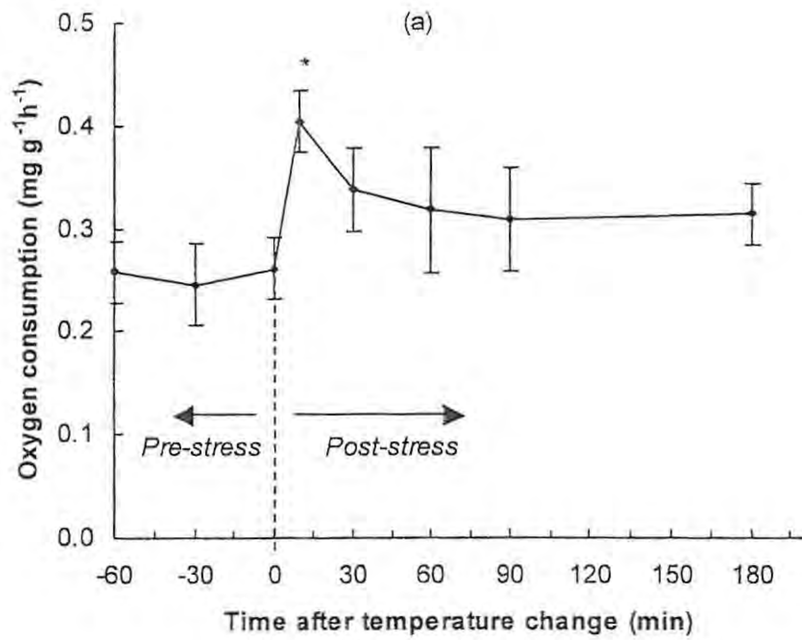
### Effect of acute temperature changes on oxygen consumption rate

Acute temperature elevation induced a stress response in juvenile *P. commersonnii* that resulted in a significant increase in metabolic rate (ANOVA: $F = 8.95$, $p = 0.001$) that peaked approximately 15 minutes after the temperature elevation (Fig 5.1 a). During the stress response, oxygen consumption increased by 60%, from 0.25 ± 0.03 mg $g^{-1}h^{-1}$ to 0.4

$\pm$ 0.06 mg $g^{-1}h^{-1}$, before declining to 0.34 $\pm$ 0.02 mg $g^{-1}h^{-1}$ after 3 hours. An acute temperature decrease resulted in a significant reduction in the oxygen consumption rate (ANOVA: $F = 2.6$, $p < 0.05$, Fig. 5.1 b).

**Effect of oxygen depletion on metabolic rate**

Juvenile *P. commersonnii* maintained a uniform rate of oxygen consumption (0.26 $\pm$ 0.07 mg $O_2$ $l^{-1}$) at dissolved oxygen concentrations between 7.0 mg $O_2$ $l^{-1}$ and 2 mg $O_2$ $l^{-1}$. Below 2 mg $O_2$ $l^{-1}$, oxygen consumption decreased linearly with further decrease in dissolved oxygen concentration and the fish became progressively more agitated and rapidly lost equilibrium. The initial point of depression of oxygen consumption rate for juvenile *P. commersonnii* determined by piecewise linear regression analysis with breakpoint was 2.6 mg $O_2$ $l^{-1}$ ($r^2 = 0.84$). This was a close approximation to the critical dissolved oxygen concentration (2.1 mg $O_2$ $l^{-1}$) determined using a combination of criteria (Fig 5.2). The critical oxygen concentration was determined as the point of intersection of the line representing the average oxygen consumption rates with the best-fit line of the declining oxygen consumption trend.



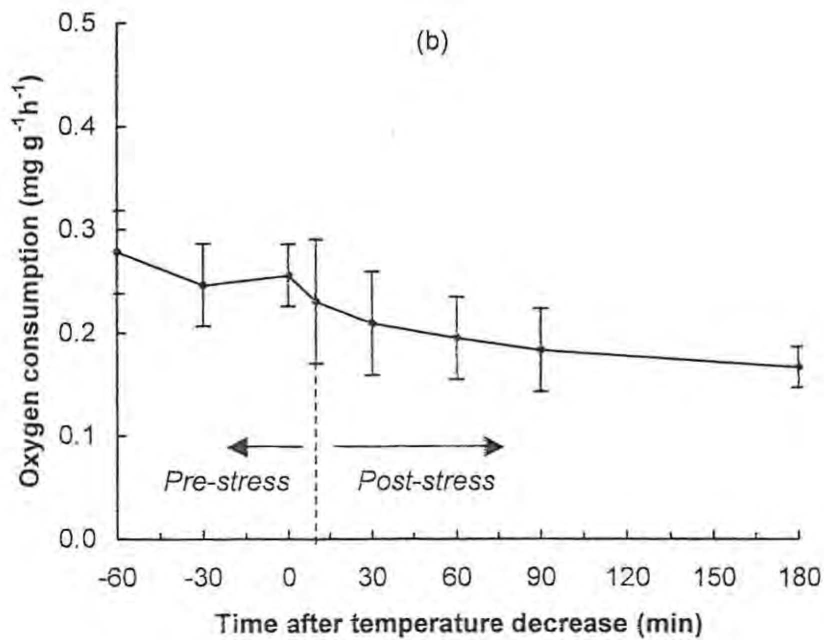




Fig: 5.1. The effects of acute temperature increase (a), and decrease (b) on the oxygen consumption rate of juvenile *Pomadasys commersonnii*: The temperature changes were effected after at least 12 hours acclimation in the respirometer. The data points are mean (± S.D., $n = 5$). The arrows indicate the pre-stress oxygen consumption rates and oxygen consumption rates after the temperature change. The asterisk indicates a significantly different oxygen consumption rate.

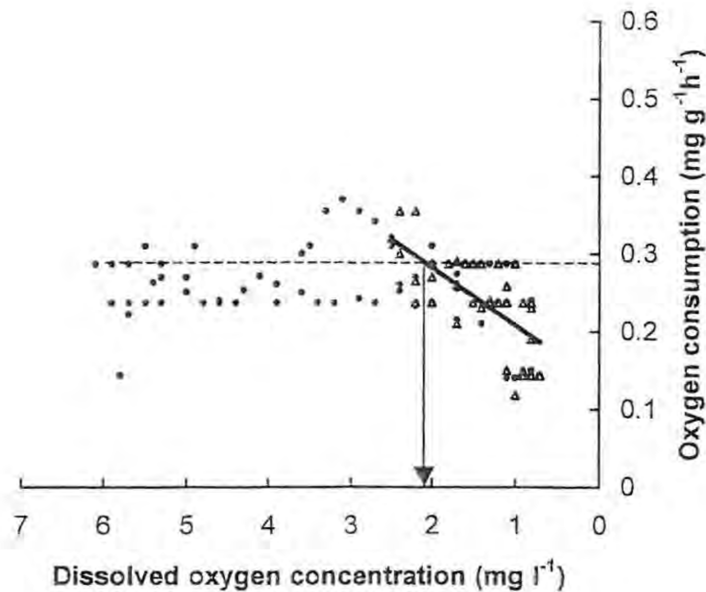



Figure: 5.2. Oxygen consumption rates of juvenile *Pomadasys commersonii* in declining dissolved oxygen concentrations. Data points are mean oxygen consumption rates ($n = 5$) recorded before the onset of observable changes in behaviour or decline in oxygen consumption rate (•), and measurements recorded after the onset of either abnormal behaviour or decline in oxygen consumption rate (▲). The critical oxygen concentration is indicated by arrow. The dashed line represents the average oxygen consumption rates of the fish.

### Effect of fish density on metabolic rate

There were no significant differences in oxygen consumption measurements performed on individual fish or groups of fish (ANOVA: $F = 2.002$, $p = 0.5$). This indicates that the oxygen consumption rate of juvenile *P. commersonnii* was not affected by the presence of conspecifics or by the stocking density within the range tested.

Table 5.1. The effects of density and number of fish on the oxygen consumption rate of juvenile *Pomadasys commersonnii*: Values are total weight of fish (± S.D., $n$ = 3), number of fish in the respirometer.

| Mass (g $l^{-1}$) | No. fish in chamber | Oxygen consumption (mg $g^{-1}$ $h^{-1}$) |
|---|---|---|
| 13.3 ± 1.2 | 1 | 0.26 ± 0.02 |
| 23.0 ± 3.8 | 2 | 0.25 ± 0.01 |
| 50.5 ± 2.1 | 5 | 0.30 ± 0.01 |

## Discussion

At the beginning of this chapter it was noted that different stressors elicit stress responses that may differ in both magnitude and duration. It was hypothesized that the magnitude of the metabolic stress response could be used to measure, and subsequently to rank different stressors according to their effect on the fish. It was suggested that in fish transport, capture and handling might be the most stressful. It was also suggested that acute temperature changes occurring within the thermal limits of a species could elicit abrupt increases in metabolic rate. Oxygen is a limiting factor (Fry 1971) that may only affect metabolic rate at a concentration that is below a given critical level. As such, it was assumed that at non-limiting dissolved oxygen concentrations, oxygen depletion would not elicit a metabolic stress response. By subjecting juvenile *P. commersonnii* to potential transport-related stressors it was possible to estimate the magnitude of the metabolic stress response resulting from the different stressors, and to categorize them according to their relative metabolic costs.

Table 5.2 summarizes the changes in metabolic rate of juvenile *P. commersonnii* after the application of various transport-related stressors, and the metabolic costs of the stressors to the fish. At 25 $^{o}$ C, the average oxygen consumption of undisturbed juvenile *P. commersonnii* was 0.26 mg $O_2$ $g^{-1}h^{-1}$. The metabolic rate of the fish, however, increased to 0.60 mg $O_2$ $g^{-1}h^{-1}$ after capture and handling, reflecting a more than 130% increase in oxygen consumption above pre-stress levels. Oxygen consumption increased to 0.40 mg $O_2$ $g^{-1}h^{-1}$after acute temperature elevation, an increase of about 60% above pre-stress levels. The cost of metabolism attributed to the effects of capture and handling was, therefore, twice as much as that attributed to acute temperature elevation. The elevation of temperature had been effected at a rate of 0.3 $^{o}$ C $min^{-1}$ that was considered slow enough by Becker and Genoway (1979) to parallel the internal body temperature of the fish, but too fast to allow for physiological adjustment by the fish. The increase in metabolic rate was, therefore, most likely as a result of the heat stress and not just a reflection of the controlling effect of temperature on metabolic rate. Acute reduction of temperature resulted in a decrease in metabolic rates, whereas oxygen depletion at dissolved concentrations above the critical level did not affect the metabolic rates of the juveniles of this species. Similarly, increases in fish packing density did not affect the metabolic rates of the fish. Thus, capture and handling and acute temperature elevation resulted in increases in the metabolic rates of juvenile *P. commersonnii*, the magnitude of which depended on the nature of the stressor. Although heat stress resulted in increased metabolic costs to juvenile *P. commersonnii*, capture and handling was the most metabolically costly of the variables investigated. The fish did not incur a metabolic expense as a result of acute decrease in temperature. On the contrary, the acute temperature reduction resulted in a reduction in the metabolic rate of the fish, a

phenomenon that had been attributed to a reduced enzymatic activity by Neill and Bryan (1991).

Table 5.2. Changes in the oxygen consumption rate of juvenile *Pomadasys commersonnii* subjected to potential stressors related to fish transport, and the metabolic cost of the different stressors. The metabolic cost was determined as the percent increase in the oxygen consumption rate of the fish after stressor application.

| Stressor | Oxygen consumption rate (mg $g^{-1}h^{-1}$) | | Metabolic cost (%) |
|---|---|---|---|
| | Before | After | |
| Capture and handling | 0.26 ± 0.05 | 0.60 ± 0.06 | 130 |
| Heat stress | 0.25 ± 0.03 | 0.40 ± 0.06 | 60 |
| Cold shock | 0.25 ± 0.03 | 0.18 ± 0.04 | -17 * |
| Fish density | 0.26 ± 0.02 | 0.30 ± 0.01 | 15 * |
| Oxygen depletion | 0.26 ± 0.07 | 0.26 ± 0.07 | - |

* The asterisk indicates non-significant changes in oxygen consumption rate

Note: The negative symbol indicates a decrease in metabolic rate after the temperature reduction

Capture and handling and heat stress may involve considerable amounts of fright and discomfort that results in a response akin to the general adaptation syndrome proposed by Selye (1973). The metabolic responses to the two stressors are, therefore, likely to be similar in many respects. Following the application of both capture and thermal stress there was a rapid increase in oxygen consumption rate of juvenile *P. commersonnii*. In both cases the stress response lasted approximately 15 minutes. The rapid elevation in metabolic rate immediately after application of the stressor followed by the gradual

adjustment until a steady state was regained was typical of the GAS response of stenothermic poikilotherms (Jobling 1994).

Temperature controls the velocity of organic reactions in fish, directly affecting their respiratory metabolic rate (Fry 1971). Generally, a decrease in temperature results in a reduced metabolic rate due to a lowered metabolic demand (Fry 1971; Brett and Groves 1979). Although an acute decrease in temperature is reported to result in a transient elevation of metabolic rate similar to heat stress (Jobling 1994) there was no such response in juvenile *P. commersonnii*. The effects of temperature on the physiology of fishes changes have mostly been studied by abrupt transfer of fish acclimated at one temperature to the various test temperatures (Chow *et al.* 1994; Currie *et al.* 1998). The transfer, however, involves a degree of stress as a result of capture and handling. During this study reduction of water temperature was instituted through displacement by adding chilled seawater to the water bath. This ensured that physical stress was eliminated as a possible source of additional stress to the fish. It is, therefore, suggested that a temporary elevation in oxygen consumption as a result of temperature reduction, as suggested by Jobling (1994), may have been due to physical stress rather than the effect of a decrease in temperature.

