Determining growth status in teleost larvae using flow cytometric cell cycle analysis

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DECLARATION

I, Don Bromhead, hereby declare that this thesis is my own work and contains no material which has been accepted for the award of any other degree or diploma in any university, and to the best of my knowledge, contains no material previously published or written by any other person except where due reference is made in the text of the thesis.

Don Bromhead

22nd of June 2001
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ABSTRACT

This study assessed the suitability of using flow cytometric analysis of cell cycle fractions in larval teleost brain, as a tool for the analysis of whole larval growth. Initially, a protocol was presented which optimised the methodological criteria for the preparation of cells, flow cytometric data collection and data modelling. This protocol also allowed for the simultaneous analysis of both cell proliferation and cell death (apoptosis) and the calculation of a net tissue growth index (NTGI; the fraction of cells dividing minus fraction of apoptotic cells). This protocol was then applied to: 1) Assess the influence of environmental factors, including temperature, salinity, photoperiod and prey availability, as well as the effect of developmental and physiological factors, upon brain cell proliferation and net brain tissue growth of larval teleosts; and 2) Determine which cell fraction (S, G2M), fraction of cells dividing (FOCD) or NTGI, might best correlate with overall larval growth rate (mm/d). Most experiments investigated brain cell proliferation in larval snapper, Pagrus auratus.

The first investigation looked at the relationship between salinity and cell proliferation in brain tissue of snapper larvae from age 6 through to 21 days. The effect of salinity upon fraction S phase was most apparent in larvae 12 days of age or older. The developmental and length data supported the conclusions from the cell cycle data, that growth and condition was optimal at 30 ppt. Cell proliferation was lowest, and apoptosis highest, at 10 ppt. Mean growth rate was most closely correlated with mean fraction of cells in S phase ($r^2=0.63$). The fraction of cells in G2M showed no significant correlation with either growth rate or fraction S. There was some evidence for cells arresting in G2M in response to disease which affected larvae late in the experiment.

The second investigation looked at the relationship between temperature and cell proliferation in brain tissue of snapper larvae from age 6 through to 14 days. The optimal temperature for brain cell proliferation in larval snapper decreased as the larvae got older, despite the fact that overall growth (mm/d) and development was most rapid at the higher temperatures, regardless of larval age. Consequently, the fraction of cells in S phase and FOCD showed a low correlation with mean growth rates. Survival, however, was lowest at the highest temperatures. There was evidence for brain cell
cycle arrest in G2M at the highest temperature extremes. Apoptosis was higher at lower temperatures initially but was lowest in larvae reared at 15°C by day 14.

The third investigation comprised of two experiments. The first experiment investigated whether there was any diel variation in cell proliferation of pre-feeding, mixed feeding and exogenous feeding snapper larvae, with the latter also subjected to changed feeding time and extended photoperiod treatments. The second experiment investigated how starvation might affect patterns of diel variation in brain cell proliferation in golden perch (*Macquaria ambigua*) larvae, and also looked at diel variation at two developmental stages, mid- and post-flexion. There was little evidence for circadian rhythms in cell proliferation in either species except for post-flexion golden perch. A shortened photoperiod resulted in lower growth and fraction of cells in S phase for snapper larvae. The first experiment (snapper) may have been affected by poor larval quality, but demonstrated strong diel variation in G2M, and a lower level of variation in S phase fractions. However, variation in the fraction of cells in S phase was also related to feeding (gut fullness), swim bladder inflation, and photoperiod length. The latter result was supported by trends in length data. Golden perch larvae aged 9 to 12 days exhibited a sub-daily rhythm (12-15 hr) in cell proliferation that was disrupted in starved larvae. Overall the pattern and extent of diel variation appeared to depend on species and developmental stage. The fraction of brain cells in S phase showed the least variability and highest correlation with feeding and length measures.

The final experiment investigated the relationships between starvation, mortality, overall growth and the fraction of brain cells synthesising DNA in 20- to 27-day-old snapper larvae. From day 1 of the experiment, larvae were either fully fed (FF), starved 1 day (ST1), starved 2 days (ST2) or starved 3 days (ST3) then refed until day 7. Despite seemingly lower overall levels of brain cell proliferation (when compared to snapper in salinity and temperature experiments), the fraction of brain cells synthesising DNA was significantly lower in ST3 larvae on day 4 than in other treatments and exhibited a lag in recovery to FF levels of nearly 48 hr after refeeding. The high degree of correlation between the mean fraction of cells synthesising DNA and mean growth rate suggested that the fraction of cells synthesising DNA is a sensitive indicator of larval growth rate. The mean fraction of cells in S phase was also negatively related to mortality levels.
The relationship between brain cell proliferation and overall larval growth, as well as the effect of environment and development upon these, is discussed in some depth. Overall it was clear that both brain cell proliferation and overall growth (mm/d) in larval snapper were influenced in a similar manner by environmental conditions (except perhaps when environmental temperatures were unusually high). However it was also clear that the fraction of cells synthesising DNA (S phase) was a more sensitive index of overall growth than G2M or any index that included G2M. This was most likely a result of the capacity of cells to arrest in G2M as a result of adverse physiological and environmental factors. The level of cell proliferation varied depending on the developmental status and age of the larvae. The effect of environmental factors upon brain cell proliferation also varied with age and development. However, even in older (>20 day) larvae, with low S phase fractions, this index was able to discriminate between fed and starved larvae by 3 days, and showed a high degree of correlation with mean growth rates. Consequently, the fraction of brain cells synthesising DNA (S phase) is presented as being a highly sensitive and suitable indicator of larval growth and should have potential as a tool for investigating processes affecting larval recruitment into juvenile and adult stocks.
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<table>
<thead>
<tr>
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<th>Description</th>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromo-deoxyuridine</td>
</tr>
<tr>
<td>CCT</td>
<td>Compressed cycle time</td>
</tr>
<tr>
<td>CDI</td>
<td>Cell division index</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin dependant kinase</td>
</tr>
<tr>
<td>CGO</td>
<td>Circulatory cell gated out</td>
</tr>
<tr>
<td>CID</td>
<td>Change in deviance</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>D</td>
<td>dark hours in a photoperiod (i.e. 12D)</td>
</tr>
<tr>
<td>DF</td>
<td>Degrees of freedom</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>Deoxyribonuclease</td>
</tr>
<tr>
<td>DO</td>
<td>Dissolved oxygen</td>
</tr>
<tr>
<td>EMEM</td>
<td>Eagles minimum essential medium</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>FACS</td>
<td>Flow analytical cell sorter</td>
</tr>
<tr>
<td>FAp</td>
<td>Fraction of cells which are apoptotic</td>
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<tr>
<td>FCM</td>
<td>Flow cytometer</td>
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<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FF</td>
<td>Fully fed (or fed daily)</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein-isothiocyanate</td>
</tr>
<tr>
<td>Fl</td>
<td>Flexion</td>
</tr>
<tr>
<td>FL1</td>
<td>Fluorescence (channel 1)</td>
</tr>
<tr>
<td>FL1-H</td>
<td>Fluorescence (channel 1) height of pulse signal</td>
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<td>FL2-H</td>
<td>Fluorescence (channel 2) height of pulse signal</td>
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<tr>
<td>FOCD</td>
<td>Fraction of cells dividing</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward light scatter</td>
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<tr>
<td>FSC-H</td>
<td>Forward light scatter (pulse signal height)</td>
</tr>
<tr>
<td>G0</td>
<td>Gap 0 (quiescent phase)</td>
</tr>
<tr>
<td>G01</td>
<td>Fraction of cells in G0 and G1 phases</td>
</tr>
</tbody>
</table>
G1 – Initial growth phase of cell cycle
G2 – Second gap/growth phase of cell cycle, after DNA synthesis
G2M – fraction of cells in G2 and M phases of cell cycle
GF – Gut fullness
GH – Growth hormone
GR – Growth rate (mm per day)
IFRS – Inland Fisheries Research Centre
L – Light hours in photoperiod (i.e. 12L)
LSD – Least significant difference
M – Mitosis phase
MPF – Mitosis promoting factor
n – sample number
NSW – New South Wales
NTGI – Net tissue growth index
OD – Oil droplet
PBS – Phosphate buffered saline
PI – Propidium iodide
PNR – Point of no return
ppt – Parts per thousand
ppm – Parts per million
PS – Phosphatidyl-serine
PSRC – Port Stephens Research Centre
RCS – Reduced chi-square value
REML – Restricted maximum likelihood
RNA – Ribonucleic acid
RNAse – Ribonuclease
S – DNA synthesis phase
SB – Swim bladder
SED – Standard error of difference
SL – Standard length
SSC – Side light scatter
ST1 – Starved 1 day
ST2 – Starved 2 days
ST3 – Starved 3 days
CHAPTER 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

For over two decades researchers have attempted to find appropriate and accurate indices to assess the condition and growth of larval and juvenile fishes in the field, with the aim of applying such techniques to the optimization of rearing processes. However, many of the indices proposed to date are either too time-consuming, intrusive, or have measured core components (Wainman and Applegate, 1984). Nuclear and visceral indices are currently considered to be accurate and practical and efficient methods of assessing larval condition and growth (Beggs, 1982; Ricklefs et al., 1991). Recently, Tucker and Sim (1992) proposed that a new optical dual cycle analysis, they offer a fast and sensitive tool for investigating larval condition and growth. Basset et al. (1994) detailed the potential of this technique as an accurate indicator of larval performance and, thus, a more comprehensive exploration of rearing methods. The research conducted in this thesis aims to investigate 1.3 the recent ecotoxicological trends required to the influence of physiological and environmental factors on larval survival and condition of fish larvae; and (ii) the influence of larval condition and growth on the survival of larval fish. Consequently, we begin this research with a description of the development of larval condition and growth research, and section 1.2 will describe the theoretical and practical aspects of applying these techniques, allowing analysis of the assessment of condition and growth in larval fish.

1.2 CONDITION AND GROWTH OF FISH LARVAE

1.2.1 Introduction

Indicators of larval condition and growth have been used in a wide range of applications in larval research. The increasing push for the development of these indices has come from researchers interested in understanding larval growth and survival probabilities and relating this to eventual recruitment into the adult population. Such a predictive ability would hold enormous significance for fisheries management and conservation. However, the development and application of these indices is still subject to some important issues to be discussed.
1.1 GENERAL INTRODUCTION

For over two decades researchers have attempted to find appropriate and accurate indices to assess the condition and growth of larval and juvenile fishes in the field, with the aim of applying such techniques to the elucidation of recruitment processes. However, many of the indices proposed to date are either too time consuming, insensitive, or have unresolved error components (Ferron and Leggett, 1994). Nucleic acid based indices are currently considered to be among the most accurate and efficient methods of assessing larval condition and growth (Bergeron, 1997; Buckley et al., 1999). Recently, Theilacker and Shen (1993) proposed that a new method, cell cycle analysis, may offer a fast and sensitive tool for investigating larval condition and growth. Bromhead et al. (2000) further highlighted the potential of this technique as an accurate indicator of these parameters, warranting a more comprehensive experimental investigation. The research presented in this thesis aims to investigate: (a) the ideal methodological criteria required; (b) the influence of physiological and environmental factors on larval cell cycle; (c) the sensitivity of cell cycle to the nutritional condition of fish larvae; and (d) the relative merits and failings of the technique. Consequently, as background to the research described in this thesis, Section 1.2 describes the past history and current status of larval condition and growth research, and Section 1.3 will describe the theoretical and practical aspects of applying flow cytometric cell cycle analysis to the assessment of condition and growth in larval fish.

1.2 CONDITION AND GROWTH OF FISH LARVAE

1.2.1 Introduction

Indicators of larval condition and growth have been used in a wide range of applications in larval research, but the predominant push for the development of these indices has come from researchers interested in assessing larval growth and survival probabilities and relating this to eventual recruitment into the adult population. Such a predictive ability would hold enormous significance for fisheries management and conservation. However, the development and application of these indices to recruitment assessment are currently subject to three major areas of debate, concerning:
1) The practicality or utility of such indices in assessment of future recruitment to fish stocks;
2) The suitability of the parameter being measured as an indicator of condition or growth; and
3) The accuracy and reliability of the techniques in measuring the parameters (indices) they attempt to measure.

The following review will explain these issues in some detail, focussing in particular on the last two, thus highlighting the current need for a technique which is fast, sensitive, accurate, precise and appropriate for the assessment of larval condition and growth.