When fishes are exposed to reductions in dissolved oxygen concentration a variety of physiological responses may occur that result in modifications in ventilation rate, thereby enabling the fish to maintain a uniform metabolic rate (Jobling 1994). These non-conformer fishes exhibit oxygen consumption rates that are independent of ambient dissolved oxygen concentration; at least until critical dissolved oxygen concentration is reached (Jobling 1982). Below this level, normal aerobic respiration can no longer be

maintained and oxygen consumption decreases with further decline in dissolved oxygen concentration (Fry 1971). Under such conditions, the fish frequently show avoidance responses just before the onset of respiratory distress (Jobling 1994; Heath 1995; Schreck *et al.* 1997). *P. commersonnii* exhibited uniform oxygen consumption rates that were independent of the ambient dissolved oxygen concentration and can, therefore, be classified as a non-conformer. Oxygen consumption of juvenile *P. commersonnii* was not affected at dissolved oxygen concentrations above 2 mg $l^{-1}$. Below this level, however, the oxygen consumption rate decreased with further decreases in dissolved oxygen concentration. With respect to oxygen depletion, therefore, the metabolic response of juvenile *P. commersonnii* followed the same patterns as those reported for many teleost fishes (Jobling 1982; Robertson *et al.* 1988, Iwama and Mazur 1993, Pankhurst *et al.* 1992; Crocker and Cech 1997; Maxime *et al.* 2000). Since juvenile *P. commersonnii* began to show signs of distress like gasping and intense struggling at a concentration of 2 mg $l^{-1}$, the critical oxygen concentration for juveniles of this species was assumed to be between 3 and 2 mg $l^{-1}$.

Metabolic costs are known to increase with the intensity of struggling (Davies and Schreck 1997) and are usually reflected in increases in metabolic rate. Although juvenile *P. commersonnii* did not elicit an increase in metabolic rate below the critical oxygen concentration, this does not necessarily mean there were no metabolic costs associated with hypoxia. On the contrary, the intense struggling by juvenile *P. commersonnii*, which might have been associated with an avoidance response, indicated that the fish spent considerable amounts of energy during the phase of extreme hypoxia. Although speculative, it may be suggested that this energy may have been generated by the fish

through anaerobic respiration and could therefore be reflected as a post-exercise oxygen debt (Jobling 1994) that can only be measured during the recovery period.

There were no significant differences in oxygen consumption by juvenile *P. commersonnii* measured either individually or in groups. Oxygen consumption rate was therefore not affected by the presence of conspecifics or by stocking density within the range tested. Results from several studies are also inconclusive with regard to the effect of density on metabolic rate. For example, when examining respiratory metabolism data on 15 species, Parker (1973) found that individuals in a school of fish experienced a reduction in oxygen consumption. Staurnes *et al.* (1994), on the other hand, found no density-related differences in oxygen consumption during transport simulations with Atlantic cod, *Gadus morhua*. The seemingly density-independent oxygen consumption observed in juvenile *P. commersonnii* need to be ascertained for other species.

Because of the activity-related cyclic fluctuations in oxygen consumption rate of fish (Brett 1964; Du Preez *et al.* 1986; Deacon and Hecht 1995), metabolic changes can occur as a result of heightened activity and might not necessarily represent a stress response. The absence of a metabolic response by juvenile spotted grunter to hypoxia and packing density, therefore, does not mean that these factors do not cause stress in the fish. Indeed, Davis and Schreck (1997) suggested that metabolism associated with either exercise or hypoxia was the primary cause of the elevated oxygen consumption observed during acute handling of juvenile coho Salmon. Wieser *et al.* (1985) and Lackner (1988) further suggested that intensive struggling by fish probably resulted in a metabolic disturbance similar to that caused by exhaustive exercise in which whole body lactate concentrations were nearly five times higher than in undisturbed fish and similar to levels in exhausted

fish. The lack of a metabolic response to hypoxia could also be because the fish is not able to extract oxygen from the water at critically low external partial pressure (Jobling 1994).

In summary, the results obtained in this study confirmed that the highest energetic cost to juvenile *P. commersonnii* was incurred as a result of capture and handling. Heat stress resulted in considerable elevations in the metabolic rate of the fish, whereas acute temperature reduction, oxygen depletion and crowding did not affect oxygen consumption rate. Oxygen consumption by individual fish was not affected by the presence of their conspecifics. The hypothesis on which this work was based was that changes in metabolic rate could be used to measure the stress response in fish and that the magnitude of the metabolic stress response could be used to rank different stressors according to their physiological impact on fish. It was demonstrated that by using metabolic rate measurements it was possible to measure the relative metabolic costs of various transport-related stressors and to rank them according to their metabolic costs to the fish. The findings outlined in this chapter have therefore demonstrated the effectiveness of metabolic rate as a measure of the acute stress response in fish.

# CHAPTER 6

## Effect of 2-phenoxyethanol on the stress response of juvenile *P. commersonnii*

### Introduction

In fisheries and aquaculture, anaesthetics are used to reduce the activity of fish during capture and to immobilize them for easier handling (Ross and Ross 1984; Marking and Meyer 1985). Some anaesthetics, e.g., 2-phenoxyethanol (Teo and Chen 1993), have also been reported to reduce the metabolic rate of fishes. However, anaesthetic procedures have been shown to cause elevations in plasma cortisol similar to those normally associated with acute stress (Barton and Peter 1982). For example, Robertson *et al.* (1988) reported an increase in the magnitude of the cortisol stress response in red drum, *Sciaenops ocellatus*, transported using Tricaine (MS-222). Cho and Heath (2000) also reported an increase in plasma cortisol levels in juvenile chinook salmon, *Oncorhynchus tshawytscha*, treated with the same chemical. An elevation in plasma cortisol level was also observed in Matrinxa, *Brycon cephalus*, treated with Benzocaine, a common fish anaesthetic (Urbinati and Carneiro 2001). Because they reduce metabolic rate, but at the same time cause an increase in plasma cortisol concentration, many fish anaesthetics may act both as a stressor, and a remedy for the effects of acute stress on fish. Consequently, before recommending their use for prolonged anaesthesia, it is prudent to evaluate their effects on the stress response in fish. An overview of fish anaesthesia, and the physiological effects of the

commonly used fish anaesthetics is necessary to be able to evaluate the effects of 2-phenoxyethanol on the stress response of juvenile *P. commersonnii*.

**The use of anaesthetics in fisheries and aquaculture**

Anaesthetics are chemical or physical agents that produce sedation, surgical anaesthesia or narcotic death depending on the dose and duration of exposure to the agent (Rang *et al.* 1995). The level of anaesthetization depends on the purpose for the anaesthetization. Anaesthetics may calm or cause loss of mobility and sensation, or both, with or without loss of consciousness (Summerfelt and Smith 1990) by preventing the initiation and conduction of nerve impulses (Ross and Geddes 1979; Ross and Ross 1984; Summerfelt and Smith 1990; Rang *et al.* 1995). Behavioural changes that occur in fishes during anaesthesia are characterized in Table 6.1. The terminology and definitions that are commonly used to describe anaesthesia and related phenomena in fish are listed in Table 6.2.

A good fish anaesthetic should be water soluble or easily dissolved in any other non-toxic solvent solution (Summerfelt and Smith 1990). It should be efficient at low doses, the toxic dose considerably exceeding the effective dose, in order to provide a wide safety margin (Ross and Ross 1984). The anaesthetic should require a short induction time, and its effects should be easily reversible (Ross and Ross 1984; Summerfelt and Smith 1990). It should also be readily available and cost-effective. For purposes of this study, the effective sedative concentration is defined as the concentration that induces sustained sedation (Stage II, Table 6.1) for up to 24 hours. At this stage of anaesthesia fish show a total loss of reactivity to external stimuli,

except to strong pressure, while maintaining normal equilibrium. They also exhibit a slight decrease in opercular movement.

Table 6.1. Behavioural changes that occur in fishes during anaesthesia: Levels of anaesthesia considered valuable to fisheries work are in bold type (Adapted from MacFarland 1960).

| Definable levels of anaesthesia | | Behavioural responses of fish |
|---|---|---|
| Stage | Description | |
| 0 | Normal | Reactive to external stimuli, equilibrium and muscle tone normal |
| I | Light sedation | Slight loss of reactivity to external stimuli (visual and tactile) |
| **II** | **Deep sedation** | Total loss of reactivity to external stimuli except strong pressure, slight decrease in opercular movement |
| III | Partial loss equilibrium | Partial loss of muscle tone, fish react only to very strong tactile and vibrational stimuli, rheotaxis present, but swimming capability seriously disrupted, increased opercular movement |
| **IV** | **Deep anaesthesia** (Total loss equilibrium) | Total loss of muscle tone, fish react only to deep pressure stimuli, opercular movement below normal |
| **V** | **Loss of reflex reactivity** | Total loss of reactivity, respiratory rate very slow, heart rate slow |
| VI | Medullary collapse | Respiratory movements cease, followed several minutes later by cardiac arrest |

Table 5.2. The terminology and definitions that are commonly used with fish anaesthetics.

| Terminology | Definition | Source |
|---|---|---|
| Anaesthetics | A chemical or physical agent that produce sedation, surgical anaesthesia or narcotic death depending on the dose and duration of exposure to the agent | Rang *et al.* 1995 |
| Anaesthesia | The loss of sensation resulting from pharmacological depression of nerve functions | Summerfelt and Smith 1990; Rang *et al.* 1995 |
| Efficacy | The effectiveness of an anaesthetic: defined in terms of induction time, recovery time and survival rate | Summerfelt and Smith, 1990 |
| Effective concentration | The concentration that induces deep anaesthesia within a given period of time | MacFarland, 1960; Ross and Ross, 1984 |
| Safety margin | The difference between the concentration needed for effectiveness and that which is toxic | Ross and Ross, 1984; Summerfelt and Smith, 1990 |
| Induction time | The time taken to reach a given stage of anaesthesia | " |
| Exposure time | The total time the fish is in contact with the anaesthetic solution | " |
| Recovery time | The time taken by the fish to resume full mobility after it is removed from the anaesthetic solution | " |

The efficacy of an anaesthetic can be altered or mediated by both biological factors such as fish species and body size, lipid content, etc., and environmental factors including temperature, pH, salinity and mineral content in the water (Ross and Geddes 1979). The induction time depends on the rate of uptake of the anaesthetic, which in turn is related to the gill surface: body-weight ratio (Hughes 1989; Oikawa *et al.* 1994). Induction time can, therefore, vary considerably between species. Also

within a given species, there is a direct relationship between the effective concentration and fish size (Oikawa *et al.* 1994). Anaesthetics seem to act principally on the cell membrane (Rang *et al.* 1995). By dissolving in the membrane lipid, anaesthetic drugs alter the function of the membrane. The interaction of anaesthetic molecules with hydrophobic domains of membrane proteins (ion channels, receptors etc.) may also affect their functions by disrupting the normal mechanisms by which the ion permeability of the membrane is controlled. Changes in temperature may affect the physico-chemical passage of the anaesthetic into the fish (Ross and Ross 1984) and thereby affect the efficacy of the anaesthetic (Summerfelt and Smith 1990).

A wide variety of drugs have been used as anaesthetics for fish. However, their use has been limited by lack of knowledge about their specific effects at the physiological level or because of other undesirable effects (Ross and Ross 1984; Guilderhus and Marking 1987; Summerfelt and Smith 1990). Several chemical anaesthetics have been used for sedation of fish and shellfish with variable success. They include Tricaine, Quinaldine, Benzocaine, and 2-phenoxyethanol (Guilderhus and Marking 1987; Summerfelt and Smith 1990; Teo and Chen 1993;Urbinati and Carneiro 2001). Others are Etomidate, Metomidate, clove oil, Magnesium sulphate, Magnesium chloride and carbon dioxide (Marking and Meyer 1985; Malmstrom *et al.* 1993; Guo *et al.* 1995; White 1995; Cho and Heath 2000). Of these chemicals, only Tricaine and carbon dioxide are used with food fish (Summerfelt and Smith 1990).

Tricaine (MS-222) or 3-aminobenzoic acid ethyl ester methanosulphate is a water and lipid soluble chemical that is highly effective in fish sedation (Guilderhus and

Marking 1987). Like other fish anaesthetics, MS-222 is excreted from fish by diffusion through the gills or by coupling to specific cell membrane transport systems (Hunn and Allen 1974). However, because of its acidic nature, MS-222 causes irritation to the fish due to low water pH. Tricaine has also been linked to an increase in plasma cortisol during fish transport (Robertson *et al.* 1988). Other consequences of the use of MS-222 are hypoxia, hypercapnia and changes in blood electrolytes (Ross and Ross 1984; Summerfelt and Smith 1990).

Quinaldine is only slightly soluble in water but is readily soluble in acetone and ethanol. Although its low water solubility may prove useful in the prevention of overdose, it has the potential for accumulation in lipid-rich areas of the body such as the brain (Summerfelt and Smith 1990). Quinaldine does not suppress stress effects, is irritable to mucous membranes and may cause damage to gills (Summerfelt and Smith 1990). It also has a longer induction time than MS-222 (Guilderhus and Marking 1987). Benzocaine is hardly soluble in water but readily soluble in acetone or ethanol (Summerfelt and Smith 1990). Although it has fewer side effects than MS-222 (Ross and Geddes 1979), it is less effective than MS-222 and Quinaldine (Ross and Lindsay 1979; Ross and Ross 1984). It is also reported to have resulted in high mortality, especially in shellfish (Summerfelt and Smith 1990; White 1995). 2-phenoxyethanol is moderately soluble in water but freely soluble in methanol. Its sedative properties are reportedly good at low concentration, but recovery from its effects is sometimes abrupt (Summerfelt and Smith 1990). A wide margin of safety has been reported for the drug (Weyl *et al.* 1996). It can also remain active in solution for more than 3 days (Ross and Ross 1984). Although 2-phenoxyethanol has lately

come into much use in fish anaesthesia, information on its efficacy and safety is scanty.

Several attempts have been made to determine the efficacy of 2-phenoxyethanol as a fish anaesthetic but with conflicting results (Gilderhus and Marking 1987; Teo *et al.* 1987; Teo and Chen 1993; Guo *et al.* 1995; Weyl *et al.* 1996; Deacon *et al.* 1997; Kaiser and Vine 1998; Tort *et al.* 2002). Different concentrations of the anaesthetic have been reported to be effective for the same species (Gilderhus and Marking 1987; Teo *et al.* 1989; Teo and Chen 1993; Weyl *et al.* 1996; and Deacon *et al.* 1997). Many questions therefore arise with respect to use of this anaesthetic in fisheries. For instance, what is the effective concentration of 2-phenoxyethanol and what is the sustainable sedative concentration of the anaesthetic? Does fish size affect this dose? Does 2-phenoxyethanol have an impact on the stress response in fish?

This study was based on two general hypotheses: (1) that changes in metabolic rate could indicate the existence and the magnitude of stress in fish, and as such metabolic rate could be a good indicator of the stress response, and (2) that 2-phenoxyethanol could reduce the effects of stress on fish and could, therefore, be a suitable anaesthetic for use in fish transport procedures. The suitability of metabolic rate as an indicator of the stress response in fish was demonstrated in Chapter 4, and was further tested in Chapter 5 to measure the effects of various potential transport-related stressors on the stress response of juvenile *P. commersonnii*. To test the second hypothesis, this chapter examines the effects of 2-phenoxyethanol on the stress response of juvenile *P. commersonnii*. Since it was established that changes in metabolic rate could indicate the magnitude of a stressor (Chapter 4), a reduction in

the magnitude of the metabolic stress response as a result of the application of anaesthetic would, therefore, indicate the usefulness of the anaesthetic in mitigation of the physiological effects of stress. On the other hand, an increase in the plasma cortisol concentration of the fish as a result of the anaesthetic would suggest that it was a source of stress to the fish. Consequently, this study was based on the hypothesis that 2-phenoxyethanol could reduce the metabolic and the plasma cortisol stress responses in fish. Determination of the efficacy of 2-phenoxyethanol was essential to establish its suitability as a fish anaesthetic.

The objectives of this study were, therefore:

- To investigate the efficacy of 2-phenoxyethanol as anaesthetic for juvenile *P. commersonnii*, and
- To study the effect of the anaesthetic on the stress response of juvenile *P. commersonnii*.

To achieve these objectives the following tasks were performed:

- Evaluation of the efficacy of 2-phenoxyethanol,
- Determination of the effective sedative concentration of this anaesthetic for juvenile *P. commersonnii*,
- Quantification of the effect of the drug on the oxygen consumption rate and plasma cortisol levels in juvenile *P. commersonnii*.

Induction time is dependent on the species and size of fish (Schmidt-Nielsen 1984; Oikawa *et al.* 1994; Weyl and Kaiser 1996; Deacon *et al.* 1997). It is, therefore, necessary that fish size be taken into account when evaluating the efficacy of a given anaesthetic. Consequently, the effect of size on the rate of anaesthetization of the fish was also investigated.

## Materials and methods

### Determination of the effective concentration of 2-phenoxyethanol

To determine the effective concentration ($C_E$) of 2-phenoxyethanol for (1) deep anaesthesia and (2) long-term sedation of juvenile *P. commersonnii*, five concentrations of the anaesthetic (Assay ≥ 99% GC, Sigma Chemicals; Switzerland): 0.1, 0.2, 0.3, 0.4 and 0.5 ml $l^{-1}$ were tested. Experimental fish were obtained by beach seine netting from the Great Fish River estuary and acclimated to captive conditions before experimenting (see Chapter 2). To determine the effective concentration of 2-phenoxyethanol the fish were netted and placed individually into a 45 cm (L) x 30 cm (W) x 25 cm (D) glass aquarium containing 20 L of a prepared solution of 2-phenoxyethanol. The aquarium was shaded using dark polythene sheets to minimize disturbance to the fish from external sources, e.g., movements of the experimenter. The fish were observed throughout the anaesthetization process, and the time taken to reach deep anaesthesia (Stage IV- McFarland 1960, Table 6.1), was recorded. The fish were deemed to have reached deep anaesthesia when they had attained a total loss of equilibrium and the fish were turned upside-down and opercular movement had become laboured and erratic. At this point the fish were transferred into fresh

seawater and recovery time was recorded for each fish. The same method was used for each of the five concentrations. A total of 30 fish (wt = 6.5 ± 0.2 g, mean ± S.D.) were tested at each concentration. All experiments were performed at a water temperature of 25 ± 0.7 ° C, which is the preferred temperature for juveniles of this species (Deacon and Hecht 1995). The effective concentration for juvenile *P. commersonnii* was considered to be the concentration of 2-phenoxyethanol that induced deep anaesthesia in at least 75% of the fish within 5 minutes, and which had a recovery time of less than 10 minutes. In addition, there should be no mortality 24 h after the 30 min anaesthetization at this concentration (see Guilderhus and Marking 1987; Summerfelt and Smith 1990; Malmstrom *et al.* 1993).

Earlier work by Deacon *et al.* (1997) showed that 0.2 ml $l^{-1}$ 2-phenoxyethanol was the lowest concentration for inducing anaesthesia in juvenile *P. commersonnii*. In preliminary test trials during this study, 0.2 ml $l^{-1}$ 2-phenoxyethanol gave adequate sedation (Stage II-McFarland 1960, Table 6.1) to juvenile *P. commersonnii* for more than 60 minutes without loss of equilibrium. Consequently, 24-h experiments were conducted at this concentration to assess the effect of long-term anaesthesia on the fish. Ten fish were randomly selected from a holding tank and transferred into a 0.2 ml $l^{-1}$ solution of 2-phenoxyethanol in a 100 L partially shaded aquarium. The tank water was mixed and aerated by air diffusers installed at the centre of the tank. Water temperature in the tank was maintained at 25 ± 1 ° C, using thermostatic heaters. Since the effective sedative concentration was defined as the concentration of anaesthetic that can induce sustained sedation (see Table 6.1) for up to 24 h, the fish were kept in the sedative solution for that period, at the end of which mortality and the number of fish that had lost their equilibrium was recorded. The trials were

conducted in triplicate. Mortality and loss of equilibrium were the criteria for assessing the suitability of the anaesthetic for long-term anaesthesia.

### Effect of fish size on anaesthesia induction time

In efficacy experiments conducted at 25 $^{0}$ C, 0.3 ml $l^{-1}$ solution of 2-phenoxyethanol had a 50% induction rate (Stage IV-McFarland 1960) in less than 10 min. This concentration was used to investigate the effect of fish size on the rate of anaesthesia since a lower concentration could not give suitable anaesthesia, whereas a higher concentration could result in an induction time too fast to monitor. Juvenile *P. commersonnii* were obtained by beach seine from the Great Fish River and acclimated to captive conditions before experimenting (see Chapter 2). 39 fish weighing between 8 – 27 g were used for this experiment. To study the effect of fish size on the rate of anaesthesia experimental fish were netted and placed individually into a 45 mm (L) x 30 mm (W) x 25 mm (D) partially shaded glass aquarium containing 20 L of a 0.3 ml $l^{-1}$ solution of 2-phenoxyethanol. The progress of anaesthesia was monitored continuously and the time taken by each fish to reach deep anaesthesia (Stage IV- McFarland 1960, Table 6.1) was recorded. The fish were deemed to have reached deep anaesthesia the moment they became dorso-ventrally inverted.

### Effect of 2-phenoxyethanol on the metabolic stress response

The procedures used to study the effect of 2-phenoxyethanol on the metabolic stress response were identical to those used to estimate the metabolic stress response

(Chapter 4). Sixty juvenile *P. commersonnii* were transported from Port Alfred to the laboratory in Grahamstown where they were kept in 300-L plastic holding tanks at 15 fish per tank. The fish were acclimated for 7 days before experimenting. All experimental trials were conducted at 25 ± 0.7 ° C. In efficacy trials using 2-phenoxyethanol, 0.2 ml $l^{-1}$ 2-phenoxyethanol was found to be the effective concentration for long-term (> 60 min) anaesthetization of juvenile *P. commersonnii*. Consequently, this concentration was used to study the effect of the anaesthetic on the metabolic stress response. The effect of 2-phenoxyethanol on the metabolic stress response was studied by measuring the oxygen consumption of juvenile *P. commersonnii* that were subjected to a simulated capture and handling stressor. Individual *P. commersonnii* (wt = 15.5 ± 2.1 g) were introduced into the measuring chamber at least 12 h before conducting the study. Before measuring oxygen consumption, the water flow through the measuring chamber was stopped. An aliquot of 2-phenoxyethanol was added to the water bath to make a 0.2 ml $l^{-1}$ solution. Air diffusers within the tank continually aerated and maintained a homogenous mixing of the solution. After mixing the solution, the fish were subjected to the simulated capture and handling disturbance by shaking the measuring chamber for less than 2 minutes while under water. After the simulated capture and handling, an aliquot of 2-phenoxyethanol was also injected into the measuring chamber to make a 0.2 ml $l^{-1}$ solution. Oxygen consumption was subsequently recorded every 15 seconds for the first 30 min and thereafter at half-hourly intervals for 90 min. At each interval, oxygen measurements were recorded every 15 seconds for up to 20 min. The measuring chamber was flushed for 10 min between measurements. Flushing was done by opening the outflow of the respirometer chamber and draining the solution into a tank below the water bath. A pre-mixed 0.2 ml $l^{-1}$2-phenoxyethanol solution

was added to top up the water bath as required. A schematic representation of these procedures is given in Fig 6.1.

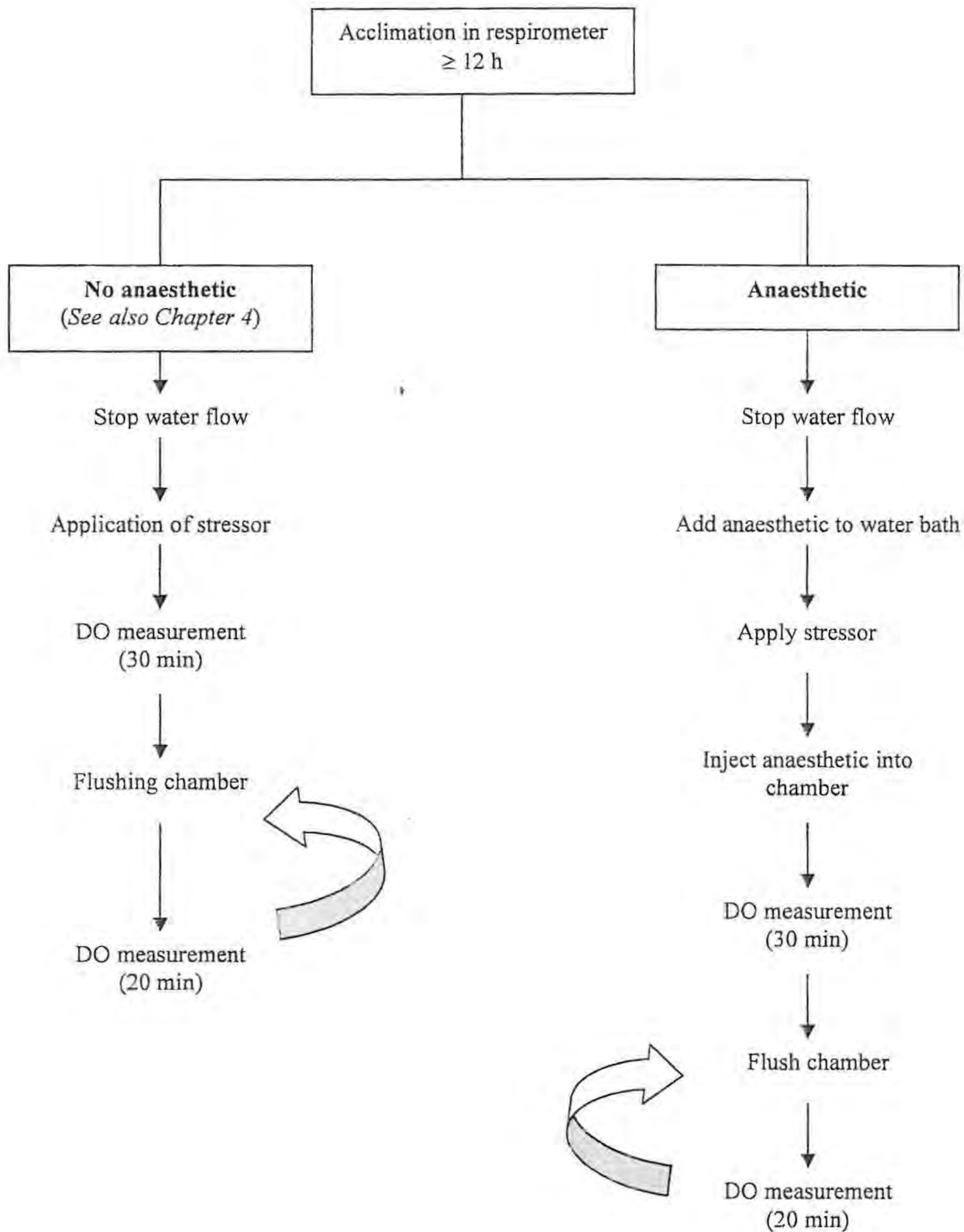



Fig 6.1. Summary of the procedures used to study the effect of 2-phenoxyethanol on the metabolic stress response of juvenile *Pomadasys commersonnii*.