1.2.2 Recruitment and fisheries oceanography

One of the most important goals in current fisheries, marine and conservation research is the development of methods by which to predict fluctuations in year-class strength of fish stocks (Lasker, 1987; Bailey and Houde, 1989). To predict stock fluctuations, one must be able to measure recruitment: the number of larvae or juveniles that survive to adulthood or to an age vulnerable to harvesting (Lasker, 1987; Fogarty et al., 1991). The survival capacity of fish increases as they develop and mature, therefore processes which affect recruitment numbers are generally accepted as acting primarily upon the early life history stages (Ferron and Leggett, 1994). Indeed, the average mortality rate of larvae in the ocean is very close to 1.0, or as Pepin et al. (1999) eloquently put it, "the average larva is dead". In other words, a very minor percentage of larvae will survive through to adulthood, and even a tiny change in the mortality rate can have orders of magnitude greater effect on the number of recruits added to the adult population (Buckley et al., 1999). Such small changes in larval survival thus have large implications for fisheries and their ability to gain maximum yield from stocks while still maintaining viable populations.

Recruitment is a complex process and there are many factors that are thought to contribute to its variation. These include water temperature (Buckley et al., 1990), disease (Steinhart and Eckmann, 1992), major meteorological and oceanographic phenomena (Houde, 1989; Bailey et al., 1995; Lough et al., 1996; McFadzen et al.,
1997; Chicharo et al., 1998; Coombs et al., 1999), egg incubation conditions (Buckley et al., 1990; McGurk et al., 1993; Canino, 1994; Baynes and Howell, 1996), and maternal condition (Kerrigan, 1997). However, while the impact of each of these factors varies (Houde, 1989), two of the most consistent and influential factors are believed to be food availability (starvation) (Lett and Kohler, 1976), and predation (Leggett and Deblois, 1994; McCormick, 1998; Rooker et al., 1998).

Researchers interested in recruitment forecasting have focused on starvation-based mortality, or the relationship between nutritional condition and larval survival probability. However, given that predation is also an important factor, this approach can only be valid if lowered nutritional condition (or reduced growth rate) of larvae directly increases their vulnerability to predation (Miller et al., 1988; Rice et al., 1993). Supporting evidence has led to the formation of the “bigger is better” and “stage duration” theories which hold that, starved and/or smaller larvae (i.e. slower growing larvae) are less able to avoid predators, and are exposed to a higher predation pressure for longer periods (reviewed by Leggett and Deblois, 1994). However, evidence has also been put forward that suggests that these condition or size related biases in susceptibility of larvae to predation are not apparent (Litvak and Leggett, 1992; Cowan et al., 1996; Paradis et al., 1996; Elliott and Leggett, 1998), leading to considerable debate in the area (Elliott and Leggett, 2000; Suthers, 2000). It appears more likely that the relationships between larval size, condition and growth, and their vulnerability to predation, vary with species and size (Cowan et al., 1996; Chick and Van Den Avyle, 2000). A much greater level of investigation is required before any generalised conclusions regarding the relevance of these factors to recruitment analysis can be made.

1.2.3 The Biology of larval condition and growth

1.2.3.1 Defining growth and condition

Growth of fish larvae (and indeed any organism) is achieved by the simultaneous occurrence of two processes: synthesis and degradation. These processes operate at every level of the larva, be that molecular, cellular or tissue, and manifest as an increase
or decrease of size and mass of the whole organism. While numerous factors contribute, the balance between synthesis and degradation will predominantly rely on the intake and supply of nutrients to the various tissues as fuel for metabolism and growth. This external supply removes the need to breakdown endogenous energy stores. This relationship has allowed larval researchers to use these processes as targets for the estimation of nutritional condition and subsequent larval growth.

Growth is a process that can be measured at a molecular, cellular or whole organism level, and can be defined in unit terms. Condition, on the other hand, is a qualitative concept, more so than a quantifiable parameter. Originally defined as an indication of “fatness or general well-being” (Le Cren, 1951), it has been more recently defined for larval fish as the physiological status (or health) of a larva which is primarily related to its nutritional intake (Ferron and Leggett, 1994) and which will consequently affect the “fitness” or survival probability of that larva (Pepin et al., 1999). However, the term has been used fairly broadly in this regard and not always appropriately in relation to its definition. This has prompted recent calls (Suthers, 2000) for “condition” to be better defined. Suthers (1998) defined the set of indices commonly regarded as “condition” indices into three groups: storage, growth, and starvation indices. “Growth” indices target synthetic processes (e.g. RNA based, protein, cell cycle) that indicate recent nutritional intake but imply little about the endogenous reserves of a larvae, upon which its survival capacity depends in the absence of external food source. “Starvation” indices, such as cell and tissue histological parameters, target degradation processes and can indicate both an arrest in synthetic processes and the degradation of endogenous fuel reserves, and thus an indication of survival capacity. “Storage” indices target endogenous energy stores (e.g. lipids, protein, carbohydrates) and relate closely to the survival capacity of the larvae in times of nutritional stress. Based on these observations, the definition of condition should be amended to: the survival capacity of a larva, as relates to its nutritional status, which will depend on both its recent nutritional intake and the extent of its endogenous energy reserves.

Survival capacity relates directly to survival probability, which may aid in recruitment predictions. Any index that does not account for both recent nutritional intake and endogenous energy reserves is not in the sense of the current definition a “condition” index. Histology-based “starvation” indices come the closest to this definition, but their...
subjective nature and high labour requirements make other growth and storage indices more attractive. Growth indices can still be used to make inferences about condition. For example, low growth may imply little recent nutritional intake, and an increasing reliance of the larvae upon endogenous reserves, and therefore decreasing condition (or survival capacity).

1.2.3.2 Temporal and developmental considerations

The following section describes those factors that will influence the growth and condition of fish larvae, and in doing so, makes three basic points. Firstly, that changes in growth of fish are dictated by genes, environment, and the interaction between these. These factors operate over a wide range of temporal scales, further adding to the complexity of the growth dynamics. Secondly, the relationship between condition and growth changes with development and with environmental conditions. Thirdly, condition and growth of larvae is not “static” in nature, but is influenced by, and will influence, the condition of previous and subsequent stages, respectively.

While growth in fish larvae may manifest as an increase in size or mass, within this lies a rapid continuum of ontogenetic and physiological change. The timing and duration of developmental phases are driven by both genetic and environmental factors (Balon, 1981). In larvae these stages can be divided into yolk dependent, mixed feeding, and pre- and post-flexion (feeding dependent) (Mathers et al., 1993). Each stage varies in its growth rate, type of growth, sensitivity to starvation and therefore susceptibility to changes in condition (Buckley et al., 1999). Protein metabolism (and hyperplasia) dominating in the early stages, lipid and fat deposition (hypertrophy) in the latter (Ehrlich, 1974; Segner and Verreth, 1995). However, different tissues and organs will contribute to overall growth at different times (Mathers et al., 1994), different rates, and often by different modes (hypertrophy v. hyperplasia). If individual tissues are being used to assess growth and condition of larvae, then such factors will affect the relationship between the parameters.

As discussed above, measures of growth have been used to infer condition based on the assumption of a close relationship between the two. However, as Dutil et al. (1998) point out, the overall condition of a fish increases in parallel with its mass, as
endogenous reserves are built up. Hence a decrease in growth of first feeding larvae can be taken to relate fairly closely to nutritional condition and survival capacity, as these larvae have little endogenous energy reserves. As the larvae develop and accumulate endogenous energy reserves, the same growth rate would have less severe implications for its condition. This observation also demonstrates that particular measures of growth may only be appropriate for estimation of condition in certain stages (Malloy and Targett, 1994).

The condition of one developmental stage can affect the condition and growth capacity of the next. In relation to larval fish, both condition and growth status of the adult stock and the environmental conditions surrounding the eggs from which larvae hatch will have a very large influence on the condition, growth and survival capacity (Buckley et al., 1990; Canino, 1994; Baynes and Howell, 1996; Kerrigan, 1997; Gronkjaer and Schytte, 1999). Furthermore, starvation (causing lowered condition) in post-hatch larvae can reduce larval ability to cope with starvation in the future (Pedersen et al., 1990).

1.2.3.3 Environmental considerations

Environmental influences upon larval growth and condition can be classified into two types, cyclical and random. Cyclical variables include those associated with the diel, lunar and seasonal cycles driven by the sun and moon. These have a strong association with water temperature and food availability, two of the most important factors to affect instantaneous and recent growth rates (Buckley, 1984). Hence, changes in growth parameters, such as in RNA/DNA ratio (Mugiya and Oka, 1991; Rooker and Holt, 1996) or growth promoting hormones (Bates et al., 1989), have been linked to diel light/dark cycles, the lunar cycle (Farbridge and Leatherland, 1987) and seasonal cycles in growth of juvenile and adult fish (Le Cren, 1951; Lambert and Dutil, 1997; Arkhipchuk, 1999). Diel fluctuations in physiological parameters have the potential to confound interpretations of larval growth that are based on measures of biochemical growth parameters. This will be discussed further in Chapter 6.

Non-cyclical environmental factors, such as those associated with hydrographic or meteorological events, also affect water temperature and prey availability and play a large role in the growth and condition of larval fish and fish in general. Advection of
larvae away from nursery grounds to areas of low prey density (Lough et al., 1996; McFadzen et al., 1997; Coombs et al., 1999), mixed or stratified sites (Lough et al., 1996; Clemmesen et al., 1997), or position in the water column (Gronkjaer et al., 1997; Gronkjaer and Schytte, 1999), can affect larval access to food. Temperature as an influencing factor requires mention to the same degree as nutrition. The majority of fish are poikilotherms, and environmental temperature directly affects the rate at which synthetic and degradative processes occur, and the resultant growth rate. Indirectly, temperature can affect larval condition and growth by influencing the embryonic development of eggs, both maternally and after spawning and fertilisation (Blaxter and Hempel, 1963; Buckley et al., 1990; Canino, 1994; Baynes and Howell, 1996) as well as the time available to develop feeding skills before the point of irreversible starvation is reached (Baynes and Howell, 1996). These are discussed further in Chapter 5.

Other environmental factors that have been shown to have a direct influence on condition and growth include pollution (Rosenthal and Alderdice, 1976; Wang et al., 1993; Benton et al., 1994), disease (Peterson and Brown-Peterson, 1992; Steinhart and Eckmann, 1992), meteorological events such as storms (Lough et al., 1996), salinity (Jurss et al., 1986), and dissolved oxygen (Rosenthal and Alderdice, 1976; Peterson and Brown-Peterson, 1992; Dutta, 1994).

This section has highlighted the fact that fluctuation in, and the relationship between, growth and condition, will vary on both environmental and developmental based temporal scales, and in a species-specific manner. This highlights the difficulty in developing and applying techniques which will accurately reflect the survival potential of larvae based on growth and condition.

### 1.2.4 Condition and growth indices

#### 1.2.4.1 Classes of condition and growth indices

The proliferation of larval "condition" indices, based on a wide variety of techniques and theoretical approaches, poses perhaps the biggest obstacle to the cross comparison of data between laboratories and across species, and their eventual use in recruitment
studies (Buckley et al., 1999). Few of these indices have been presented as a true condition measure, and those which have come closest have unfortunately not fulfilled the practical criteria of speed and ease of processing (Ferron and Leggett, 1994). While the various types of morphological, histological, otolith and biochemical indices have been successfully used to identify larval condition or growth status, many have deficiencies which make them less attractive or appropriate. Morphological indices have a long latency of response, preservation-associated shrinkage problems, and differences in morphological features between laboratory reared and wild larvae (Ferron and Leggett, 1994). Histological indices are hindered by their relatively slow processing speed, dependency on the subjective judgement of the investigator and variable timing of development of the target tissues (Margulies, 1993; Ferron and Leggett, 1994; McFadzen et al., 1997). Otolith based indices are promising but the possibility of the un-coupling of growth and increment deposition, in response to certain environmental conditions, requires further investigation (e.g. Maillet and Checkley, 1989). Biochemical indices have predominantly focused upon nucleic acids and lipids. The latter is complicated by differences in lipid levels between reared and wild larvae (Hakanson, 1989; Hakanson, 1993), changing lipid content with development (Brightman et al., 1997), and the contribution of gut content lipid to overall estimates (Lochmann et al., 1996). Currently, the predominant indices in use are those using nucleic acids.