Five replicate trials were conducted in this study. Oxygen consumption rate for each fish was calculated using the measurements recorded during the last five minutes of the measurement period (details given in Chapter 2), while the overall oxygen consumption rate was calculated using the mean (per interval) oxygen consumption rates according to the formula:

$$mo_2 = Vr * \delta Cwo_2 / \delta t \quad \text{(Steffensen 1989)}$$

Where, $mo_2$ = oxygen consumption rate (mg $O^2$ $g^{-1}l^{-1}$); $Vr$ = volume of water in the respiratory chamber (L); $\delta Cwo_2$ = the difference in the oxygen concentration of the water at the beginning and end of measurement; $\delta t$ = duration of measurement.

Measurements for unanaesthetized fish had been obtained during the estimation of the metabolic stress response (Chapter 4). These measurements were used as the reference values since they had been obtained from juvenile *P. commersonnii* from the same batch of fish, following similar procedures as those used to measure the effect of 2-phenoxyethanol on the metabolic stress response.

**Effect of 2-phenoxyethanol on plasma cortisol levels**

These experiments were conducted to study the effect of 2-phenoxyethanol on the cortisol stress response in juvenile *P. commersonnii* and to test whether 2-phenoxyethanol is a stressor to the fish. The test fish were obtained from the Great Fish River at the same time as those used to measure the metabolic stress response. At approximately 30 g (the largest size used to measure oxygen consumption) the

fish intended for cortisol measurements had been transferred to net cages in a sheltered part of the Kowie estuary in Port Alfred. A random sample of 150 fish was taken from this population and moved to the Grahamstown laboratory where they were divided into ten 300-L holding tanks to facilitate the sampling process. The fish were held for a minimum of 7 days before experimenting. The experiments to evaluate the effect of 2-phenoxyethanol on plasma cortisol levels were conducted as follows:

1. Experimental fish were captured without prior anaesthetization and kept in seawater without anaesthetic. The concentration of plasma cortisol in these fish was expected to reflect the effect of capture stress on the plasma cortisol levels of the fish. Since the capture process was identical to the simulated capture and handling stressor described in Chapter 4, the measurements obtained as part of the cortisol stress response were used as reference data for this study.

2. Experimental fish were captured without prior anaesthetization and transferred into a 0.3 ml $l^{-1}$ 2-phenoxyethanol solution, where they were anaesthetized for 60 minutes after which blood samples were taken for cortisol analysis. These fish had already experienced stress before the anaesthetization.

3. 2-phenoxyethanol was added to a tank with undisturbed fish. The fish were anaesthetized in the resultant 0.3 ml $l^{-1}$ 2-phenoxyethanol solution for 15 minutes, after which they were subjected to a simulated capture and handling procedure during which they were netted and taken out of the anaesthetic solution, a process

that lasted for less than 2 min, before they were put back into the solution. Blood samples for cortisol analysis were subsequently taken after 15, 30 and 60 min.

4. 2-phenoxyethanol was added to a tank with undisturbed fish. The experimental fish were anaesthetized for 60 minutes in the resultant 0.3 ml $l^{-1}$ solution before sampling for cortisol.

The procedure used to sample blood for cortisol measurement was similar to that used to estimate the cortisol stress response (see Chapter 4). After the application of a suitable treatment regime, one fish was immediately sampled for blood and represented the control cortisol level for the particular treatment. The remaining three fishes were transferred into separate partially shaded aquaria where they were anaesthetized in 0.3 ml $l^{-1}$ 2-phenoxyethanol solution. Blood samples were taken from these fish 15, 30 and 60 minutes after the treatment. To obtain blood samples, the fish were netted and dipped into a lethal 5 ml $l^{-1}$ 2-phenoxyethanol solution. Blood samples were taken immediately after cessation of opercular movement by severing the caudal peduncle. This process was repeated for ten batches of four fish.

To study the effect of 1 h anaesthesia on plasma cortisol levels (Study 4 above), 10 fish were transferred into a 300-L partially shaded plastic holding tank and left to acclimate for at least 12 h. An aliquot of 2-phenoxyethanol was then added to the tank to make a 0.3 ml $l^{-1}$ 2-phenoxyethanol solution. Diffusers in the tank aerated and maintained homogeneity of the solution. The fish were anaesthetized in the solution for 60 minutes before blood sampling. The fish were sampled one after another in a process that lasted less than ten minutes for the whole batch. Blood samples were

obtained by severing the caudal peduncle as outlined above. Cortisol measurements were done following the procedures outlined in Chapter 4.

### Statistical analysis

Linear regression analysis was used to investigate the relationship between fish size and the time taken to induce deep anaesthesia in the fish. One-way ANOVA with repeated measures was used to study the effect of anaesthesia on the metabolic rate and the plasma cortisol concentrations in the fish, after testing for normality of the data and the homogeneity of their variances using the Shapiro-Wilks and the Levene's test, respectively. Where the data were not normally distributed, a log transformation was performed before the analysis. Statistically different oxygen consumption rates and cortisol concentrations were distinguished by the Scheffé test for significant differences.

## Results

### Effective concentration of 2-phenoxyethanol for the induction of anaesthesia

The lowest concentration of 0.1 ml $l^{-1}$ 2-phenoxyethanol was ineffective in inducing anaesthesia in juvenile *P. commersonnii* since no fish were anaesthetized within the requisite 30 min. The fish elicited normal swimming activity and maintained a normal equilibrium throughout the 30-min observation period. They also showed a quick reaction to a slight tapping of the aquarium walls with the sharp end of a plastic ruler. At a concentration of 0.2 ml $l^{-1}$ less than 25% of the fish reached deep

anaesthesia after 30 minutes exposure to the anaesthetic (Fig 6.1). In the 0.3 ml $l^{-1}$ anaesthetic solution 50% of the fish reached deep anaesthesia within 10 min and all fish were fully anaesthetized within 30 min. In the 0.4 ml $l^{-1}$ 2-phenoxyethanol solution > 75% of the fish were fully anaesthetized after five minutes and all fish reached deep anaesthesia within 10 min. The average recovery time was less than 3 minutes at all concentrations tested. Attempts to determine the rate of anaesthetization at a concentration of 0.5 ml $l^{-1}$ were abandoned because the experimental fish became dorso-laterally inverted immediately on exposure to the anaesthetic solution. This concentration of the anaesthetic was therefore considered too high for juveniles of this species. The effective concentration of 2-phenoxyethanol was defined as the concentration that induced deep anaesthesia in at least 75% of the experimental fish within 5 minutes and which had a recovery time of less than 10 minutes, without any mortality after 30 min anaesthetization. Since these criteria were met at a concentration of 0.4 ml $l^{-1}$, this concentration was therefore determined to be the effective concentration for anaesthetizing juvenile *P. commersonnii.*

No mortality was recorded in fish anaesthetized for 24 h with 0.2 ml $l^{-1}$ 2-phenoxyethanol. At the end of that period all fish remained quiescent eliciting little physical activity under moderate disturbance indicating a state of full sedation. None of the fish showed signs of loss of equilibrium and all fish resumed normal swimming activity in less than 3 minutes after transfer into fresh seawater. Consequently, this concentration of the anaesthetic was considered to be the effective concentration for 24-h anaesthetization of juvenile *P. commersonnii.*

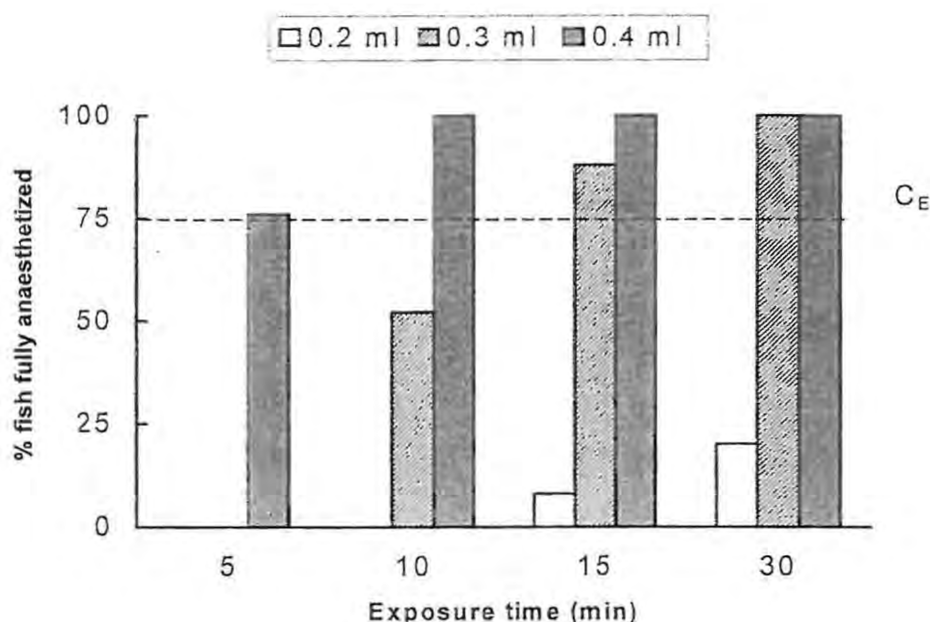



Figure 6.1. The effective concentration ($C_E$) of 2-phenoxyethanol (ml $l^{-1}$) for juvenile *Pomadasys commersonnii*: $C_E$ was determined as the concentration in which at least 75% of the experimental fish were fully anaesthetized (Stage IV-McFarland 1960) within 5 min. Different bars indicate the proportions (%) of fish anaesthetized at different concentrations after 5, 10, 15 and 30 min. the dashed line ($C_E$) represents the threshold value for the effective concentration of the anaesthetic.

### Effect of fish size on the rate of induction of anaesthesia

There was no significant correlation between fish weight and the rate of induction of anaesthesia ($r^2 = 0.001$: $F = 1.028$, $p = 0.3$, Fig 6.2). The average duration taken by fish ranging in size between 8.8 – 26.5 g to reach deep anaesthesia at a concentration of 0.4 ml $l^{-1}$2-phenoxyethanol was 4 min 22 sec ± 1 min 57 seconds (mean ± S.D., $n$ = 39).

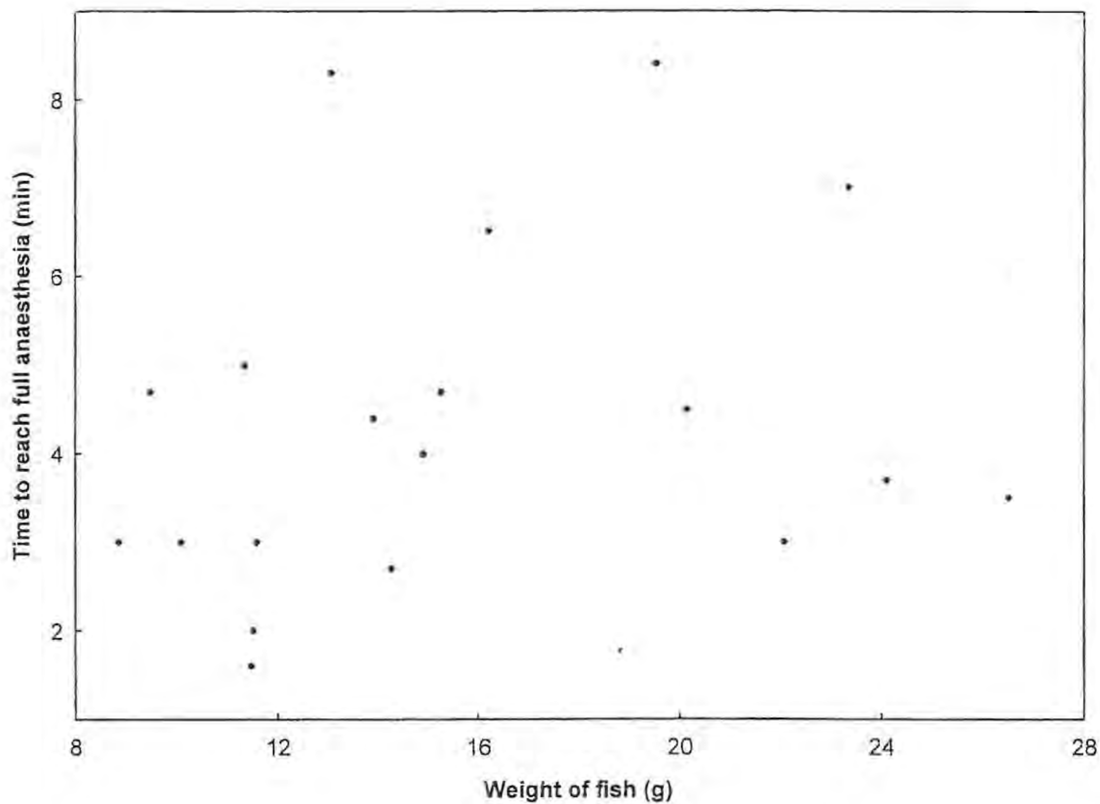



Figure 6.2. The effect of fish size on anaesthesia induction in juvenile *Pomadasys commersonnii*: The fish were anaesthetized using a 0.3 ml $l^{-1}$ 2-phenoxyethanol solution. The data points are the time taken by the fish to reach full anesthesia (Stage IV-McFarland 1960).

### Effect of 2-phenoxyethanol on metabolic rate

Capture and handling resulted in an increase in metabolic rate of juvenile *P. commersonnii* that lasted for approximately 15 minutes (Fig. 6.3). Oxygen consumption rate was 150% higher in the un-anaesthetized fish compared to the fish that were anaesthetized after the disturbance. From an initial rate of 0.26 ± 0.03 mg $O_2$ $g^{-1}h^{-1}$ (mean ± S.D., $n$ = 5), the oxygen consumption rate of un-anaesthetized fish increased to 0.6 ± 0.06 mg $O_2$ $g^{-1}h^{-1}$, whereas oxygen consumption of anaesthetized fish increased to 0.36 ± 0.01 mg $O_2$ $g^{-1}h^{-1}$. The fish that were anaesthetized prior to

capture and handling (undisturbed-anaesthetized fish) exhibited an average oxygen consumption rate of 0.17 ± 0.01 mg $O_2$ $g^{-1}h^{-1}$, a rate that was comparable to that of resting fish (0.16 ± 0.02 mg $O_2$ $g^{-1}h^{-1}$, see Chapter 3). Oxygen consumption in the anaesthetized fish returned to the pre-stress level in approximately 30 minutes, whereas the level in the un-anaesthetized fish did not normalize within 90 minutes.

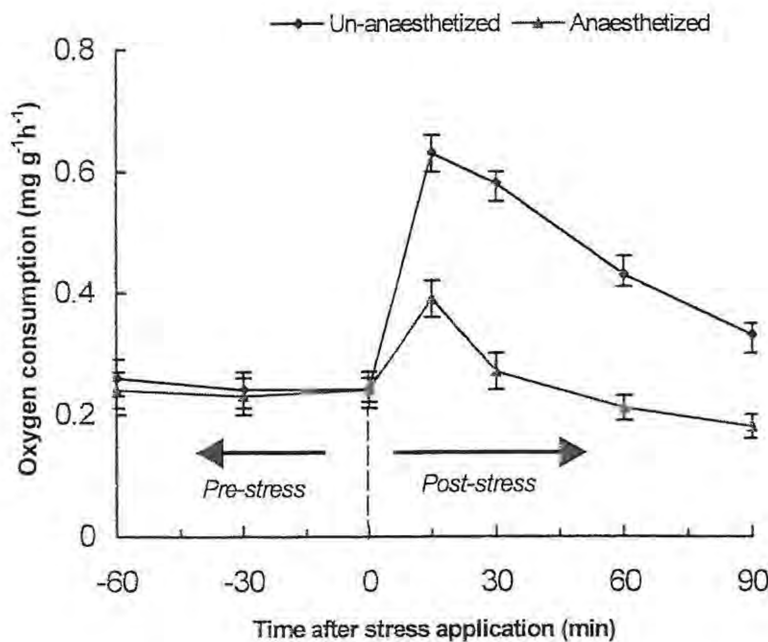



Figure 6.3. Mean (± S.D.) oxygen consumption of juvenile anaesthetized and un-anaesthetized *Pomadasys commersonnii*. The fish were anaesthetized after they were subjected to a 2-min capture and handling stress.

**Effect of 2-phenoxyethanol on cortisol levels**

Analysis of variance showed that plasma cortisol levels in both the un-anaesthetized fish, and in the fish anaesthetized after capture and handling increased significantly following the capture and handling disturbance (ANOVA: $F = 5.38$, $p < 0.001$). From

179.1 ± 83.5 (mean ± S.D.) nmol $l^{-1}$, cortisol concentrations in the un-anaesthetized fish increased to 473.2 ± 184.5 nmol $l^{-1}$, whereas cortisol concentrations in the fish that were anaesthetized after the disturbance increased from 158.9 ± 85.3 to 370.5 ± 174.4 nmol $l^{-1}$, a more than 200% increase in the levels of circulating cortisol in the fish in both cases. The cortisol stress response reached peak levels approximately 15 minutes after capture in both the stressed-anaesthetized and stressed-un-anaesthetized fish. However, pre-stress cortisol levels were re-established in the un-anaesthetized fish in approximately 30 minutes, whereas the plasma cortisol concentration in the anaesthetized fish remained significantly higher (Scheffé test, $p < 0.05$) than pre-tress levels even after the 30-min period. Scheffé multiple range tests also showed that at the time of capture plasma cortisol concentrations in juvenile *P. commersonnii* that were anaesthetized prior to capture were significantly higher than those of resting fish ($p < 0.001$). The cortisol concentrations in the fish anaesthetized prior to capture were, however, comparable to those measured in the stressed-un-anaesthetized fish 15 min after capture (Scheffé test, $p = 0.95$). This suggests that the former was already exhibiting a stress response before the capture.

Undisturbed anaesthetized juvenile *P. commersonnii* had more than five times higher circulating plasma cortisol than the un-anaesthetized controls after 1 h of anaesthetization (Fig 6.5), suggesting that anaesthesia with 2-phenoxyethanol was a major cause of the elevated cortisol levels in the anaesthetized fish. Analysis of variance showed that the method of application of 2-phenoxyethanol, i.e. whether the anaesthetic was applied before or after capture, or not at all had an effect on plasma cortisol levels in the fish ($F = 41.98$, $p < 0.001$). The exposure time also contributed significantly ($F = 7.89$, $p < 0.001$) to the differences in cortisol concentration between

these groups of fish. Although cortisol levels were different between all the three treatment options, it is interesting to note that the lowest cortisol concentrations were measured in the un-anaesthetized fish, while the highest concentrations of cortisol were measured in the fish that were anaesthetized before capture.

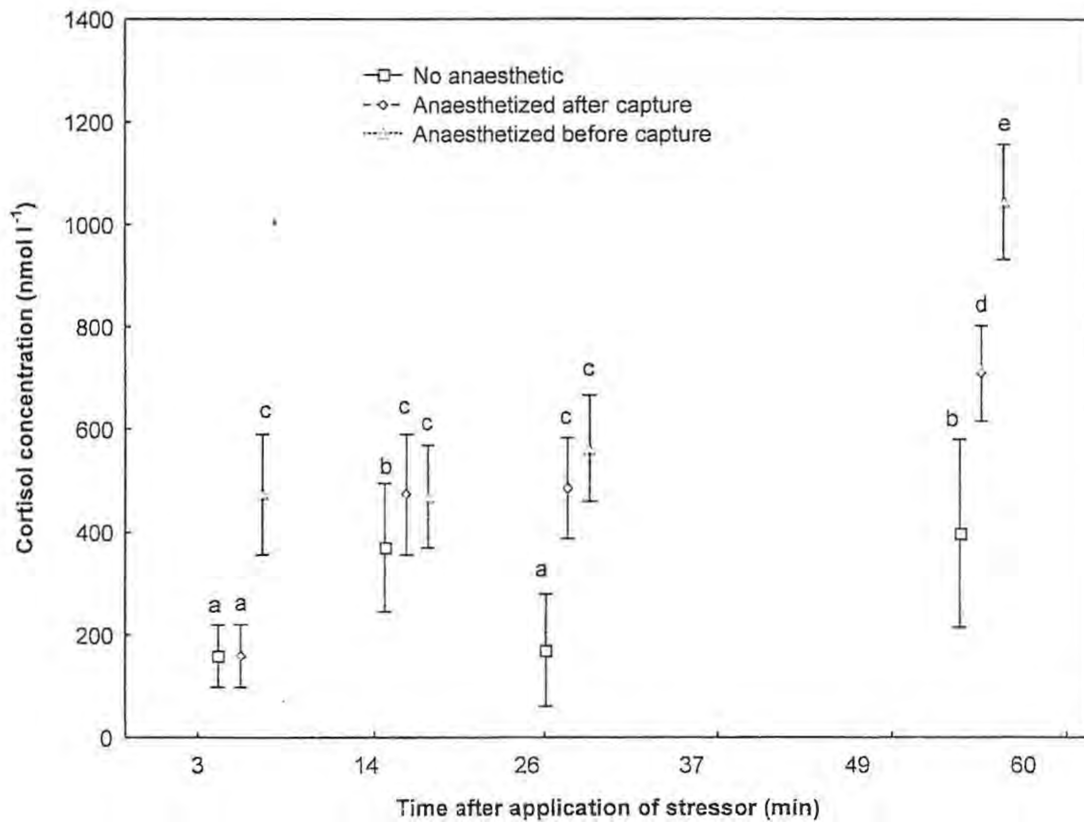



Figure 6.4. Mean ± S.E., and 95% confidence interval ($n$ = $10$) for plasma cortisol concentration in stressed non-anaesthetized juvenile *Pomadasys commersonnii*, and in juvenile *P. commersonnii* anaesthetized with 0.3 ml $l^{-1}$ 2-phenoxyethanol before and after capture and handling. Homologous groups are indicated by similar captions.

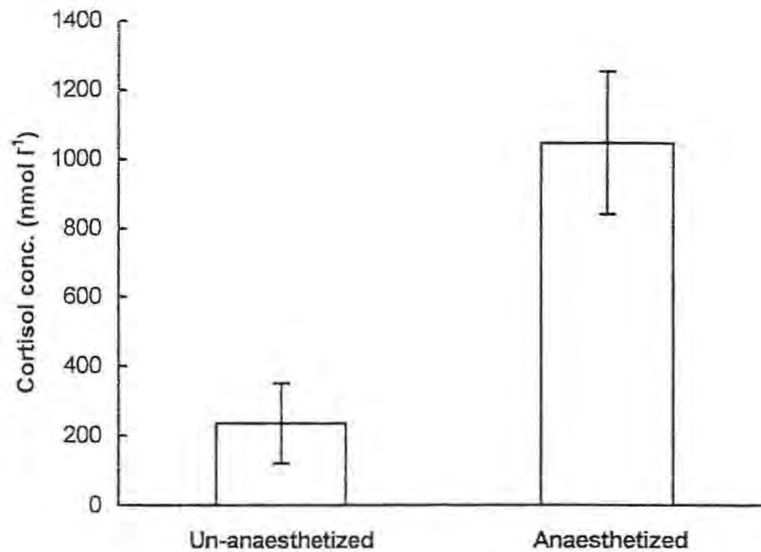



Figure 6.5. Mean (± S.D., *n = 10*) plasma cortisol concentration in undisturbed un-anaesthetized juvenile *P. commersonnii*, and in undisturbed juvenile *Pomadasys commersonnii* that were anaesthetized for 1 h.

## Discussion

*The efficacy of 2-phenoxyethanol and the effect of fish size on anaesthesia induction rate*

The main factors to consider in the anaesthetization of fish are the efficacy of the anaesthetic, the level of anaesthesia desired, and the size of the fish to be anaesthetized (Ross and Ross 1984). At 25 $^{o}$ C, the concentration of 2-phenoxyethanol required to fully anaesthetize (Stage IV, McFarland 1960) juvenile *P. commersonnii* was 0.4 ml $l^{-1}$, and the most appropriate concentration for deep sedation (Stage II, McFarland 1960) of the fish for at least 24 h was 0.2 ml $l^{-1}$. A maximum time of 3 minutes was required by the fish to fully recover from the effects of the anaesthetic. 2-phenoxyethanol, therefore, met the criteria for a good

anaesthetic for juvenile *P. commersonnii*. The efficacy of anaesthetics varies with temperature. For example, Teo and Chen (1993), reported variations in the degree of suppression of oxygen consumption rate of *Poecilia reticulata* by 2-phenoexthanol, with variations in temperature. Weyl *et al.* (1996) also reported differences in the concentration of 2-phenoxyethanol needed to anaesthetize juvenile goldfish, *Carassius auratus*, at different temperatures. During this study, water temperature was maintained at the preferred level for *P. commersonnii*, approximately 25 $^{\circ}$ C (Deacon and Hecht 1995).

Anaesthetics are mainly absorbed by fishes through the gills (Hughes 1989), but can also be absorbed by fish through the skin (Ferreira *et al.* 1984). The rate at which anaesthesia is induced in fish is related to the gill surface: body-weight ratio (Hughes 1989). The rate of induction of anaesthesia can, therefore, vary considerably between different sizes of fish within the same species. In this study, however, fish size had no significant effect on the rate of anaesthesia induction. In an experiment with larger *P. commersonnii* (600 – 1800 g), P.D. Cauley (SAIAB, Personal communication), observed an average induction time of less than 4 min at a concentration of 0.3 ml $l^{-1}$ 2-phenoxyethanol, suggesting that size does not affect the rate of induction of anaesthesia in this species. According to Rang *et al.* (1995), general anaesthetics act by binding to specific sites on the cell membranes of organisms, thus preventing impulse transmission, and thereby reducing activity. These binding sites are likely to be species rather than size dependent. Consequently, once a certain threshold concentration of anaesthetic molecules is available in solution the cross membrane transfer will occur at a constant rate and anaesthesia will be effected at the same rate irrespective of the size of the organism.