The following section details the use of the RNA/DNA ratio, an index which relates most closely to the method being investigated in the following chapters, and which is currently the most widely used and accepted in larval condition and growth research. There are a number of types of nucleic acid indices, which in general focus on either RNA, as an indicator of protein synthetic and growth capacity, or DNA, as an indicator of cell number, size or cycle status (see Wang et al., 1993; Suthers et al., 1996; Bergeron et al., 1997; Suresh and Sheehan, 1998; Bromhead et al., 2000). However, the RNA/DNA ratio is the predominant index in use, based on its sensitivity, accuracy, speed of processing and cost effectiveness (Buckley et al., 1999).
1.2.4.2 The RNA/DNA index

Growth in young fish is achieved primarily through protein synthesis, which is regulated by the level of RNA (Bergeron, 1997). RNA varies with the physiological status and metabolic requirements for protein synthesis and growth (Buckley et al., 1999), and is dependent on adequate nutritional intake. Based on the idea that the amount of DNA per cell is relatively constant, the ratio of total RNA content to total DNA content (RNA/DNA) is taken as an estimate of the protein synthetic (growth) capacity per cell. The reliance of RNA production on nutritional intake allows RNA/DNA to be used as an indicator of nutritional condition, and current growth capacity of fish larvae. Section 1.2.4.3 will detail research that has questioned some of the assumptions that underlie the use of this ratio.

The historical development of nucleic acid based indices in ichthyology has been extensively reviewed (Buckley and Bulow, 1987; Ferron and Leggett, 1994; Bergeron, 1997; Buckley et al., 1999). The one-dye fluorometric method is currently considered the fastest and most sensitive measure of RNA/DNA. Initially proposed by Raae et al. (1988), Westerman and Holt (1988) and progressively modified by Clemmesen (1993) and Wagner et al. (1998), the one dye method uses ethidium bromide, or thiazole orange (Gremare and Vetion, 1994; Elliott and Leggett, 1998), to measure total nucleic acid content. The sample is then treated with RNase to allow determination of DNA content, and the difference between the two is attributed to RNA. DNase treatment can determine if there is any residual or background fluorescence not attributable to nucleic acids (Westerman and Holt, 1988). This method is faster and requires less tissue than the two-dye method. The speed of processing was dramatically improved by Wagner and colleagues (1998), who simplified the extraction and purification method (Caldarone and Buckley, 1991), through the use of sarcosil, and used a 96 well plate fluorescence reader to enable the measurement of up to 80 samples in less than 2 minutes. However, this technique requires much greater attention to procedural detail, with issues such as recovery efficiency of nucleic acids, replicate precision, residual fluorescence, purity of standards, and the use of controls being very important considerations (Buckley et al., 1999).
1.2.4.3 RNA/DNA: Sources of variation

Numerous factors, abiotic and biotic, endogenous and environmental, will play a role in determining the level of RNA production and activity at any one moment in time. There is growing evidence that some of these factors can cause the relationship between growth and RNA to become dissociated, which concerns investigators using RNA based indices. Because the RNA/DNA ratio is currently the “benchmark” index, an analysis of influencing factors and of flaws in its theoretical basis will highlight the criteria which must be met for any new index being developed.

Use of the RNA/DNA index is based on a number of underlying assumptions, which may or may not be justified under all circumstances (Miglavs and Jobling, 1989). The first assumption holds that RNA content will vary in relation to the recent nutritional intake of the larvae, and thereby provide an index of its nutritional status. This assumption requires that the amount of DNA per cell is constant, allowing total DNA content to be used to standardise the amount of RNA to the size of the larvae, or the number of cells in the sample. This assumption provides the link between the index and the nutritional status of the larvae, but is only true once larvae are fully prey dependent, with oil and yolk reserves exhausted (Mathers et al., 1993). The analysis of DNA content to standardise for cell number or larval size does not take into account the fact that DNA cannot indicate growth that occurs via acellular mechanisms, such as bone or lipid deposition (Suthers, 1998), or the fact that cycling cells have an increased DNA content prior to actual division. Up to 40% of cells in larval *Galaxias olidus* brain tissue were found to be synthesising DNA at any one time (Bromhead, 1996).

A second assumption underlying the RNA/DNA index holds that RNA content will be related to protein production, and therefore the somatic growth rate of the larvae, which is then used to infer condition. For this to be true, RNA level must be directly related to somatic growth, and protein production must vary directly with RNA level. RNA/DNA is essentially a measure of a synthesis-related process, and these assumptions do not take into account the fact that somatic growth is the product of both synthetic and degradative processes (Miglavs and Jobling, 1989). For RNA to be directly related to somatic growth it must be directly related to protein accrualment (as opposed to protein synthetic rate). Numerous studies of protein dynamics in fish have shown that protein...
accruement is dependent not only on RNA level, but also RNA efficiency (Houlihan et al., 1995), ribosomal number and activity (Smith, 1981; Lied et al., 1983; Loughna and Goldspink, 1984; Miglavs and Jobling, 1989), protein synthesis, degradation and retention efficiency (Mathers et al., 1994) all of which can vary and react in different ways to different factors. Larval fish have been shown to increase protein production in the absence of an increase in RNA content, implying an increase in RNA efficiency (Mathers et al., 1993; Houlihan et al., 1995; McLaughlin et al., 1995). This increase in RNA efficiency has also been observed as a compensatory response of fish to refeeding after a period of starvation (Miglavs and Jobling, 1989), or in response to anoxic conditions (Smith et al., 1999).

Conversely, fluctuations in RNA in the absence of changes in somatic growth have also been observed to occur in response to temperature. Numerous authors have observed that RNA levels become dissociated from growth rate in cold acclimatised fish (Goolish et al., 1984; Mathers et al., 1993; Escot and Granadolorencio, 1997). Protein synthesis rate and retention efficiency decreases at colder temperatures (Somero and Doyle, 1973; Matthews and Haschemeyer, 1978). In response, RNA concentrations increase in order that overall protein synthesis can remain at a level that allows for maintenance of enzymic activity and metabolism, despite RNA activity being lower (Goolish et al., 1984; Foster et al., 1992). However, it remains to be seen if larval fish have similar compensatory responses (Mathers et al., 1993). A dissociation of RNA levels from growth rate has also been documented in fish reared at higher temperatures, which Brightman et al. (1997) suggest is most likely due to increases in both the efficiency of ribosomes in initiating protein synthesis and in the rate of chain elongation.

1.2.4.4 Considerations of size and developmental status

There is currently some debate over the most appropriate method by which to normalise RNA content to size. One view holds that most of the variation in RNA/DNA between fed and starving larvae is in fact due to differences in their dry weight (DW), and therefore RNA should be normalised to DW so that only variation due to their condition is left (Suthers, 1998). An opposing argument holds that RNA/DNA, which essentially normalises for cell number rather than size, should not be further normalised for size for three reasons (Buckley et al., 1999). Firstly, starvation will result in a decrease in
weight, and normalising RNA for DW will potentially remove some of the treatment effect, thus dampening the signal. Secondly, it could mask differences in condition between larger and smaller larvae, the latter being more susceptible to starvation mortality (Buckley et al., 1999). Thirdly, evidence suggests that size does not play a large role in how RNA/DNA ratios should be interpreted in relation to a larva’s survival chances (i.e. critical ratio very similar over wide range of sizes) (Clemmesen, 1994; Buckley et al., 1999). In addition, some studies have found no relation between size and RNA/DNA (Suneetha et al., 1999).

Regardless of which method is used, there is still variation in RNA content that is associated with developmental status of larvae. In general, RNA/DNA levels are stable or decrease slightly (Westerman and Holt, 1994; Clemmesen and Doan, 1996; Brightman et al., 1997) during the yolk-sac stage and then increase sharply once feeding is established (Robinson and Ware, 1988; Buckley et al., 1999). This increase may be a real physiological increase, or due to weaker, non-feeding larvae with lower condition, dying (Buckley et al., 1999; Pepin et al., 1999). This increase levels off in later larval development (Rooker and Holt, 1996; Gronkjaer et al., 1997; Buckley et al., 1999). Differences in genetics and chance stochastic processes will mean that individual larvae develop at different rates, and a population of larvae of the same age will possess a large amount of variability in the developmental status of its individual larvae. Therefore it is not unexpected that there is a high degree of individual variability in RNA/DNA of larvae sampled from the same population.

The responsiveness of RNA/DNA to starvation has been shown to be stage dependent, and this stage dependency shown to vary with species (Richard et al., 1991; Clemmesen, 1994; Bisbal and Bengtson, 1995; Suneetha et al., 1999). During the early post-hatch stages, RNA varies in its relationship to food availability and growth, depending on whether a larva is utilising endogenous reserves, is mixed feeding, or feeding dependent. RNA/DNA is generally found to be more sensitive once feeding dependency is fully established (Mathers et al., 1993; Clemmesen and Doan, 1996) and can indicate starvation within 3 days of the onset of this stage (Buckley et al., 1999). This sensitivity may decrease in post-metamorphosis larvae and juveniles (Richard et al., 1991; Clemmesen, 1994). A final development-related point refers to tissue growth, which during larval development is non-allometric. Tissues and organs will appear at
different stages, grow at different rates, and react in different ways to various factors. However, the practice of measuring whole body RNA/DNA will integrate all the tissue specific responses, which is likely to complicate the interpretation of whole body measures of RNA/DNA (Theilacker and Shen, 1993), and may explain the high degree of individual variability in RNA/DNA. All of these observations support the idea that comparisons of RNA/DNA in larvae should only be done between larvae of the same developmental stage (Richard et al., 1991; Bergeron, 1997; Buckley et al., 1999) and preferably in a single tissue (Theilacker and Shen, 1993), that has a defined feeding specific response in RNA levels to starvation (Bergeron, 1997).

1.2.5 Summary of section 1.2

This section has detailed the need for condition and growth indices in larval research, and described the current status and problems associated with the predominant larval growth index, the RNA/DNA ratio. As Westerman et al. (1999) noted, the use of metabolism-based biochemical indicators of growth may be limited by the fact that they do not measure the parameter which is most predominantly associated with increases in somatic growth in larvae, this being cell proliferation. Section 1.3 (below) details the theory behind targeting cell proliferation as a larval growth index and describes current flow cytometric methods for determining fractions of dividing cells in tissues.

1.3 CELL GROWTH AND DIVISION

Growth in mature vertebrate organisms is generally dominated by hypertrophy of certain tissues and accumulation of endogenous reserves. Growth in younger rapidly developing stages, however, is characterised also by increased hyperplasia, or cell division. The cell division cycle in eukaryotes is generally accepted as being highly conserved, based on similarities between the mechanisms and components of the cell cycle in unicellular organisms such as yeast, and those of mammals (Murray and Hunt, 1993). Only a few fish-based studies of cell cycle have been published (Kajiura et al., 1993; Balamurugan and Haider, 1998; Yamashita, 1998). While these have further confirmed the highly conserved nature of the eukaryotic cell cycle, most of the following discussion of cell cycle is based on studies of mammalian cell systems. In
post-embryonic stages, cell populations can be said to comprise dividing (or cycling) and non-dividing fractions (Soprano and Cosenza, 1992). The latter population may be either quiescent (i.e. resting, but capable of re-entering the cell cycle), terminally differentiated, or apoptotic (Murray and Hunt, 1993) (Figure 1.1). The following section discusses how each of these cell states relates to the growth of tissues, and organisms in general.

1.3.1 The cell cycle

The cell cycle is a complex multi-phase process, which has only recently been elucidated to any significant degree. It is initiated by the binding of growth factors to receptors in the plasma membrane, triggering a cascade of reactions within the cell which culminate with cell growth, DNA synthesis and eventual division. The entire cycle and the events that it comprises are typically divided up into a number of phases (Figure 1.1).

The two main phases are inter-phase and mitosis (Murray and Hunt, 1993), each of which can be further subdivided according to specific events that occur sequentially in each stage. Temporally speaking, inter-phase takes up most of the cell cycle, and is divided into three sub-phases: Gap 1 (G1), Synthesis (S) and Gap 2 (G2) (Howard and Pelc, 1953). G1 is a cell growth phase characterised by increased RNA and protein synthesis (John, 1981), and events which prepare the cell for replication of its DNA. G1 progresses into the S (or synthesis phase) where replication of the chromosomes occurs. This is followed by G2, in which the cell prepares for mitosis. Mitosis comprises five stages: prophase, pro-metaphase, metaphase, anaphase and telophase, in which chromosomes condense, nuclear membrane breaks down and spindle structures are formed which draw the replicated chromosomes to opposite poles of the cell. This is followed by the physical division (cytokinesis) of the cell into two daughter cells. Entry into the cycle is dependent on those factors that act as mitogenic signals.

Signals capable of triggering a mitogenic response can be physical (i.e. spatial or contact based) or chemical (Baserga, 1985). Physical signals are generally predominant after injury and loss of cells, with nearby cells stimulated to “fill in the gap”. Chemical
signals are generated by a group of molecules referred to as growth factors. These polypeptide molecules form a large class of molecules within the endocrine system (Olashaw et al., 1992), which is itself stimulated by external factors such as photoperiod, temperature and feeding. Growth factors, which can be stimulatory or inhibitory, bind to specific receptor molecules in the plasma membrane, and act not only to initiate the cell mitogenic response but also to regulate the cell cycle through each of its phases.

While the internal mechanisms driving the cell cycle are not yet fully understood, the key regulating components are now reasonably well defined. The three main players are the cyclins, the cyclin dependent kinases (CDK), and the complexes that these combine to form at certain stages (e.g. mitosis promoting factor, MPF). Cyclins act with CDKs to phosphorylate key protein substrates, inactivate tumour suppressor genes and allow the progression of the cell through each stage in the cell cycle (Dirks and Rutka, 1997; Schafer, 1998). There are three main groups of cyclin, these being the G1-, S-, and M-phase cyclins. Each group of cyclins has a corresponding group of CDKs, which must bind its respective cyclin in order to be activated. And while CDK levels are relatively constant, the levels of cyclins rise and fall with their respective stages of the cell cycle, thus periodically activating and inactivating their respective CDK and allowing progression of the cell through its cycle.

### 1.3.2 Non-cycling states

Providing that there is still sufficient mitogenic stimuli following cell division, newly formed cells can continue to cycle. However, checkpoints exist in the cell cycle which act to prevent the progression of the cycle if conditions are not appropriate, and thus avoid errors in DNA replication and chromosome segregation. Cells can be forced to leave the cell cycle either temporarily or permanently, and enter a stage designated G0 (Murray and Hunt, 1993). Cells in this stage are dormant with respect to division but still physiologically active, carrying out their role in their respective tissues. Cells, which leave the cycle temporarily, are said to be quiescent and will remain in this state until the appropriate mitogenic stimuli are present. In most normal multicellular eukaryote cells, the main regulatory checkpoint occurs at the G1-S transition, and is
Figure 1.1 - Model of a generalised multicellular eukaryote cell cycle. During active cell growth and division, cells pass through G1 (growth phase), S (DNA synthesis phase), G2 (second gap phase), and M (mitosis) before dividing physically (cytokinesis). Newly divided cells may continue to cycle, terminally differentiate into a non proliferative cell type, or temporarily arrest in G0 (quiescent phase) until nutrient and growth factor levels are high enough to stimulate and support the division process. A checkpoint, R, exists in the cell cycle to ensure that the cell is ready for a new cycle. A second checkpoint (G2M checkpoint) ensures DNA synthesis and proper chromosome segregation. If cells are found to be damaged, or be physiologically impaired, or have reached the end of their physiological lifespan (at G2M or R checkpoints) then they may become apoptotic. Adapted from Soprano and Cosenza (1992).
generally referred to as the restriction point (R). R acts to coordinate the cell cycle with growth thus preventing the formation of progressively smaller cells by ensuring daughter cells pass a threshold size (Campisi and Pardee, 1984). Progression through R also depends on adequate nutrition (amino acid supply) and serum or growth factors (Campisi and Pardee, 1984; Pardee, 1989). It is generally accepted that essential amino acids provided from diet are critical to continuation of cell cycle due to their role in the formation of proteins involved in late gene transcription (Eagle, 1955; McKeehan and McKeehan, 1981).

G1 phase is sensitive to the nutrient status of the immediate environment, while subsequent stages are relatively immune to nutrient deprivation. Evidence from mammalian cell culture experiments has demonstrated that once cells pass R and enter S-phase, they are committed to completing the division cycle, and subsequently arresting in G0 (Zetterberg and Larson, 1985). This latter stage insensitivity results from protective mechanisms, such as internal energy store reserves, which prevent lack of external nutrients from reducing ATP and stalling cell cycle during critical periods such as DNA synthesis (Murray and Hunt, 1993). Nutrient deprivation induced arrest has been used by researchers to synchronise mammalian cell cultures at G1 (e.g. Lindeman et al., 1997). G2 phase arrest in response to nutrient deprivation has been noted in fission yeast (Murray and Hunt, 1993) and S-phase arrest in human lung cancer cell lines (Tinnemans et al., 1995), however these results may not be relevant to normal somatic cell in multicellular eukaryotes.

Quiescent cells can re-enter the cell cycle, however, many cells which arrest post-division do so permanently in order to fulfil a specific physiological function in their host tissue or organ. Examples of such terminally differentiated cell types include G1 arrested neurones, and G2 arrested epidermal cells. Terminally differentiated cells from different tissues have different life spans, which affects the rate of replacement division in each tissue. For example, adult mammalian digestive epithelial cells survive for three days and require constant replacement. In contrast, liver cells can survive for up to a year and therefore cell division is much less frequent (Murray and Hunt, 1993). Some cells will arrest as a result of DNA damage or abnormal physiological function, and these cells may become apoptotic. Apoptosis, also known as programmed cell death, is generally recognised as serving two main functions, these being the removal of
abnormal or potentially cancerous cells, and the removal of unwanted and excess tissue during development of organs and tissues (Maclean and Hall, 1987; Murray and Hunt, 1993). The main morphological features of apoptosis are cytoplasmic and nuclear condensation, cell shrinkage, and release of membrane bound vesicles. Calcium dependant nucleases also cleave double-stranded DNA, creating small kilobase fragments (Wyllie et al., 1980). Cell division functions not only to allow growth but also to replace dead cells (Maclean and Hall, 1987) and overall growth occurs when the number of cells produced is greater than the fraction dying (Baserga, 1985).

1.3.3 Flow cytometric cell cycle analysis

The research presented in this thesis centres on the development and application of methods that are not yet accepted or familiar to most larval fish researchers. For this reason, it is important to detail both flow cytometry and its application to cell cycle analysis. A flow cytometer (FCM) is an instrument, which enables multiple cellular parameters to be rapidly and accurately quantified in individual cells. Initially, flow cytometry was developed as a diagnostic tool in cancer research, but today is used in any field which has an interest in cellular function or growth, including immunology, microbiology, parasitology, toxicology and marine biology (Shapiro, 1988).

1.3.3.1 The flow cytometer

Analysis of cell parameters by flow cytometry is a four-step process involving sample collection, preparation, processing by FCM, and data analysis. Sample preparation of cells involves their dissociation into a single cell suspension and the chemical treatment with a fluorescent probe(s), which will bind specifically to the cell constituent(s) that one wishes to measure (Shapiro, 1988). The FCM draws the cell suspension from the sample tube via a siphon. Hydrodynamic focussing principles are then employed to create a laminar flow within the tubing which effectively restricts the cells to flowing single file in the very centre of the stream. Cells are then passed through the “observation” point, or point at which a laser beam (illumination source) intersects with the cell stream (Figure 1.2A) (Shapiro, 1988). As each cell passes through this point it will emit fluorescent light signals (from the probe bound to constituent of interest), as well as scatter the incident light. Fluorescent signals are detected by fluorescence
collection lenses (photomultiplier tubes) while scattered light of different angles are collected by forward scatter and side scatter collection lenses (photovoltaic photodiodes or solar cells). Each of these types of detector generate current in response to light absorption. These “pulses” in current caused by the signals emitted by each cell, can be converted to digital data relating to the amount of light detected (Figure 1.2B). The height and area of a fluorescence-generated pulse is related to the amount of fluorochrome(s) bound within each cell, and therefore the relative amount of the constituent(s) to which the dye selectively binds. Pulses created by light scatter signals relate to the size of a cell and its internal complexity, which are useful in separating or identifying cell types. Analysis of a particular parameter from a cell sample is often done by representing the parameter in a frequency distribution (histogram), and employing statistical or modelling programs to analyse changes in distribution of parameters between samples or relative to other parameters. Standardisation of procedures is extremely important for accurate flow cytometric analysis, both in sample preparation, instrument set-up and data analysis (Shapiro, 1988).

1.3.3.2 DNA based cell cycle analysis using flow cytometry

The use of cell cycle analysis to estimate growth in single or multicellular organisms, is already accepted within a number of fields. These include microbiology (Muratahori and Fujishima, 1996; Liu et al., 1997; Liu et al., 1999), cell biology (Wheatley et al., 1994; Kumada et al., 1995; Kominami et al., 1998), developmental biology (Freeman, 1995), oceanography (Vaulot et al., 1995) and medicine.

The amount of DNA in a cell increases during S phase and is twice that of G1, from the end of S phase until cytokinesis. Therefore, a measure of the amount of DNA in a cell can be used to indicate mitogenic stimulation. A number of fluorochromes have been developed which are specific for DNA (e.g. Diamidino-2-phenylindole) or for double stranded nucleic acids (e.g. propidium iodide), the latter of which can be used for DNA assessment after RNAse treatment (Robinson, 1993). The amount of fluoro-chrome that binds will be proportional to the amount of DNA in the cell, and this is indicated by the intensity of fluorescence emitted by each cell as it passes through the FCM laser.

Standardisation of flow rates, cell concentrations, fluoro-chrome concentration and instrument set-up, as well as use of controls to ensure stable cell ploidy and centring of
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Figure 1.2 - Schematic of a basic flow cytometer. Dissociated and stained cells are siphoned into the flow chamber where they are hydrodynamically focussed into a single cell stream. This stream intersects at right angle with a laser beam, which causes the bound dye to fluoresce. These fluorescent and normal light signals, are detected by fluorescence and light detectors, which B) generate “pulses” of current in response light absorbed from the emissions given off by the cells as they pass through the laser beam. The width (or duration) of a pulse gives information regarding the size of the cell, or fluorochrome bound particle, and the height (amplitude) or area (integral) of the pulse will be proportional to amount of fluorescent material bound within the cell, and therefore the amount of the constituent that it is bound to (Shapiro, 1988).
peak acquisition, are important issues which must be accounted for to ensure accurate DNA analysis by flow. Once the raw data is acquired, it can be converted to a DNA frequency histogram (Figure 1.3). In normal diploid populations, this is characterised by a G0G1 (or G01) peak, an S phase “trough”, and a G2M peak. By using modelling programs which calculate the area under these peaks, the proportion of cells in S and G2M can be determined, and used as an indicator of the proliferative (growth) status of that cell population, tissue or organism from which the cells originated.

Models vary in their complexity, and the suitability of a model is determined based on the shape of the histogram. If a histogram has ideal shape and little debris or aggregates, then a simple model is recommended. These generally calculate the area under the left and right hand halves of the G01 and G2M peaks respectively, multiply them by two, with the remainder attributed to S phase. More complex models can be used which employ curve fitting “least squares” methods on an iterative basis. In other words, successive approximations are made with parameters in the model being revised and re-fitted. These models allow cell aggregates to be gated out and debris to be modelled and subtracted. The goodness of fit is estimated by the reduced chi-square statistic (RCS), which is “a measure of the deviation of the fitting data from the actual data” (Rabinovitch, 1994). The precision of the actual staining and measurement of the cells is given by a coefficient of variation (CV), which is equal to 100XSD/(mean of peak) and describes the G01 peak width. Given that G01 cells will all have exactly the same amount of DNA (within species), an ideal histogram would have all G01 cells falling within a single channel. However, very slight variations in cell to cell staining and FCM signal detection mean that in real situations, the peak has a gaussian distribution about its mean. Ideally, for accurate analysis it is recommended that CV be less than 8% (Shankey et al., 1993) or even 5% (Shapiro, 1988).

1.3.3.3 Previous larval growth research utilising cell cycle analysis

The analysis of cell cycle as a means to determine the growth and condition of fish larvae is a relatively recent development, and has been investigated by only a few researchers. Early research papers were by Theilacker and Shen (1993a,b), followed by Moore and colleagues (1994), and a methods chapter (Moore and Morss, 1996). More
Exponential modelling of debris

Gaussian modelling of G01 and G2M

Trapezoid modelling of S phase

Overlap

Figure 1.3 - Cell cycle analysis of DNA histograms involves applying models to fit the data and estimate the number of cells in each phase of the cycle. A typical DNA histogram may have some debris, some aggregates, and possibly an apoptotic population, as well as the normal G01, S and G2M components. Debris is typically modelled using an exponential (or related) function. G01 and G2M peaks are modelled using gaussian distributions, while S phase is typically modelled using a trapezoid distribution. Modelling programs generate two statistics, the CV and RCS, which help the researcher to determine if the model is appropriate for the data and has applied a good fit. The CV is a measure of the G01 peak width, which will be large if the staining procedure and instrument settings are inappropriate or inefficient. The RCS gives a measure of the “goodness of fit” of the model to the actual data.
recently, there has been a promising publication on cyclins (Westerman et al., 1999), and papers by Bromhead et al. (2000) and Theilacker and Shen (2001). The following discussion focuses on research in larval cell proliferation which has used flow cytometry.