Capture and handling resulted in a marked elevation in oxygen consumption rate and the level of circulating plasma cortisol. At the peak of the stress response, oxygen consumption was almost twice as high in the unanaesthetized fish compared to the fish anaesthetized after the disturbance, suggesting that anaesthetization with 2-phenoxyethanol may have reduced the effect of the disturbance on the metabolic rate of this species. Increases in metabolic rate have been linked with the struggling associated with attempts by fish to escape from a perceived stressor (Barton and Schreck 1987; Davis and Schreck 1997; Radull *et al.* 2002). Consequently, if fish were captured without causing them to struggle, such fish would be expected to have metabolic rates that are similar to fish exhibiting routine activity. Juvenile *P. commersonnii* anaesthetized prior to capture exhibited oxygen consumption rates that were comparable to those of the undisturbed fish. These fish had been rendered inactive and thereby unable to struggle during capture. General anaesthetics such as 2-phenoxyethanol exert inhibitory effects on the central nervous system that result in a reduction in activity (Rang *et al.* 1995). The sluggish movements shown by the anaesthetized fish thus suggested that the role of the anaesthetic might be linked to the reduction of physical activity. The reduction in activity and, thus, in the amount of energy required for metabolism could also explain the faster resumption of routine metabolism in the anaesthetized as compared to the non-anaesthetized fish. The reduced activity levels and oxygen consumption rate of anaesthetized fish supports the suggestion by Davis and Schreck (1997) that metabolism associated with exercise may be the primary cause of the elevated oxygen consumption observed following acute handling of fish.

*The effect of 2-phenoxyethanol on the cortisol stress response*

The primary stress response of fishes is measured by the concentration of plasma cortisol (Thomas 1990; Mommsen *et al.* 1999). By comparing the plasma cortisol levels in anaesthetized and unanaesthetized juvenile *P. commersonnii*, therefore, it was possible to assess whether 2-phenoxyethanol was a stressor to the fish. Anaesthetization of juvenile *P. commersonnii* with 0.3 ml $l^{-1}$ 2-phenoxyethanol resulted in an elevated stress response as shown by the increased plasma cortisol concentration in the fish. When subjected to capture and handling stress, unanaesthetized fish took less than 30 min to recover, whereas stress levels remained high in the anaesthetized fish indicating that they may have been experiencing sustained stress. Furthermore, at the time of capture cortisol levels in fish that were anaesthetized prior to capture were the same as those measured in the disturbed fish at the peak of the stress response, suggesting that the anaesthetized fish were already experiencing considerable stress at the time they were captured. Undisturbed juvenile *P. commersonnii* that were anaesthetized for 1 h also had cortisol levels that were five times higher than those measured in undisturbed-unanaesthetized fish. The elevated levels of plasma cortisol in the anaesthetized fish suggested a manifestation of 2-phenoxyethanol as a stressor.

The concentration of circulating cortisol in the blood reflects the net effect of biosynthesis and secretion of the hormone on the one hand and the rate of plasma clearance of the hormone on the other (Mommsen *et al.* 1999). The secretion of cortisol is a neuro-endocrine process controlled by the hypothalamus-pituitary-

interrenal (HPI) axis (Sumpter 1997). The initiation of the cortisol response is therefore dependent on the stimulation of the Central Nervous System (CNS). Anaesthetics cause loss of mobility and sensation by preventing the initiation and conduction of nerve impulses (Rang *et al.* 1995) and as such should prevent the initiation of the cortisol response. The increase in the concentration of circulating plasma cortisol in the anaesthetized and unanaesthetized fish, therefore, supports the observation by Robertson *et al.* (1987) and Cho and Heath (2000), that the degree of CNS depression that may be achieved by light anaesthesia might not necessarily alleviate the primary cortisol response in fish. Exposure to sedating doses of anaesthetics has also been shown to increase plasma cortisol in other fish species (Strange and Schreck 1978; Davis *et al.* 1982; Carmichael *et al.* 1984; Robertson *et al.* 1987; Tort *et al.* 2002). Robertson *et al.* (1987), for example, found that immobilization of red drum, *Sciaenops ocellatus,* with MS-222 prior to capture followed by long-term exposure to sedative doses of the anaesthetic evoked increased plasma cortisol concentrations in the fish. They further reported that anaesthetizing the fish prior to capture and subsequently transporting them in anaesthetic-free water reduced the plasma cortisol response at capture and at the end of transport. Tort *et al.* (2002) also reported an increase in the levels of plasma cortisol in sea bream, *Sparus auratus*, and rainbow trout, *Oncorhynchus mykiss*, treated with 2-phenoxyethanol and clove oil. Barton and Peter (1982) on the other hand found no differences in cortisol concentrations of rainbow trout treated with MS-222 prior to capture and the untreated controls. It is, therefore, unclear whether or not anaesthetics act directly on the stress hormone production.

At 25 °C the concentration of 2-phenoxyethanol required to fully anaesthetize juvenile *P. commersonnii* (Stage IV-McFarland 1960) was 0.4 ml $l^{-1}$ since more than 75% of the fish were fully anaesthetized within 5 minutes at this concentration. On the other hand, the most appropriate concentration for 24-h sedation of the juvenile *P. commersonnii* appeared to be 0.2 ml $l^{-1}$. Although an increase in plasma cortisol concentration is accompanied by a similar increase in metabolic rate, evidence presented by Davis and Schreck (1997) suggest that cortisol has no major effect on metabolic rate. While this may suggest that fish can be transported safely in sedative doses of 2-phenoxyethanol, cortisol levels as high as those measured in the anaesthetized fish may have an immunosuppressive effect, and may leave the fish susceptible to infections and disease. For example, the average mortality of rainbow trout, *O. mykiss*, inoculated with *Aeromonas salmonicida* increased from about 40% in unstressed fish to about 60% in acutely stressed fish (Angelidis *et al.* 1987). Maule *et al.* (1989) also reported reduced disease resistance in Chinook salmon, *O. tshawytscha*, challenged with *Vibrio anguillarum* after a transport stressor.

In summary, at the beginning of this Chapter it was suggested that the effects of 2-phenoxyethanol on stress levels in fish might be assessed using the magnitude and duration of the metabolic and cortisol stress responses. By subjecting juvenile *P. commersonnii* to capture and handling stress and subsequently administering 2-phenoxyethanol it was possible to assess the effect of the anaesthetic on the metabolic rate of the fish, and thereby to assess the metabolic stress response of the fish. The higher metabolic rate in the fish that were not anaesthetized after capture and the longer time it took them to resume normal metabolism compared to the anaesthetized fish, indicates that 2-phenoxyethanol effectively reduced the effect of

capture and handling on the metabolic stress response in this species. Application of 2-phenoxyethanol did not, however, reduce the cortisol stress response in the fish. On the contrary, prolonged anaesthetization with 2-phenoxyethanol increased the concentration of plasma cortisol in the fish. It is evident therefore, that 2-phenoxyethanol can cause stress in fish. However, because of its inhibitory action on the central nervous system, 2-phenoxyethanol is able to reduce physical activity in the fish, and thereby reduce oxygen consumption rate.

# CHAPTER 7

## General discussion and a critical review of the use of metabolic rate as a measure of the stress response in fish

### Introduction

Few concepts have evoked as much discussion and disagreement as that of stress when applied to biological systems (Pickering 1981). The main problem seems to arise from the very definition of stress. Stress in fish has been defined in various ways, mainly depending on the context of the investigation (Barton and Iwama 1991). Different workers, therefore, tend to look at stress from different viewpoints. Consequently, views on issues such as what constitutes an ideal indicator of stress in fish and how best stress in fish can be measured differs depending on the investigator. The following quotations cited by Barton (1997) serve to illustrate the difficulties encountered when dealing with issues involving stress:

"Everybody knows what stress is and nobody knows what it is" (Selye 1973).

"There are few concepts that have provoked as much discussion and disagreement as that of stress when applied to biological systems" (Pickering 1981).

"A reliable measurement of stress is critical; however, a reliable, acceptable measurement of stress has not been found, perhaps because the concept is applied to so many different phenomena" (Moberg 1985).

"I am not certain whether one who undertakes this task (of defining the concept of stress) either has an enormous ego, is immeasurably stupid, or is totally mad" (Levine 1985).

An overview of the effects of stress in fish (Chapter 1) indicated that adverse environmental conditions and different aquaculture procedures cause varying levels of

stress in fish. Intervention strategies such as the application of anaesthetics, which are commonly used to alleviate the effects of stress during the execution of these procedures, have also been implicated in causing stress in fish (Ross and Ross 1984; Summerfelt and Smith 1990). The importance of stress in fish health is such that quantification of stress effects is necessary in order to evaluate the potential long-term effects of different stressors. An effective stress mitigation strategy also requires knowledge of the efficacy of a given anaesthetic and its effect on the stress response in fish. Quantification of stress, however, requires the use of a stress indicator that is easy to measure and which can be readily interpreted in terms of the potential long-term effects to the organism. Although various indicators have been used to measure stress (Adams 1990; Schreck 1990; Thomas 1990), the identification of a universal indicator of stress in fish has not been possible because of the different views on the concept of stress. Since there is a metabolic cost to stress that is proportional to the intensity of the stressor (Barton and Schreck 1987; Davis and Schreck 1997), metabolic rate has been proposed, in this study, as an alternative / complementary method to quantify stress in juvenile fish. This study hypothesized that changes in metabolic rate could be used to measure the degree of the stress response in fish. It was also suggested that differences in the magnitude of the metabolic stress response could be used to evaluate the effects of different stressors. Previously, metabolic rate had been used to estimate the metabolic cost of acute physical stress in juvenile steelhead (Barton and Schreck 1987) and coho salmon (Davis and Schreck (1997), respectively. However, these studies were aimed at determining the energetic cost of the stressors, in contrast to the aim of the present study, which was to evaluate the suitability of metabolic rate as an indicator of the stress response.

Stress indicators must be validated before they can be used to assess life-threatening situations (Schreck 1990). Implicit to the validation of metabolic rate as a suitable indicator of stress in fish was the development of a profile of the metabolic stress response of juvenile *P. commersonnii*, a sub-tropical marine fish (Chapter 4). It was shown that the metabolic stress response in this species was similar to what Jobling (1994) described as 'a typical response of teleost fishes to acute stress'. This response included a transient increase in metabolic rate of the fish followed by a gradual decrease to pre-stress levels. The response of fishes to acute stressors has commonly been measured using plasma cortisol concentration (Mommsen *et al.* 1999). The corticosteroid stress response was therefore used to validate the suitability of the metabolic stress response as a measure of stress in fish. To evaluate the efficacy of metabolic rate for measuring stress, differences in the magnitude of the metabolic stress response were used to assess the effect of different transport-related stressors on the physiology of the fish (Chapter 5) and to assess the effect of 2-phenoxyethanol on its stress physiology (Chapter 6). A prerequisite to this assessment was the estimation of baseline metabolic rates of the fish (Chapter 3), a task that required the standardization of the methods used for measuring oxygen consumption (Chapter 2).

This study has contributed towards understanding the stress response in juvenile *P. commersonnii*. It has also contributed towards understanding the effects of a commonly used anaesthetic, 2-phenoxyethanol, on the physiology of this species. By comparing the changes in metabolic rate of juvenile *P. commersonnii* that resulted from the effect of capture and handling with the changes in plasma cortisol concentrations due to the same stressor, it could be shown that metabolic rate is a good stress indicator. Similarly, by comparing the magnitudes of the metabolic response of the fish to different simulated stressors it was shown that it is possible to use metabolic rate to quantify the effects of

stress on aspects of fish physiology. It was also shown that 2-phenoxyethanol reduces the effect of acute stressors on metabolic rate, probably through the inhibitory effects on physical activity. However, 2-phenoxyethanol caused an increase in the levels of circulating plasma cortisol in the fish. It is, therefore, argued that 2-phenoxyethanol may be both a stressor and stress mitigator.

Consistent with the conceptual model (Fig 7.1) an energetic cost was associated with the stress response. Consequently, by comparing the magnitude of the metabolic stress response, it was possible to rank different transport-related stressors according to their relative energetic costs. The inter-linkage between the primary stress response and the energetic component of the stress response makes it possible to interpret the effects of different stressors in the context of their long-term effect on survival and development of the fish. By anaesthetizing fish under different conditions of stress, it was possible to evaluate the effect of 2-phenoxyethanol on the metabolic stress response. This study, therefore, assists in understanding the stress physiology of fishes, particularly in relation to the quantification of the effects of acute stressors. The results from these studies also have practical implications for the use of anaesthetics during fish transport.