Theilacker and Shen (1993a) published the first results from a preliminary laboratory investigation into the use of flow cytometry and cell cycle analysis in larval growth analysis. A more comprehensive investigation and paper followed (Theilacker and Shen, 1993b). This work proposed that flow cytometric analysis of larval brain cells (stained with acridine orange) could be used to determine: (a) DNA content per cell and therefore the fraction of dividing cells, as a measure of growth; and (b) RNA content per cell as a measure of condition. Their choice of a single tissue avoided complications in interpretation that might result from integrating tissue-specific responses. They targeted brain tissue in particular due to the ease of cell dissociation, the high RNA content of this tissue, and previous research which indicated that brain cell RNA and division were both related to larval feeding history (Theilacker and Shen, 1993a). These claims were made despite histological evidence that brain tissue is somewhat defended from effects of starvation (O'Connell, 1976; Theilacker, 1978) and that brain growth is relatively slow compared to overall growth in fish (Brandstatter and Kotrschal, 1990).

They classified cells in G0 and G1 as region 1, representing RNA synthesis, and those in S, G2 and M as region 2, representing DNA synthesis, and later classified as the fraction of cells dividing (FOCD). They attempted to distinguish the quiescent G0 cells from G1 cells by treating the sample with RNase, and using the resulting RNA negative population as an indicator of the G0 cells distribution. They then subtracted this using a modelling program to determine the fraction of cells in G1 (Theilacker and Shen, 1993). Their results indicated that both RNA activity and cell division could be used as indicators of nutritional status. They showed that the number of cells in G1 was higher in fed larvae or refeeding larvae when compared to starved larvae, and that the move of cells from G1 to S may be delayed in starved-refed larvae. In summary they concluded that larvae that had low cell division and high fraction of G1 cells in brain tissue, would indicate current feeding but recent starvation. However, they also had a number of cautionary comments. They stated that more studies are required to determine "how RNA level responds to feeding rate and meal size over time, to describe the ontogenetic
pattern of cycling cells, to determine the duration of the cell cycle, and to explore effects of food limitation after initial period of good feeding. Additionally, the index needs to be calibrated for the effects of temperature and fish size” (Theilacker and Shen, 1993b).

The same authors recently published a paper which investigates and compares both brain cell and muscle nuclei as potential candidates for flow cytometric cell cycle analysis of growth in fish larvae (Theilacker and Shen, 2001). They reasoned that the number of dividing muscle cells was an appropriate indicator on the basis that muscle growth in larval fish was more likely due to increase in cell number than in cell size. Results indicated that both brain and muscle cell division was sensitive to the feeding conditions of the larvae, that larger larvae had higher fractions of dividing cells, and that FOCD is positively related to growth rate (mm/d). They also demonstrated that 3.5 times more muscle cells were dividing in 5 day fed than 5 day starved larvae (compared to 2.5 times in brain), indicating that muscle was increasing in mass faster than brain tissue. In addition, muscle and brain FOCD were correlated with each other for all treatments, and there was some evidence that brain cell division was defended from the effects of nutrient deprivation, when compared to muscle. They developed a model that successfully classified 79% of larvae as either “fast” or “slow” growing, based on size and FOCD.

The pioneering research of Theilacker and Shen (1993a, b) demonstrated cell cycle based analysis of larval growth to be an approach with considerable promise, but also highlighted numerous issues which required further research. We investigated the application of the technique in brain tissue isolated from both lab reared and wild larvae of a freshwater species, *Galaxias olidus* (Bromhead et al., 2000). We concentrated on the DNA based cell division analysis, using the ratio of G2M cells to G0/G1 cells to calculate a cell division index (CDI). The study determined that: (1) brain cell division was influenced by temperature; (2) a strong length dependant relationship was evident in larvae from different sites; and (3) that there was significant fluctuation in mean CDI of larvae sampled over 24 hours. CDI and gut content analysis from this and later field collected samples suggested a physiological link between the timing of growth and digestive processes over a diel period. The final experiment confirmed Theilacker and Shen’s (1993) observation that the proportion of larval brain cells dividing decreases in
response to starvation and increases again, with a lag phase, upon refeeding. While this pattern of nutrition-related response in CDI was the same at two different temperatures, mean daily CDI was consistently lower at 12°C than at 20°C. However the experiment did not run a control of constant fed larvae. The application of the laboratory data to the assessment of growth and condition in wild larvae was complicated by a difference in length distributions of the two data sets. Only a very tentative prediction was made based on an extrapolation of the regression lines (Bromhead 1996, Bromhead et al. 2000).

These results highlighted a number of unresolved questions that require further research before the flow cytometry method can be applied in the field assessment of larval condition. For example, how does the relationship between brain cell division, and environmental factors such as temperature, nutrition and photoperiod, change as larvae develop and mature? What is the temporal sensitivity of the index to changes in feeding conditions? What is the relationship between photoperiod (or time of day), feeding and variation in CDI? Is it possible that some cells were arresting in G2 in response to starvation? If so, to what extent and how does this affect the sensitivity of the index? Was the sub-diploid population observed in some histograms composed of apoptotic cells? If so, could this population be quantified and incorporated as an indicator of cell loss (tissue degradation)? Based on these questions, and those raised by Theilacker and Shen (1993), it is clear that more basic research is required in the fine-tuning of methodologies and modelling, in understanding dynamics within the cell cycle, and in defining the relation between cell cycle and intrinsic and environmental parameters.

The following chapters detail the use of DNA-based cell cycle analysis, as measured by flow cytometry. However, it is useful to briefly consider and compare these with other methods of cell proliferation analysis which have been used in studies of larval fish condition and growth. The in vivo incorporation and immuno-histochemical staining of uridine base substitutes, such as BrdU, CldU, and IdU (Aten et al., 1994; Moore et al., 1994; Poot et al., 1994) in living cells and organisms has enabled the more definite separation of cells synthesising DNA. However, such substance have some toxicity to the test organism (Moore and Morss, 1996), thus they may not be of use in defining growth response in healthy organisms, nor can they be used to assess growth in wild sampled organisms. However, a BrdU-based cell proliferation assay was developed by
Moore and colleagues (1994) for use in larval fish. Another promising method for defined analysis of separate cell cycle phases is the flow cytometric analysis of cyclins, which has arisen through the development of antibodies to different cyclins expressed selectively in particular phases of the cell cycle (Darzynkiewicz et al., 1994). These include cyclin B1 which controls entry into mitosis, and cyclin E whose expression peaks at the G1/S transition (Norbury and Nurse, 1992; Sherr, 1993). The advantage of targeting these components is that they are very specific for cycling cells (Darzynkiewicz et al., 1994). Cyclin analysis has recently been used to determine cell proliferation in larval fish by Westerman et al. (1999). They describe a quantitative spin-filter assay of sucl-precipitated MPF activity, which when used in a simple growth experiment, demonstrated that MPF activity in fast growing red drum (Scianops ocellatus) larvae, was significantly higher than in intermittently fed larvae.

1.3.4 Brain tissue

Brain tissue is considered an appropriate tissue target for the assessment of growth and condition in fish larvae for the following reasons. Firstly, the brain is particularly amendable to flow cytometric cell cycle analysis (Theilacker and Shen, 1993b; Bromhead, 1996) due to its ease of cell isolation and preparation. Secondly, the brain is the second largest tissue mass in post-hatch larvae, and has a high RNA content. Thirdly, cell proliferation (hyperplasia) is the dominant growth mode in the brain of fish (Zupanc, 1999), with little or no demonstrated hypertrophy, and is a continuous process that occurs throughout a fishes life (Zupanc, 1999). Histological evidence suggests the brain is one of the last tissues to be affected by starvation (O'Connell, 1976; Theilacker, 1978), in which case any effect observed in the brain will reflect an even greater effect upon the whole organism.

Until recently, brain growth in teleosts was not particularly well studied or understood. The last 10 years has seen an explosion in research of brain cell proliferation (Zupanc, 1999; Zupanc and Ott, 1999) and death (Soutschek and Zupanc, 1995; Soutschek and Zupanc, 1996) in adult fish. These have demonstrated that, in adult teleosts, cell proliferation activity occurs throughout many areas of the brain at some level, but is particularly active within the cerebellum (Zupanc and Horschke, 1995). Newly divided cells can then migrate from proliferative zones to their target site where they may
differentiate into neurones or remain glial cells. However, many are thought to die within 4-7 weeks. Unfortunately, there are very few studies that investigate the origin and proliferation of brains cells in larval fish. Most studies of brain growth in larval fish have been morphometric in nature (Packard and Wainwright, 1974; Toyoda and Uematsu, 1994; Tomoda and Uematsu, 1996).

1.4 STUDY SPECIES

The species chosen as subjects for the following research were snapper, *Pagrus auratus*, and golden perch, *Macquaria ambigua*. While chosen partly for their accessibility, these two species come from different habitats (marine and freshwater) and have quite different development strategies. It was hoped that this might help determine the between species applicability of the method being investigated, as well as highlight any developmental related issues that might need be addressed.

1.4.1 *Pagrus auratus*

Formerly thought to be two separate species, *Chrysophrys auratus* (northern hemisphere) and *Pagrus major* (southern hemisphere) were re-classified by Paulin (1990) as a single species *Pagrus auratus*, commonly known in Australia as snapper. The distribution of snapper ranges from the warm temperate or subtropical waters off Australia and New Zealand, up through Asia to China and Japan (Paulin, 1990). They are an important species for fisheries and aquaculture in a number of countries, particularly Japan, where large scale restocking programs have been attempted for this species (Foscarini, 1988; Francis, 1997). They have been identified as an excellent aquaculture prospect in Australia (Bell *et al.*, 1991; Battaglene and Talbot, 1992) and are currently subject of large-scale inland saline water trials (S. Fielder, personal communication). They are a carnivorous species that migrate to shallow coastal waters to spawn. The larvae hatch relatively early and undeveloped, feeding initially on zooplankton (Pankhurst *et al.*, 1991). In culture, larvae absorb their yolk sac by day 2 and oil globule by day 6 by which time external feeding has commenced (Battaglene and Talbot, 1992). Metamorphosis is complete by around day 25 or 8.6 mm TL (Battaglene and Talbot, 1992). Following metamorphosis, larvae adopt a more benthic
feeding strategy, migrating to seagrass beds in shallow bays and estuaries (Foscarini, 1988; Hecht et al., 1996). Juveniles retreat to increasingly deeper waters each successive winter as they get older. Snapper reach maturity after 3 to 4 years. They have been subject to a large-scale recruitment study off New Zealand (Francis, 1997).

1.4.2 *Macquaria ambiguа*

Commonly known as golden perch or yellow-belly, *Macquaria ambiguа* is a native freshwater species which is distributed throughout most of the Murray-Darling system (see Arumugam and Geddes, 1987; Arumugam and Geddes, 1992). There is still much to be learned about the biology and ecology of this species, particularly regarding its early life history stages. Adults migrate upstream to spawn in response to rising floodwaters. It is thought that the buoyant eggs are then carried downstream to high nutrient floodplains, which act as nursery grounds for larvae. This species is currently threatened by damming and weir constructions which may prevent spawning migrations and reduce suitable nursery habitat caused by flooding (Arumugam and Geddes, 1987).

1.5 AIMS

This chapter has outlined the current status of larval condition and growth research, highlighting some of the theoretical and methodological limitations of currently used indices such as RNA/DNA ratio. It also introduced and detailed the theory behind the cell proliferation approach, and why such an index might provide a highly sensitive and appropriate alternative measure of larval growth. The discussion of previous investigations into this approach has highlighted a number of unresolved questions and areas requiring further investigation. Based on this discussion, the current investigation aims to:

a) Investigate and optimise the methodological and analytical criteria required for the sensitive and accurate determination of cell cycle fractions in brain tissue isolated from larval teleosts. This includes developing a secondary indice to assess cell loss (death) and net tissue growth (Chapter 3);
b) Investigate the effect of abiotic environmental factors including temperature, salinity and light (photoperiod) upon brain cell proliferation and death, and the implications of these relationships for the assessment of larval growth using this index (Chapters 4, 5 and 6);

c) Determine whether proliferation of larval brain cells exhibits diel variability, the nature of this variability (cyclical or random) and possible triggering factors. Do patterns of variability vary with developmental status or between species? (Chapter 6);

d) Further investigate the relationship between the nutritional status and brain cell proliferation, focusing on the latency of responses to starvation and refeeding, sensitivity to varying duration’s of starvation, and correlation with overall larval growth rates (Chapter 7);

e) Assess the importance of developmental status, larval age and size, for the interpretation of cell proliferation data. Do overall levels of cell proliferation change with development? Does the effect of environmental factors upon brain cell proliferation change as larvae develop? (Chapters 4, 5, 6 and 7);

f) Assess the relationship between different components of the cell cycle (S and G2M phases) with overall larval growth rates. Which cell cycle fraction provides the most sensitive indicator of overall larval growth? (Chapters 4, 5, 6 and 7); and...

g) Assess the overall potential of this approach for the assessment of larval growth and its possible use in studies of recruitment dynamics of wild fish species (Chapter 8).
CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 INTRODUCTION

The following chapter describes the methods and materials used in the course of this research. Many of the methods described are based on standard laboratory methods and cycle fractions required considerable preliminary testing, standardisation and modifications, in order to appropriately suit them to the new aims and subjects to which they were being applied. Details of these preliminary experiments are presented in Chapter 3. The finalised protocols and testing methods therefore are described in the current chapter, and are used in the main body of experimental work to investigate the relationship between cell cycle and intermolecular and intramolecular factors (Figure 2.1). Larvae of snapper, Parapercis colias, were used as experimental subjects for all investigations but where indicated. Fish were bred at Port Stephens and algae grown at Port Stephens Research Centre (PSRC) whereas Snapper were stocked at Taylor Beach, Port Stephens, on the northern coast of NSW, Australia. Larvae were reared in conjunction with modes being run by NSW Fisheries (see Chapters 4 and 5), or from separate experiments run using the PSRC facilities (Chapter 2).

2.2 LARVAL SUPPLY AND REARING

### 2.2.1 Supply

All snapper larvace used in this study, as well as the facilities and equipment for their rearing, were generously supplied by Steven Fisher of NSW Fisheries Port Stephens Research Centre (PSRC). Larvae at Taylor Beach, Port Stephens, on the northern coast of NSW, Australia. Larvae were reared in conjunction with modes being run by NSW Fisheries (see Chapters 4 and 5), or from separate experiments run using the PSRC facilities (Chapter 2).
2.1 INTRODUCTION

The following chapter describes the methods routinely used throughout the course of this research. Many of the methods associated with flow cytometric analysis of cell cycle fractions required considerable preliminary testing, standardisation and modification, in order to appropriately adapt them to the new aims and tasks to which they were being applied. Details of these preliminary experiments are presented in Chapter 3. The finalised protocols and rearing methods however, are described in the current chapter, and are used in the main body of experimental work to investigate the relationship between cell cycle and environmental and endogenous factors (Figure 2.1). Larvae of snapper, *Pagrus auratus*, were used as experimental subjects for all investigations bar those in Chapter 6, in which larvae of golden perch, *Macquaria ambiguа*, were also investigated.

2.2 LARVAL SUPPLY AND REARING

2.2.1 Snapper (*Pagrus auratus*)

2.2.1.1 Supply

All snapper larvae used in this thesis, as well as the facilities and equipment for their rearing, were generously supplied by Stewart Fielder of NSW Fisheries Port Stephens Research Centre (PSRC), located at Taylors Beach, Port Stephens, on the north-east coast of NSW, Australia. Larvae were sampled in conjunction with trials being run by NSW Fisheries (see Chapters 4 and 5), or from separate experiments run using the PSRC facilities (Chapters 6 and 7).

In general, the basic supply and rearing details of larvae for all of these experiments were as follows. Mature females of the Australian snapper, *Pagrus auratus*, were artificially induced to spawn in a 17 ML broodstock holding tank, located at the Port Stephens Research Centre, N.S.W., Australia. Spawning induction was achieved by intramuscular implantation of 400 µg of Leuteinising Hormone Releasing Hormone Analogue (LHRHa; Peptech Animal Health Pty Limited, North Ryde, N.S.W) within
Figure 2.1 - Overall design of experimental work. Initial research aimed to refine the techniques being applied. This was dealt with in 4 sections (A), aimed at improving cell extraction and preparation protocols, cytometric data collection, and analysis (modelling or gating programs). These protocols were then applied to large-scale experimental trials (B), which aimed to investigate the intrinsic and extrinsic factors, which might be related to cell growth and division. The end aim is to present conclusions as to the suitability of this index in measuring larval condition and growth, and to future applications in assessing growth and condition of larvae in the field (C).
cholesterol pellets. Viable eggs were collected in a 500 µm sump net and transferred by dip-net to a 750 L incubating tank (22°C) filled with sterilised seawater, until larvae had hatched. Larvae were then collected by draining the incubating tank through a submerged 53 µm harvest screen. Larvae were then stocked at the required density in 100 L “experimental” tanks. Stocking involved initial acclimation in floating buckets containing 30% native tank water and 70% incubation tank water, over a period of 45 minutes to 24 hr, depending on the treatments being applied (see individual chapters).

2.2.1.2 Experimental tank design

The design and set-up of larval snapper experimental tanks was the same between experiments (except Chapter 6), and followed that recommended by Fielder and Bardsley (1999). Any changes to this are noted in the relevant chapters. The marine hatchery in which most experiments were conducted contains 32 identical 100 L, conical bottom fibreglass tanks (Figure 2.2a). Each tank is housed in its own black plastic covered box, to prevent light exchange between tanks. Tanks walls were painted black to allow larvae to visualise prey, while the conical base was white. Each tank operated independently, using water recirculated through an external biological filter comprising filter wool on top of bio-balls and sterilised crushed sea shells. The outflow pipe was located in the centre of each tank and had a 200 µm screen to prevent loss of larvae. Each tank was lighted by an artificial source operating at 12 µmol s⁻¹ m⁻² on a 12-hour light-dark cycle. Water temperature was maintained at 21.5 ± 0.7°C for the duration of the experiments. Surface skimmers were installed in all tanks to remove surface oil and debris and allow swim-bladder inflation (Battaglene and Talbot, 1994).

2.2.1.3 Maintenance and Feeding

Snapper larvae were first fed on day 4 after hatching. Food consisted of live rotifers, Branchionus plicatilis, which had been boosted on a diet of micro-algae Pavlova lutheri, and Tahitian Isochysis galbana, as well as a supplement of DHA Super Selco (Inve Aquaculture NV, Oeverstraat 7-01200, Belgium). Prior to feeding to snapper larvae, rotifers were acclimatised to the environmental parameters (e.g. temperature, salinity) that they would encounter within the experimental tanks, to prevent shock and
Figure 2.2 – Basic tank design used in experiments for: A) snapper *Pagrus auratus* larvae; or B) golden perch *Macquaria ambigua* larvae. Note that Chapter 6 uses a different tank system for snapper (see Chapter 6).
dropout after transfer (Fielder et al., 2000). Larvae were generally fed once daily, between 10 am and 11 am, at a rotifer density of 1/mL in each tank. Salinity, water temperature, dissolved oxygen, pH and water levels were checked daily. Approximately 10% of water was exchanged each day, and bottom debris was siphoned off each afternoon. Salinity, which tended to increase as a result of evaporation, was kept constant by addition of lower salinity water during water exchanges.

2.2.2 Golden Perch (*Macquaria ambigu*a)

2.2.2.1 Supply

Mature females of the Australian Golden Perch *Macquaria ambigu*a were artificially induced to spawn in 2.5 ML brood-stock holding tanks, located at the Inland Fisheries Research Station (IFRS) outside Narrandera, N.S.W, Australia (courtesy of Stephen Thurstan and NSW Fisheries). Viable eggs were collected and transferred to 250 L incubating tanks until hatching. After examining a sample of larvae to ensure that they were disease free, larvae were collected on day 4 after hatching by draining through a bottom valve, and placed in oxygenated plastic transport bags. Larvae were transported 3 hours to the freshwater aquarium housed at the Department of Botany and Zoology, Australian National University in Canberra (ANU animal ethics approval number: SBTZ 0999). Larvae were initially acclimatised to and housed in a 250 L stock tank, at 25°C, 3 ppt salinity, with water conditions, aeration, and lighting conforming to those in the experimental tanks to which they would eventually be transferred. Larvae were left for 48 hours to recover from any transport stress. However, stress was considered minimal, given that larval survival during transport was near 100%.

2.2.2.2 Experimental tank design

The aquarium housed 2 tank stands, each with 3 levels. Experimental tanks were located on the middle shelf. Four 250 L rectangular glass tanks had dividers inserted to create 8 x 125 L tanks into which larvae could be stocked (Figure 2.2B). Blackout covers were fitted to each tank, to ensure a truly "dark" night period, as golden perch larvae are capable of feeding in extremely low light levels. The top cover could be removed during
the day to allow light in for feeding, while the side of the tank remained blacked out, to provide a background against which the larvae could visualise prey. Aside from light source, each tank operated independently, with no filtration and one airstone. Lighting was provided by fluorescent roof lights, partially screened out by top covers on each tank, to provide the low light levels preferred by *Macquaria ambiguа* larvae for feeding (Steve Thurstan, personal communication). Each tank had its own siphon for sampling and cleaning, and individual beakers present for *Artemia* counts and surface oil skimming, to minimise the likelihood of disease spreading between tanks.

A further 4 tanks (250 L) were set up as supply tanks on the top shelves, with two supplying 35 ppt saltwater for dilution in *Artemia* culture, and two supplying 3 ppt saltwater to top up the 8 larval tanks during daily water exchange. All four had filtration devices fitted and above water inlets to ensure dechlorination by aeration. A 100 L tank was set up to dechlorinate and supply freshwater when needed. A smaller 20 L tank with 3 airstones was set up to hatch *Artemia* cysts, for larval feeding.

### 2.2.2.3 Maintenance and Feeding

Transported larvae were initially stocked into 2 x 125 L tanks, in 3 ppt saline water, for the acclimation period of 4 days. Slightly saline water decreases the likelihood of disease and allows *Artemia*, added as food, to survive for up to 24 hr. During this period, the larvae were monitored to ensure that they were disease free. The feeding regime followed that recommended by NSW Fisheries protocols (S. Thurstan, personal communication). On day 5, live first instar *Artemia* nauplii were introduced into the tanks at a density of 6/mL, to provide nutrition for first feeding larvae. *Artemia* densities were checked twice daily from this point on and topped up to ensure that 6/mL was maintained. From day 10, when larvae had developed more efficient hunting skills, live *Artemia* were fed to the larvae at a density of 3/mL. Larvae were stocked on day 9 at 250 ± 15 larvae per tank in the 8 larval rearing tanks. These tanks were filled with freshwater the day before and aerated for 24 hr to de-chlorinate. Salinity was then adjusted to 3 ppt by addition of small quantities of water from the 35 ppt supply tank. Salinity, pH and temperature were checked daily. A 10% water change was made for each tank every day, with bottom debris and dead *Artemia* siphoned off three times a day, and surface oil and protein skimmed off twice daily. The amount of light entering
each tank was standardised for each tank, to a range of 50-60 lux. Lighting operated on a photoperiod of 12L:12D, from 8am to 8pm each day. Water temperature was maintained at 23.5 ± 0.4°C, and pH at 7.30 ± 0.05.

Artemia cysts (Cowboy brand, Aquasonic) were stocked (1g/L) and hatched in a 20 L glass culture tank on each day of the experiment. The hatching solution consisted of 12 ppt saline water held at 28°C with constant aeration, circulation and fluorescent lighting. Hatching success was generally 90-95%. After approximately 18 hours, the heating, lighting and aeration were turned off, to allow newly hatched first instar Artemia nauplii to settle to the bottom of the culture tank. They were then siphoned off, and washed and resuspended in 3 ppt saline water, for feeding to golden perch larvae. The number of Artemia in the stock culture was calculated using diluted counts under a light microscope. Water samples were taken from each tank prior to feeding so as to estimate the required number of Artemia to be added to maintain consistent feed densities.

2.3 ANALYSIS OF CELL CYCLE FRACTIONS IN LARVAL TELEOST BRAIN TISSUE

The following protocol is based upon those originally proposed by Theilacker and Shen (1993a,b; 2001), Bromhead (1996) and Bromhead et al. (2000), but modified to take into account preliminary testing and optimisation, as well as new theoretical considerations involving apoptosis, all of which are detailed in Chapter 3. This protocol is adhered to throughout the course of the experimental work presented from Chapter 4 onwards, unless otherwise stated.

2.3.1 Morphological data collection

In nearly all experiments, various morphological and developmental data were recorded for each larva sampled. These included standard length (mm), gut fullness, the presence of tail flexion, swim bladder inflation, oil and yolk dimensions (Figure 2.3). In larvae sampled and preserved by snap freezing in liquid nitrogen, these features were recorded after larvae were thawed. Freezing is known to cause shrinkage in fish larvae, and
Figure 2.3 – Preparation of larval brain tissue for flow cytometric analysis involved thawing snapper larvae (A, B), or golden perch larvae (C), and recording numerous morphological data such as standard length (SL), flexion (Fl), swim bladder inflation (SB), oil droplet dimensions (OD), and gut fullness (GF). Larval brain (Br) could then be dissected out, the cells dissociated, fixed and stained with a fluorescent dye, propidium iodide (D). Image obtained using laser scanning confocal microscopy.
therefore it is necessary to construct a calibration curve for each species which allows a more accurate estimate of the prefreezing length of larvae (Figure 2.4). For larvae which had brain tissue excised and preserved in a cryoprotectant immediately after sampling, length and other morphological characters were recorded prior to dissection.