**The use of metabolic rate to measure stress in fish**

A conceptual framework developed by Mezeaud *et al.* (1977) and Wedemeyer and McLeay (1981) for the stress response in fishes separates the stress response into three consecutively higher levels of biological organization, the primary, the secondary, and the tertiary (Fig 7.1). The primary response represents the perception of an altered state and initiates an endocrine response that results in the release of the 'stress hormones', cortisol

and catecholamine into the blood stream (Gamperl *et al.* 1994; Sumpter 1997). These hormones are the main indicators of stress at this level of biological organization (Barton and Iwama 1991). The release of the stress hormones in turn initiates a series of responses the net effect of which is the mobilization of energy reserves for the increased metabolic requirements that are associated with stress (Pickering 1992; Randall and Perry 1992). The catecholamines affect cardiovascular and respiratory functions and increase the oxygen carrying capacity of the blood (Wedemeyer *et al.* 1990; Thomas 1990; Pickering 1992; Randall and Perry 1992; Jobling 1994; McDonald and Milligan 1997). All these processes and changes require an energetic input (Jobling 1994; Beyers *et al.* 1999). Consequently, one of the endpoints of the primary stress response is an increase in metabolic rate. Physiological and biochemical changes such as increase in blood glucose, glycogen and lactic acid levels, together with the various haematological, hydromineral and structural changes associated with the stress hormones form the secondary component of the stress response. The tertiary response comprises changes in metabolic rate, growth rate and reproductive capacity that are elicited at the level of the whole organism. The schematic diagram of the stress response in fishes (Fig. 7.1.) shows how metabolic rate is interlinked with the primary, secondary and tertiary responses. Metabolic rate, a consequence of the changes initiated at the primary level of the stress response, is manifested as a tertiary stress response in fish. Metabolic rate can, therefore, be regarded both as a primary and a tertiary indicator of stress. With reference to this conceptual framework, therefore, it is apparent that the stress response can be evaluated from an energetic perspective, and that the effect of a stressor can be reflected in the amount of energy expended to achieve an adaptive compensation to the stressor.

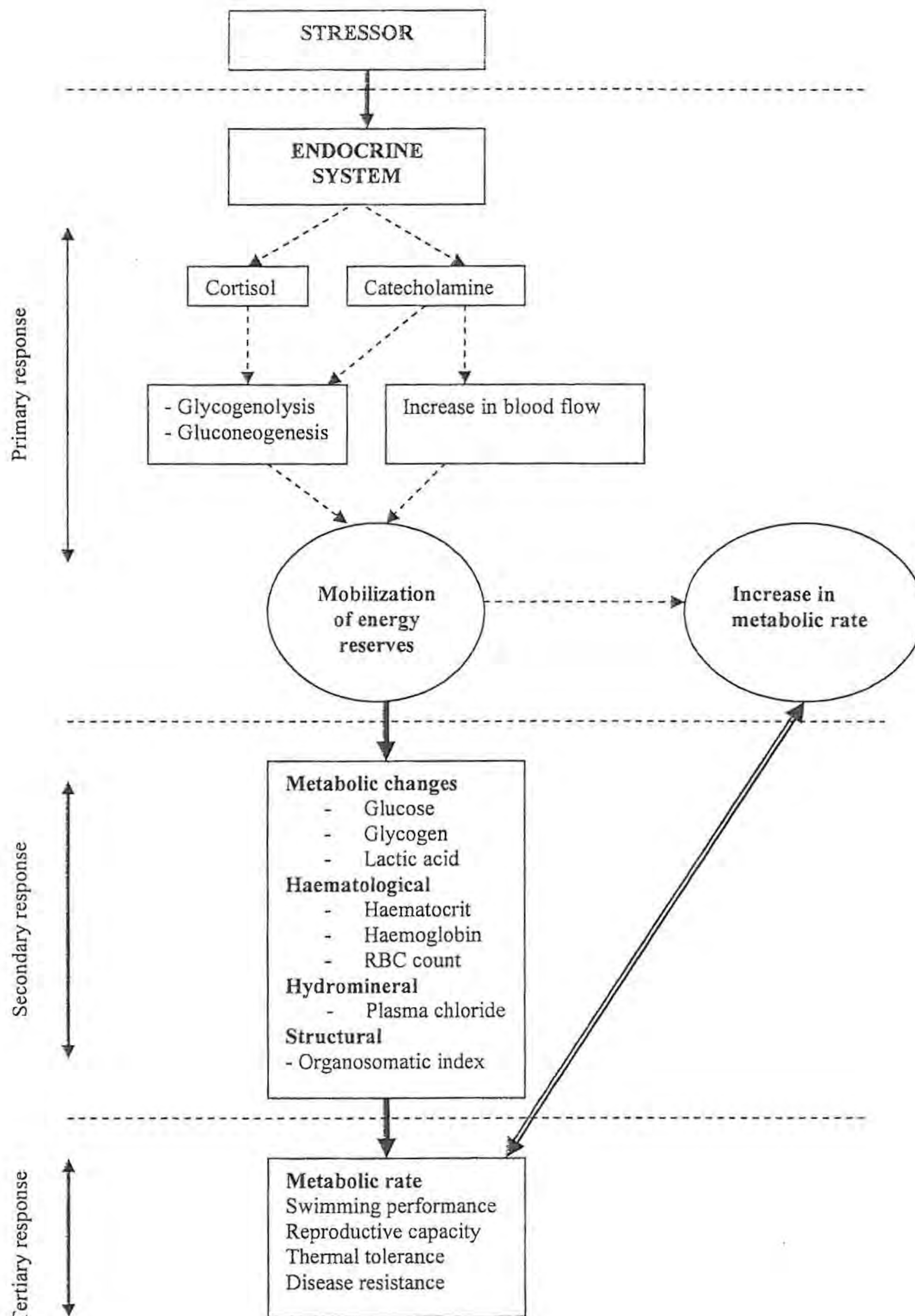



Fig. 7.1. Schematic diagram of the stress response in fishes: The diagram is an adaptation of the conceptual framework for the general stress response in fishes outlined by Wedemeyer *et al.* (1990) and Iwama *et al.* (1999) and shows how metabolic rate is interlinked with the primary, secondary and tertiary responses.

In proposing metabolic rate as an indicator of stress, it was hypothesized that metabolic rate could be used to measure the stress response in fish. It was further assumed that by using changes in metabolic rate it would be possible to rank different stressors according to their metabolic costs to the fish. It was also expected that acute temperature elevation within the thermal limits of the species would elicit an abrupt increase in metabolic rate. Because oxygen depletion is a passive process, it was expected that at non-limiting dissolved oxygen concentrations oxygen depletion would not elicit a metabolic stress response. The results obtained in chapter five demonstrated that by using metabolic rate it was possible to measure the relative metabolic costs of various transport-related stressors and to rank them according to their metabolic costs to the fish. The results also confirmed that the highest energetic cost to juvenile *P. commersonnii* was incurred as a result of capture and handling as suggested by Robertson *et al.* (1988) and Mazur and Iwama (1993) for some fish species. The results further confirmed the hypothesis that the increase in metabolic rate as a result of acute temperature elevation would be less than for capture and handling, and that acute temperature reduction, oxygen depletion and crowding would not affect oxygen consumption rate.

A comparison of the metabolic response of juvenile *P. commersonnii* to different simulated stressors (Chapter 5) showed that metabolic rate could be effectively used to measure stress in fish. The question, however, arises as to how good a measure of stress metabolic rate might be compared to other commonly used stress indicators. To address this question one needs to examine the qualities of a good stress indicator and to evaluate metabolic rate using these qualities as a guideline. From the summary of the attributes and shortcomings of the commonly used indicators of stress in fish (Table 7.1) the crucial factors to be considered when selecting an appropriate stress indicator are sensitivity to

the stressor, ease and accuracy of measurement, and the biological significance of the changes. According to these requirements, therefore, indicators that incorporate several levels of biological organization are potentially better measures of stress compared to those that involve changes at only one level of organization. The fact that the increase in metabolic rate as a result of acute stressors is a culmination of several related or interconnected events, incorporating several levels of biological organization, makes it a useful tool for measuring acute stress.

A comparison of the metabolic stress response profile for juvenile *P. commersonnii* (Chapter 4, Fig. 4.1) with a schematic profile of the cortisol stress response (Fig 7.2), and the levels of cortisol measured in the fish at various intervals after capture (Chapter 4, Fig. 4.3) showed similarities that indicated its suitability for measuring the effects of acute stressors. The stress response in juvenile *P. commersonnii* was characterized by a rapid increase in metabolic rate that was detectable within three minutes. Metabolic rate peaked after 15 min at a level approximately three times higher than pre-stress levels, before declining gradually to pre-stress levels after approximately 90 minutes. Similarly, the increase in plasma cortisol in the fish following capture was detectable within three minutes. Plasma cortisol in the fish that were sampled 15 min after capture was more than twice the pre-stress values. Pre-stress cortisol levels in the fish were re-established within 30 minutes. The rapidity with which the metabolic response was detected indicated that metabolic rate has a fast response time to acute stressors that is comparable to plasma cortisol. The fact that both the metabolic and the corticosteroid stress response reached a peak approximately 15 min after application of stress suggests that the two responses occur simultaneously. The elevation in metabolic rate as a result of capture and handling also suggests that as is the case with plasma cortisol (Barton and Iwama 1991), the

magnitude of the metabolic stress response reflects the severity of a stressor. This observation was verified by subjecting juvenile *P. commersonnii* to different stressors (Chapter 5). The metabolic rate of juvenile *P. commersonnii* increased by more than 130% after capture and handling compared to an increase of 60% after acute temperature elevation. The cost of metabolism attributed to the effects of capture and handling was therefore twice as much as that attributed to acute temperature elevation. Therefore, although capture and handling and acute temperature elevation both resulted in increases in the metabolic rates of juvenile *P. commersonnii*, the magnitude of the responses depended on the stressor.

Post-stress recovery was faster in the case of the cortisol response than the metabolic response. The delayed metabolic recovery was probably because of repayment of the oxygen debt incurred by the fish while attempting to avoid capture (Jobling 1994). When fish are forced to engage in strenuous exercise, the energy requirements of the muscles are greater than can be supplied by aerobic metabolism (Brett 1964). The immediate energetic needs of the fish are met from stores of the high-energy creatine phosphate and by the anaerobic metabolism of muscle glycogen reserves (Jobling 1994). The anaerobic metabolism results in the formation of lactate that may lead to osmoregulatory dysfunction in the fish (Wendelaar Bonga 1993). The re-oxidation of lactate and the restoration of the ionic and acid-base balance, and the replenishment of ATP and creatine phosphate and internal oxygen stores requires an oxygen supply in excess of what is required by resting fish thereby resulting in the oxygen debt. The repayment of the oxygen debt is reported to take several hours (Jobling 1994).

Table 7.1. Advantages and disadvantages of some primary, secondary, and tertiary variables that are commonly used to measure stress in fish

| Level of organization and stresss indicator | Advantages | Disadvantages |
|---|---|---|
| ***Primary*** | | |
| Endocrine: Plasma catecholamines, cortisol | Rapid response time; Sensitivity to sublethal stressors; ease of measurement; magnitude of response related to severity of the stressor (Thomas 1990) | Biological significance not often clear; baseline levels of the variables not known for many species; high variability in measurements due to sampling methods (Thomas 1990) |
| ***Secondary*** | | |
| Metabolic e.g. plasma glucose, lactic acid, liver and muscle glycogen | Easy to measure; using bio-medical equipment; involves integration at several levels of biological organization | Baseline levels of the variables not known for many species; high variability of the measurements (Schreck 1990) |
| Haematological e.g. haemoglobin, red blood cell count, white blood cell count, haematocrit, leucocrit | Same as above | High variability of the measurements; low sensitivity to many stressors (Wedemeyer *et al.* 1990) |
| Hydromineral e.g. plasma chloride, Plasma sodium, potassium, osmolality, protein | Same as above | Expensive experimental procedures; variability of the measurements |
| Structural e.g. interrenal cell size, gastric tissue morphology, organosomatic indices | Same as above | Involves sacrificing the fish; lack of sensitivity to many stressors (Hinton and Lauren 1990) |
| ***Tertiary*** | | |
| Growth; Metabolic rate; Disease resistance; Swimming performance; Reproductive capacity | Involves integration at several levels of organization | Mostly involves prolonged experimental duration |

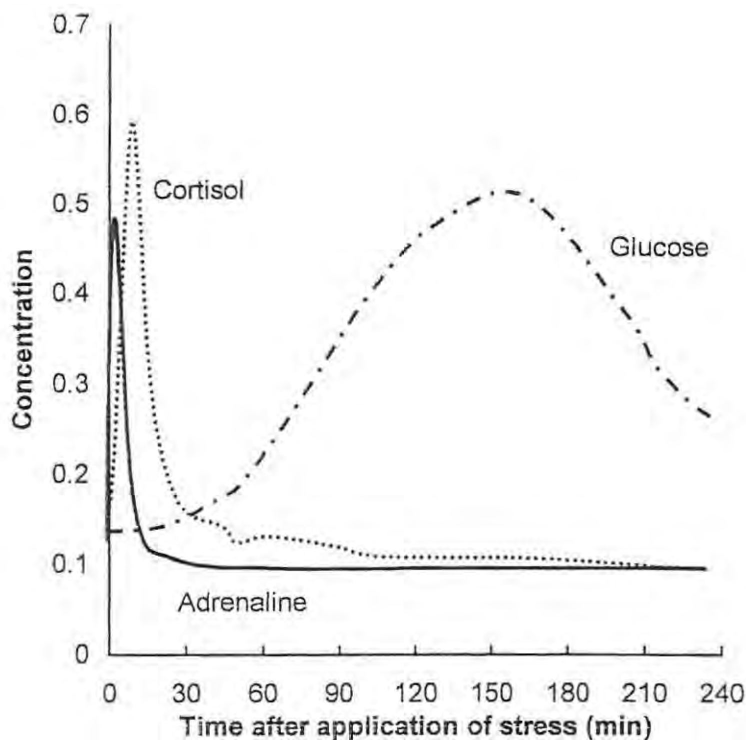



Fig. 7.2. Schematic diagram showing the relative time course of adrenaline, cortisol, and glucose concentrations in the blood plasma of fish that may be observed following a single episode of stress. Adapted from Iwama *et al.* (1999).