2.3.2 Sampling and preservation

In general, a sampling consisted of the capture of 3 to 5 larvae per tank, using a 2 mL plastic pipette, to siphon individual larvae from the water column. To avoid bias in sampling (i.e., capture of slower or smaller larvae, or larvae with varying degree of swim bladder inflation), capture was achieved by random visual selection of an individual larva at any depth or position in the tank, and pursuit until capture of identified larva. The time between visual identification and capture was generally less than 10 seconds.

When sampling was intense, involving large numbers of larvae in a short time period, snap freezing of whole larvae was the preferred method of preservation. Larval samples collected for DNA-based analysis were placed in 1 mL of tank water in a 1.5 mL cryotube (Nunc), which was sealed, then snap frozen and stored in liquid nitrogen until analysis. This simple procedure gave excellent recovery of cells for flow cytometric analysis. The use of a portable, liquid nitrogen-cooled, “dry shipper” allowed the frozen transport of large volumes of samples between Port Stephens and Canberra.

In less intensive sampling situations, larvae could be dissected and brain cell suspensions preserved in cryoprotectant, a method which yields higher subsequent cell recovery. This technique followed that of Theilacker and Shen (1993b) and yields excellent cell recovery levels of over 85% (Appendix I). Individual larvae were siphoned into wells of a 6-well sampling plate, containing sample water and 125 μg/mL MS-222, on ice. Larvae were then transferred by pipette to two successive buffer washes, then onto a cooled well slide containing 0.5 mL ice cold cryoprotectant buffer (16.6 mL of foetal calf serum (FCS), 16.6 mL of dimethyl sulfoxide (DMSO) and 66.6 mL of phosphate buffered saline (PBS), to make a 100 mL stock solution). Standard length and other morphological details (Figure 2.3) were then recorded, before the
Figure 2.4 – An analysis of the effects of freezing in liquid nitrogen upon the standard length of larvae of: A) snapper *Pagrus auratus*; and B) golden perch *Macquaria ambiguа*. Circles (•) represent original fresh lengths and crosses (+) represent larval lengths after freezing. For both species, there is significant shrinkage but this does not appear to be dependent on initial size. Golden perch would appear to undergo a greater degree of shrinkage when compared to snapper larvae. Equations were solved for “x” to determine original fresh length of larvae.
brain was removed (Section 2.3.3) and transferred immediately using a 100 µL pipette to a labelled cryotube containing 200 µL cryoprotectant on ice. The brain cells were then gently tritutrated 5 times to ensure dissociation, then the cryotubes were capped and stored in liquid nitrogen.

### 2.3.3 Preparation and staining

The following protocol was found to yield the highest recovery of intact brain cells from the very smallest larvae. Individual cryotubes containing larval samples were removed from liquid nitrogen and quickly thawed in 37°C water bath, to the point where larvae were free floating but not all ice had melted. Larvae were then quickly opened and tipped into 2 mL ice cold phosphate buffered saline (PBS) on ice, removed and washed twice more in PBS before being siphoned by pipette and placed in a drop of PBS on a cold well-slide for immediate morphological measurements and dissection. Morphological measures of size (length and oil droplet) were obtained using the dissecting microscope graticule. Length measures were later adjusted according to shrinkage compensation equations for each species (section 2.3.1). Other features were also noted (Figure 2.3A,D).

Whole larval brains (Figure 2.3B) were dissected out using two 30 gauge needles attached to 1 mL plastic syringes. Brains were dissected by first, severing occular nerves and removing eyes, then pinning the lower jaw and severing the dermal tissue between the now vacant eye sockets. The upper dorsal cranial skin was then folded backward, the neural cord severed at the base of the brain, and the entire brain removed using a 25 gauge needle attached to a 1 mL syringe. A tiny volume of PBS would be drawn into the syringe prior to extracting the brain tissue, to prevent tissue sticking to the inside of the needle. The syringe was then quickly transferred to a 5 mL flow cytometer tube (Falcon), containing 200 µL PBS, and the PBS drawn into the syringe, and expelled back into the tube. This trituration of the cells was gently repeated 5 times to fully dissociate the brain cells, prior to fixation.

Dissociated brain cells preserved in cryoprotectant were thawed quickly in a 37°C water bath, and the cell suspension transferred by pipette to individually labelled 5 mL FACS
Capture larvae

Freeze whole in tank water

Thaw (37°C)

Morphological notes

Tissue dissection

Cell dissociation

Fixation (75% EtOH) at 4°C

Centrifugation/Washing (PBS)

Staining (PI, RNase, PBS) 30 min. room temperature

FCM data collection

Modfit analysis

Analysis by REML

**Morphological Measures:**
- Length
- Yolk
- Oil
- Flexion
- Swim bladder
- Gut content

Figure 2.5 – Protocol for the collection and analysis of cell cycle data from the brains of larval fish.
tubes. Four mL of PBS were then added to each tube, and samples centrifuged at 2000 rpm for 5 minutes. The supernatant was then discarded and the cell pellet washed again in 4 mL PBS before being finally suspended in 400 µL PBS. Cells were then fixed by adding 4 mL of ice cold 90% ethanol (BDH, 99.7% v/v) while gently vortexing, to give a final concentration of near 75% EtOH. This method of EtOH addition prevents aggregation of cells in solution (Crissman and Steinkamp, 1990). The tubes were then capped to prevent evaporation of EtOH, and placed at 4°C until staining and analysis.

The following staining protocol was used to stain brain cells with propidium iodide (PI) for DNA cell cycle analysis, and is a modified procedure based on that used by Crissman and Steinkamp (1990). After fixation overnight at 4°C, the cell suspension was then pelleted at 1250 rpm (300G) for 5 minutes at 4°C and the supernatant discarded. The pellet was then resuspended and washed three times in 4 mL of PBS, before being resuspended in 150 µL of staining solution. The volume of staining solution depended on the size of the larvae, and was varied to ensure staining saturation of cells. The staining solution (1 mL) consisted of 800 µL PBS, 100 µL of 2 mg/mL PI (Sigma) and 100 µL of 1 mg/mL RNAse (Sigma). RNAse was added in order to digest any RNA in the sample, as PI binds both RNA and DNA. The samples were then left in the dark for 45 minutes at room temperature, prior to analysis by flow cytometry.

2.3.4 Data collection

The flow cytometer used in this project was a Becton Dickinson (BD) FACScan interfaced to an Apple Macintosh Quadra 840AV utilising “Cellquest” software (BD; San Jose, CA, USA) for the acquisition and storage of data. This facility is housed in the FACS Unit at the John Curtin School of Medical Research at the ANU in Canberra, ACT. Trial runs determined the ideal instrument settings for the brain cells as stained with PI (Table 2.1). The procedure used for acquiring data on the larval brain samples was as follows. Detergent was washed through the tubing for 5 minutes prior to sample analysis so as to clean out any previous user stains, which can become absorbed to the tubing within the instrument and contribute to subsequent sample fluorescence.
Table 2.1 - Ideal settings for the acquisition of data for propidium iodide (PI) stained brain cells from larval teleosts.

<table>
<thead>
<tr>
<th>Analysis program</th>
<th>Brain cells (PI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytometer</td>
<td>FACScan</td>
</tr>
<tr>
<td>Laser power (mW)</td>
<td>5.95</td>
</tr>
<tr>
<td>Laser current (A)</td>
<td>3.96</td>
</tr>
<tr>
<td>Sample pressure (V)</td>
<td>10.23</td>
</tr>
<tr>
<td>Threshold parameter &amp; level</td>
<td>FL2 – 16</td>
</tr>
<tr>
<td>FSC detector</td>
<td>E00</td>
</tr>
<tr>
<td>SSC PMT voltage</td>
<td>315</td>
</tr>
<tr>
<td>FL1 PMT voltage</td>
<td>580</td>
</tr>
<tr>
<td>FL2 PMT voltage</td>
<td>548</td>
</tr>
<tr>
<td>FL3 PMT voltage</td>
<td>150</td>
</tr>
<tr>
<td>FSC amplifier</td>
<td>3.48</td>
</tr>
<tr>
<td>SSC amplifier</td>
<td>1.49</td>
</tr>
<tr>
<td>FL1 amplifier</td>
<td>1.00</td>
</tr>
<tr>
<td>FL2 amplifier</td>
<td>1.00</td>
</tr>
<tr>
<td>FL3 amplifier</td>
<td>1.00</td>
</tr>
<tr>
<td>DDM parameter</td>
<td>FL2</td>
</tr>
<tr>
<td>FL-x area gain</td>
<td>1.00</td>
</tr>
<tr>
<td>FL-x width gain</td>
<td>4.10</td>
</tr>
<tr>
<td>Compensation FL1-%FL2</td>
<td>0.0</td>
</tr>
<tr>
<td>Compensation FL2-%FL1</td>
<td>0.0</td>
</tr>
<tr>
<td>Compensation FL2-%FL3</td>
<td>0.0</td>
</tr>
<tr>
<td>Compensation FL3-%FL2</td>
<td>0.0</td>
</tr>
</tbody>
</table>
Immediately prior to flowing, each sample was gently triturated using a 1 mL micropipette. This was found to decrease the number of aggregates (or cell clumps) recorded in solution. Samples were run on a low flow setting (12 µL/sec) at a count of approximately 200 events per second. G01 and G2M peaks were positioned at channels 200 and 400 respectively on a 1024 channel scale of relative fluorescence. When possible, 10000 cell events were collected per sample. This was ensured by setting a collection gate around the perimeter of the cell population as defined by the area and width of their fluorescent signals. These collection parameters were determined to maximise precision of the index, based on preliminary experiments presented in Chapter 3.

2.3.5 Data Analysis

The main program used for cell cycle analysis of flow cytometer (FCM) collected data was “Modfit VS.2” (Verity Software, Lonse, Topshany, Maine, U.S.A.). This software applies simple or complex modelling algorithms in order to best fit the provided data. It has a wide range of models pre-formatted to fit DNA frequency histograms typically generated by different cell systems, and suited to the method of collection and preservation of the cells. These models can be further adjusted to fit the exact requirements of the investigator. The current research uses a model specified “DIPNI.MOD” to fit the typical diploid histogram generated by larval fish brains. This model uses gaussian components to model the G01 and G2M peaks, a trapezoid function to model S phase, as well as functions to model debris and aggregates. WinMDI (Build #5; J.Trotter, Scripps Institute, USA) was used to gate out unwanted data, such as circulatory cells (see Figure 2.6). These gated files could then be re-saved in a format compatible with Modfit. As mentioned before, one of the key aims for the development of a fisheries-based tool such as larval condition indices is the speed of processing. With this in mind, a Modfit-formatted macro was modified by a computer programmer to allow the fast and consecutive modelling analysis of hundreds of FCM data files, and the automatic transfer of these data into an Excel spreadsheet for statistical analysis. The gating, modelling analysis, and data transfer of 100 files (representing 100 larvae) now takes around one hour, where previously it could take a full day (Bromhead, 1996).
Figure 2.6 – Protocol for analysis and modelling of brain cell samples from larval fish.
A) Samples are plotted in WinMDI as fluorescence (FL2-H) against size (FSC-H) and the circulatory population (R1) gated out. B) In Modfit, the files are plotted as area against width of fluorescent signals. This allows apoptotic, aggregated or ruptured cells to be gated (R2) out prior to histogram generation. C) Modfit then determines the G01 and G2M peak positions. D) It uses these to then apply the models to the data and determine the fraction of cells in each stage of the cell cycle. The level of apoptosis is determined by gating the sub-diploid population (gate R3 in A.)
The basic procedure for data analysis varied depending on the experiment, but followed a basic pattern. An FCM data file was plotted in WinMDI as FSC (size) versus FL2-H (fluorescence or DNA content). By creating a gate within this plot, unwanted cells (e.g., circulatory/blood cells) were eliminated from the analysis. This file was then saved as a listmode file and re-opened in Modfit. The appropriate start model was selected (i.e., FDIPN1.MOD) and the data plotted by its fluorescence area and fluorescence signal width. This plot was then used to gate out debris and aggregates. Modfit will then save this gated population as a gated file. Once gating was completed on all files, the macro was employed to model all files and transfer data to a spreadsheet.