The establishment of cortisol as the most commonly used measure of stress in fish stems from the fact that during stress plasma concentrations of cortisol increases proportionately to the intensity and duration of the stress (Barton and Iwama 1991). For example, intense handling and severe confinement caused an elevation in plasma cortisol in fingerling rainbow trout, *O. mykiss,* (Barton *et al.* 1980) and juvenile coho salmon, *O. kisutch*, (Davis and Schreck 1997) to levels two to four times higher than that caused by less vigorous stressors. Cortisol can also be measured easily and accurately using commercially available radioimmunoassay (RIA) or Enzyme-Linked Immunosorbent Assay (ELISA) kits (Barry *et al.* 1993). The ease and accuracy by which oxygen consumption can be measured makes it possible to measure the metabolic stress response

more accurately than the corticosteroid response. Using intermittent respirometry, the experimental conditions can be easily controlled to eliminate stressors other than the one under study. Introduction of fish into the respirometer at least 12 h before measuring oxygen consumption allows adequate time for the fish to acclimate to the respirometer and to recover from the effects of the transfer. Additional aeration and the continuous water recirculation ensure optimal water quality during oxygen consumption measurements.

The Selyean concept of stress and the idea that living organisms are constantly compensating for effects of environmental stressors suggests that an animal's physiological condition changes over time, depending on individual and environmental characteristics (Wedemeyer *et al.* 1990; Heath 1995). Establishing what is 'normal' may, therefore, not be as useful as establishing the relative change in physiological response due to a stressor. Besides, as Barton and Iwama (1997) maintain, deciding what constitutes a 'normal' resting state is itself a subjective matter. Consequently, investigations of induced stress in fish often emphasize detection of departures from the 'normal' homeostatic condition (Beyers *et al.* 1999). For example, the experiments conducted during this study revealed the relative changes in metabolic rate attributable to the different transportation variables. Establishing whether the pre-stress metabolic rates were 'normal' was, therefore, of little consequence compared with quantifying the relative changes in metabolic rates due to the different stressors. However, suitable experimental conditions were established to ensure accurate measurements of the changes in metabolic rate during the stress response. For example, the higher nocturnal relative to diurnal oxygen consumption rates exhibited by juvenile *P. commersonnii* (Chapter 3) indicated a rhythmic pattern in the diel oxygen consumption of the fish that could influence baseline

metabolic rates. 24-h measurement of oxygen consumption resulted in the establishment of the pattern in diel oxygen consumption rate of the fish that enabled the sampling time to be established between 10 h 00 and 16 h 00 to coincide with the period of least activity in the fish, and thereby avoiding the pitfalls in experimental design that could have resulted in erroneous estimations of the baseline metabolic rates. Similar to this study, other work has shown that differences in the cortisol stress response between individual fish results in wide variations in the cortisol measurements (see Mommsen *et al.* 1999). For example, Barton and Schreck (1987) reported variations in plasma cortisol concentrations of individual juvenile steelhead subjected to acute physical stress. Pottinger and Carrick (1999) and Tort *et al.* (2001) also reported consistently divergent plasma cortisol responses in rainbow trout (*O. mykiss*) subjected to a confinement stressor, and guildhead seabream (*Sparus aurata*) subjected to repeated handling. Variations have also been observed in cortisol measurements that may be attributed to the diversity of approaches in manipulating cortisol levels during tests, and to differences in sampling techniques. Such variations were pre-empted by the methods used to measure oxygen consumption rate. As a result, baseline metabolic rates for juvenile *P. commersonnii* could be determined more accurately than baseline plasma cortisol concentrations.

Because different stressors might elicit responses that differ both in magnitude and duration, temporal scaling of both the stressful situation and the response by the fish must be taken into account when measuring stress. The time at which measurements are made after the beginning or end of a stressful experience greatly influences the magnitude or extent of the observed responses and thereby influences the perceived severity and duration of the stressor. It is possible to monitor metabolic rate continuously over a long

duration using polarographic oxygen sensors installed in the respirometer thereby enabling a better evaluation of the stress response. It is however difficult, if not impossible, to continuously monitor plasma cortisol using juvenile fish.

A major attribute of a good stress indicator is its significance to the long-term survival and development of the organism. It is recognized that stress imposes a metabolic load on fish that consists of an energy demand required to cope with the disturbance and an energy cost to correct the accompanying hydromineral imbalance (Schreck 1982). Priede (1985) and Barton and Schreck (1987) estimated that as much as 25-100% of the scope for activity might be required to cope with stress. While a shift in energy resources away from other activities would allow fish to cope with the increased energy demand, the energy shift may limit a fish's bioenergetic capacity by reducing the energy available for other performance components within its scope for activity. For example, Schreck *et al.* (1989) found that the increased energy burden imposed on hatchery coho salmon from handling and hauling resulted in reduced adult returns of fish released immediately after transport as compared to control groups. It has also been demonstrated that fish experiencing stress may display a reduction in growth rate (Pickering 1993).

This study demonstrated that metabolic rate is easy to measure. It is sensitive and has a quick response time to stressor stimuli. Since the energy used to respond to a stressor so as to achieve physiological compensation represents a portion of a fish's total energy budget that is thereby made unavailable for other activities (*senso* Fry 1971) by the fish, the role of increased metabolic rates during the stress response is well explained in terms of the long-term potential effects on fish. This, therefore, suggested that changes in metabolic rate could be effectively used to quantify acute stress in fish. It is further

suggested that metabolic rate might be a more suitable way to assess the stress response in juvenile fish, than some of the commonly used physiological variables.

**Effect of 2-phenoxyethanol on stress physiology**

The efficacy of an anaesthetic depends on the intended application. To immobilize fish for easier handling, the anaesthetic should be effective at a low dose and the time taken by fish to recover from its effect should be short. To be suitable for fish transport, however, the anaesthetic should be capable of sedating the fish for a long duration and be non-toxic to the fish. It should have no persistent effects on fish physiology and behaviour. In Chapter 6, it was shown that the effective immobilizing concentration of 2-phenoxyethanol was 0.4 ml $l^{-1}$. The effective concentration of the anaesthetic for sedating *P. commersonnii* ranging in size between 3 g and 140 g was 0.2 - 0.3 ml $l^{-1}$. The recovery time was consistently less than 3 minutes. Other than irritability to fish gills (Summerfelt and Smith 1990), no side effects have been reported for this anaesthetic.

The effect of 2-phenoxyethanol on the stress physiology of juvenile *P. commersonnii* was investigated by measuring the metabolic and cortisol stress responses of the fish under different states of stress and anaesthetization. As part of the process of testing metabolic rate as an effective indicator of stress, the metabolic stress response was also used to assess the effect of 2-phenoxyethanol on the stress physiology of the fish (Chapter 6). Capture and handling resulted in an increase in metabolic rate that peaked approximately 15 minutes after capture in both the fish that were anaesthetized and those that were not anaesthetized after capture. Metabolic rate was twice as high in the fish that were not anaesthetized compared to the anaesthetized fish suggesting that anaesthetizing the fish

with 2-phenoxyethanol immediately after capture reduced the impact of capture and handling stress on the metabolic stress response. Anaesthetized fish also recovered faster from the effects of capture and handling, suggesting that 2-phenoxyethanol reduced the duration of the metabolic stress response. Fish that were anaesthetized prior to capture exhibited oxygen consumption rates that were comparable to those of resting fish. The fact that the anaesthetic reduced the metabolic rate of stressed fish but did not affect the metabolic rate of undisturbed fish indicated that the anaesthetic produced a calming effect but did not affect the basal metabolic rate of the fish.

General anaesthetics such as 2-phenoxyethanol exert inhibitory effects on the central nervous system that result in a reduction in activity (Rang *et al.* 1995). The post-stress increase in metabolic rate is usually associated with the intense physical activity involved when a fish attempts to escape capture. It is suggested that the lower metabolic rate exhibited by anaesthetized juvenile *P. commersonnii* may have resulted from the inhibitory action of 2-phenoxyethanol on fish movement. The reduction in physical activity may, consequently, have resulted in a faster repayment of the oxygen debt arising from the capture ordeal and could therefore explain the faster resumption of routine metabolism in the anaesthetized fish. However, despite these positive effects on metabolic rate, capture and handling resulted in an increase in the concentration of plasma cortisol that peaked approximately 15 minutes after capture in both the anaesthetized and un-anaesthetized fish. Pre-stress levels of plasma cortisol was re-established within 30 minutes in the un-anaesthetized fish, but plasma cortisol levels in the anaesthetized fish remained high even after the 30-min period. At the time of capture plasma cortisol concentrations in fish that were anaesthetized prior to capture were comparable to those measured in the un-anaesthetized fish 15 min after capture indicating that the former were

already experiencing stress at the time of capture. Undisturbed anaesthetized fish had more than five times higher plasma cortisol concentrations than the un-anaesthetized controls after 1 h anaesthetization, suggesting that the prolonged anaesthesia with 2-phenoxyethanol could be the reason for the increase in the plasma cortisol levels of these fish.

Acute stress in fish is commonly measured by the concentration of plasma cortisol (Mommsen *et al.* 1999), a primary level stress indicator (Wedemeyer and McLeay 1981). However, the results obtained during this study show that metabolic rate, a tertiary level indicator, could be a more useful measure of acute stress in juvenile fish. From these observations, what constitutes a good stress indicator may depend not only on the purpose for the measurement, but also on the indicator being measured or the level at which the response is being measured. The same argument may be used with regard to the effect of anaesthetics on stress physiology. Whether or not a given anaesthetic reduces stress, or is itself a stressor, may depend on the variable that is measured. Because of its inhibitory action on the central nervous system (Rang *et al.* 1995), 2-phenoxyethanol is able to reduce physical activity in the fish, and thereby reduce the oxygen consumption rate of the fish. It may thus be considered as a remedy to the effects of stress in fish. However, the increase in plasma cortisol concentration during prolonged anaesthetization using this drug suggests that the anaesthetic might be a stressor to the fish.

The question of whether or not 2-phenoxyethanol can be recommended for use as an anaesthetic for fish must also be addressed from the point of view of the intended application. The ability of 2-phenoxyethanol to reduce physical activity of the fish and thereby reduce the impact of acute stress on the metabolic stress response obviously

makes it a good agent for the mitigation of stress during capture and handling of fish and for transporting fish over short time periods. A strong relationship between elevated plasma cortisol and susceptibility to infectious desease has been documented (see review by Barton and Iwama 1991). High cortisol levels, such as those measured in juvenile *P. commersonnii* anaesthetized with 2-phenoxyethanol, are likely to have a strong immunosuppressive effect on the fish following prolonged transport that may leave the fish susceptible to infectious desease. Consequently, despite its proven efficacy, and long retention time in water 2-phenoxyethanol might not be suitable for the transportation of live fish for long periods of time.

**Conclusions and recommendations**

The results presented in this thesis demonstrate the usefulness of metabolic rate as an indicator of acute stress in fish. This was done by comparing the metabolic and the cortisol stress responses in juvenile *P. commersonnii.* The results suggest that metabolic rate measurements could be a more practical way to quantify the effects of acute stressors on juvenile fishes. By detailing the profile of the metabolic stress response in this species, this thesis makes a contribution towards understanding the physiological effects of stress in fishes. The study also contributes towards the quantification of the baseline metabolic rates of juvenile *P. commersonnii.*

The ease and accuracy with which oxygen consumption of fish could be measured made it possible to measure the stress response more accurately than by plasma cortisol concentration. Using intermittent respirometry, experimental conditions could be more easily controlled, thus, enabling a more accurate estimation of baseline rates. It was also possible to monitor metabolic rate continuously over a long duration using polarographic

oxygen sensors, thus enabling a better evaluation of the stress response. In addition, metabolic rate showed a fast response time to the acute stressors. Results from these experiments also suggested that the magnitude of the metabolic stress response reflects the severity of the stressor. Consequently, using metabolic rate, it was possible to measure the effects of different transport procedures and to categorize them according to their metabolic costs to the fish. The highest energetic cost to juvenile *P. commersonnii* was incurred as a result of capture and handling. Acute temperature elevation also resulted in considerable stress to the fish, whereas acute temperature reduction, oxygen depletion and crowding did not affect the metabolic rate of the fish. Given that the role of increased metabolic rates during the stress response is also well explained in terms of energetic requirements, and the long-term potential effects on fish, it is concluded that changes in metabolic rate can be effectively used to quantify acute stress in fish.

This study has also contributes towards understanding the effects of 2-phenoxyethanol on the stress physiology of fish. By anaesthetizing fish under different conditions of stress, it was possible to evaluate the effect of 2-phenoxyethanol on the metabolic stress response. The ability of 2-phenoxyethanol to reduce physical activity, and thereby reduce the impact of acute stress on the metabolic stress response makes it a good agent for the mitigation of stress during the capture and handling, and for transporting fish for short distances. However, the increase in plasma cortisol concentration during prolonged anaesthetization using this drug suggests that the anaesthetic might be a stressor to fish and may, therefore, not be suitable for long-term sedation.

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