Apoptotic populations were not modelled using Modfit, due to inconsistencies in the modelling fits. Instead, straight gating of the sub-diploid population was performed using the statistical functions of WinMDI analysis program. Data was plotted according to FL2-H by FSC (size), with the gate defined by those “events” occurring within the normal cell size range, but with a fluorescence intensity at least half that of normal diploid cells, so that each event represents what was once a cell with full DNA complement (Figure 2.6).

### 2.3.6 Statistical analysis

Once all data were entered into spreadsheets, two additional indices could be calculated and added to the spreadsheet data. These indices are:

\[
\text{Fraction of cells dividing (FOCD) } = \frac{(S+G2M)}{(G01+S+G2M)} \\
\text{Net tissue growth index (NTGI) } = \text{FOCD} - \text{FAp}
\]

FAp (in Eq. 2) is the fraction of cells defined as apoptotic (Section 2.3.5). These are the predominant indices by which treatment effects upon cell proliferation in brain as well as brain tissue growth will be assessed. These will be compared to the morphological growth measure of larval standard length (mm) and growth rates as calculated from length. The FOCD can also be divided into fractions according to S and G2M phases, and these phases compared to determine how they contribute to variation in FOCD.
Both S and G2M fractions will be analysed to determine if either correlates more closely to growth than does FOCD.

Statistical analysis of data contained in this thesis was done with the assistance of Ross Cunningham and Christine Donnelly (ANU Statistical Consulting Unit of the Graduate School, ANU, Canberra, ACT). Details of experimental statistics will be explained in the relevant chapters. However, in general, the current investigation used Genstat 5 (Genstat 5 Committee, 1993) to apply mixed model analysis (using REML – restricted maximum likelihood) to determine factors associated with variance in the FOCD, S or G2M fractions. While experiments were designed for the assessment of a balanced data set, there was some minor loss of data (due to death of larvae, technical problems etc). Consequently, data sets are unbalanced and therefore standard ANOVA was not an appropriate analysis method. REML analysis was chosen due to its ability to analyse unbalanced data sets and because it allowed for the mixed model analysis of both random and fixed effects (Calvin, 1993; Clarke, 1996; Yau, 2001). This allows one to account for variance in FOCD, S or G2M fractions which are due to random effects (such as between tank effects) before determining the variance due to the fixed effects of interest (e.g. treatment, age). Mixed model analysis (via REML) is the most appropriate method for analysis of data presented in this thesis.

The cell fraction indices FOCD, S and G2M are ratios (or proportions). The analyses use the empirical logit transformation of these ratios (e.g. log[FOCD]/[1-FOCD]), so as to ensure that the assumption of constant variance was met. The modelling approach used involves fitting a model with all possible variables included. The significance of each variable was then assessed by dropping individual variables, in a stepwise fashion, from the full model to create submodels. The change in deviance (CID) between the full model and the sub-model, which approximates a chi-squared ($\chi^2$) distribution, can be used to determine the significance of the variable, and therefore which variables (main effects or interactions) can be dropped from the final model, and which should be kept. The final model achieved by this process provided a parsimonious description of the relationship between the cell fractions and the variable(s) under consideration. It could then be used to predict mean values of a cell fraction (e.g. FOCD) for the levels of factors in the model, or define the slopes of the relationships between FOCD and the covariates of interest.
To demonstrate that a factor or interaction explains a significant amount of variation exhibited by the response variate (FOCD), results sections in the following chapters will generally state:

1) Change in deviance (CID): that occurs when a factor or interacting terms are dropped from the model;
2) Degrees of freedom: for the factor or interaction being tested;
3) P value: which determines if the amount of variation (in FOCD) described by the factor or interacting terms is significant; and
4) Slope: occasionally the slope of a significant relationship will also be stated.

Graphical comparison of predicted means will use a single error bar representing the “least significant difference” (LSD, which is equivalent to $2 \times$ standard error of difference, SED) which can be applied to determine whether any two predicted means differ significantly. Some basic statistical analyses were also done using JMP (i.e. comparison of means, ANOVA etc.).
CHAPTER 3

OPTIMISATION AND NEW
CONSIDERATIONS

3.1 INTRODUCTION

Previous investigations into the optimisation of cell proliferation in fish larvae have
overlooked a number of issues pertaining to both technological and ecological aspects
of this application. The methods used by Whelan and Shen (1993a,b, 2001)
Drunen et al. (1996) and Hemelrijk et al. (2000) were based on generalised methods taken
from other fields of research, such as horticulture. It is well recognised that generalised
flow cytometric protocols must be adapted and refined, so as to bear and each new
methodological application (Whelan et al., 1993). The wider flow cytometry research
community considers optimisation and determination of aspects such as cell
concentration, flow conditions, DNA stability, chromosome accessibility, flow rates and
collective parameters, among others, as a fundamental part of cell cycle analysis (Chapter
3). Mohren et al. (1996, Dreyfus et al. (1994) and Fritsche and Beumer
(1994) noted in regard to flow cytometric methodologies for analysing cell DNA
centres, so it is felt that the protocol and the best results may not evolve. The
following steps have been taken to achieve this goal: binned cell counts for each
standard larva; binned cell counts for each standard larva; and binned cell counts for
each standard larva. As observed by Whelan and Shen (1993a,b, 2001), Hemelrijk et al.
(1996) and Hemelrijk et al. (2000) which postulates as a product of cell proliferation. However, many aspects of the use of
both cell proliferation and cell death (Dranitsin, 1994). Consequently, the presence of
using a biological cell in larval brain was investigated. Taking into account factors
from the optimisation experiments, as well as preliminary biological investigations, a
modelling protocol that allows for the optimisation associated with both cell proliferation
and cell death in larval brain is presented.

3.1.1 Optimising methods to assess proliferation of larval brain cells

The most important prerequisite for a larval growth index includes the following five
features: accuracy, precision, reliability, efficiency (speed), and scalability to field
research (Whelan and Shen, 1993a,b). Hemelrijk and Lengfelder (1994), Buckley et al. (1999).
The flow cytometric protocol for fish larvae first described by Whelan and Shen
(1993a,b) was demonstrated as having the potential to meet these criteria. However, recent
3.1 INTRODUCTION

Previous investigations into the assessment of cell proliferation in fish larvae have overlooked a number of issues pertaining to both methodological and theoretical aspects of this application. The methods used by Theilacker and Shen (1993a,b; 2001), Bromhead (1996) and Bromhead et al. (2000) were based on generalised methods taken from other fields of research, such as medicine. It is well recognised that generalised flow cytometric protocols must be adapted and refined, so as to best suit each new methodological application (Robinson, 1993). The wider flow cytometry research community considers optimisation and determination of aspects such as cell concentration, fluorochrome-DNA stability, fluorochrome accessibility, flow rates and collection parameters, amongst others, critical for accurate cell cycle analysis (Shapiro, 1988; Melamed et al., 1990; Darzynkiewicz et al., 1994). As Dressler and Seamer (1994) note, in regard to flow cytometric methodologies for analysing cell DNA content, “a small deviation in quality control can lead to serious erroneous results”. The following chapter addresses a number of optimisation issues that have not previously been dealt with in relation to the measurement of brain cell proliferation in teleost larvae. It also considers the theoretical basis of the indices investigated by Theilacker and Shen (1993a,b; 2001), Bromhead (1996) and Bromhead et al. (2000) which focus on growth as a product of cell proliferation. However, tissue growth is the product of both cell proliferation and cell death (Baserga, 1985). Consequently, the presence of dying (apoptotic) cells in larval brains was investigated. Taking into account results from the optimisation experiments, as well as preliminary apoptosis investigations, a modified protocol that allows for simultaneous assessment of both cell proliferation, apoptosis and net tissue growth is presented.

3.1.1 Optimising methods to assess proliferation of larval brain cells

The most important prerequisites for a larval growth index include the following five features: accuracy, precision, sensitivity, efficiency (speed), and suitability to field research (Theilacker and Shen, 1993; Ferron and Leggett, 1994; Buckley et al., 1999). The flow cytometric protocol for fish larvae first described by Theilacker and Shen (1993b) was demonstrated as having the potential to meet these criteria. However, some
issues concerning the sensitivity and precision of the method in its application to larval growth were not addressed in this or subsequent research (Bromhead et al., 2000). To ensure accurate flow cytometric analysis of a particular cell system, it is critical that in the first instance, consideration be given to the following factors. Firstly, the optimal concentration and nature of the fluorochrome used (Shapiro, 1988). Secondly, optimal flow rates and cell concentration, such that maximal processing speed is attained without loss of instrument accuracy and precision (Shapiro, 1988). Thirdly, collection of sufficient cell numbers per sample to allow accurate and precise modelling analysis (Shankey et al., 1993; Rabinovitch, 1994). Fourthly, determining the temporal stability of the fluorochrome-DNA interaction, and finally, consideration of the modelling requirements of the particular system being analysed. Other aspects involved in each of the four stages, also require consideration and have potential to affect one or more of the prerequisites listed above.

3.1.2 Sample collection and preservation

The sensitivity and precision of a flow cytometry based cell proliferation index will be limited, in part, by the maximum number of cells that can be isolated from the smallest larvae, given that there will be an optimal minimum number of cells required for accurate modelling (Rabinovitch, 1994). The method by which cells are preserved and dissociated will affect subsequent cell recovery levels, or the number of cells that are passed through the flow cytometer. Theilacker and Shen (1993b) and Bromhead et al. (2000) proposed different methods of freeze preserving cells. Theilacker and Shen (1993b) dissociated and preserved brain cells in a cryoprotectant at -20°C. This method gave excellent cell recovery. Bromhead et al. (2000) devised a simple method for fast and efficient sampling in the field, which delayed the minute dissection and dissociation process until return to the laboratory. Larvae were placed in 1 mL river water in cryotubes and snap frozen in liquid nitrogen. The latter method is useful also for intensive sampling where large numbers of larvae are sampled in a short period. However, this method yields slightly more cellular debris (Bromhead, unpublished data), than the method of Theilacker and Shen (1993b). Hence, their method is preferred when possible. Brain cell dissociation in larval fish is most efficiently achieved by physical means (Theilacker and Shen, 1993b; Bromhead, 1996). However, the
dissociation method can result in some level of cell fragmentation and therefore affect subsequent cell recovery and index sensitivity. Theilacker and Shen (1993b) used a 100 µL pipette to gently dissociate larval brain cells in cryoprotectant. This method did not work efficiently with older larvae and juveniles which appeared to have a greater amount of connective tissue in their brain (Bromhead, 1996). Therefore Bromhead et al. (2000) used a more physical means, pushing brain tissue through 60 µm nylon filter cup. However, this method, while suitable for the age group, does produce more cellular debris and lower cell recovery rate (see Appendix I). Where possible, the trituration method of Theilacker and Shen (1993b) should be used.

3.1.3 Sample preparation and staining

Access of a DNA-targeted fluorochrome to the genomic DNA relies on the cell and nuclear membranes being permeabilised. There are a number of agents that can be used to permeabilise cells. Bromhead et al. (2000) used ethanol fixation, based on the protocol of Robinson (1993). Ethanol acts to agglutinate proteins and solubilize lipids, creating holes in the cell membrane. Theilacker and Shen (1993b) used a detergent in the staining solution to permeabilise the cells to acridine orange. However, fixation in ethanol aids in the washing and removal of degraded DNA that characterises apoptotic cells (Darzynkiewicz et al., 1992; Darzynkiewicz and Li, 1996). This allows definition of apoptotic cells on a DNA frequency histogram. Hence, the use of ethanol may depend on whether the investigator wants to consider apoptosis, in addition to cell proliferation, within their analysis.

Once cell samples have been fixed and stained, the number of samples that can be analysed in a single batch will depend partly upon how long the fluorochrome-DNA interaction remains stable. This time has yet to be determined for larval brain cells stained with propidium iodide, but is important knowledge for an investigator aiming to analyse large numbers of samples without compromising precision and accuracy of the analysis.
3.1.4 Data collection

Previous investigations (Theilacker and Shen, 1993a,b; Bromhead et al., 2000; Theilacker and Shen, 2001) have used generalised flow cytometric protocols, with little published attempt to determine the ideal collection parameters for the cell type and staining system that they are using. If the levels of signals (pulses) are incorrectly collected, this can lead to the modelling program misclassifying cells in relation to their cell cycle status. Too high cell concentration or flow rate can “clutter” signals, with the detectors not able to distinguish between individual cell signals, and this leads to erroneous classification. Thus, to maximise efficiency (speed and sample number per batch), the investigator should aim to find the highest flow rate and concentration of cells that can be analysed in a short time period without adverse effect upon the precision and sensitivity of the index.

Cell number per sample is also an important factor. The lowest number of cells that can be analysed without loss of precision or accuracy will define the sensitivity of the index. This will partly depend on the modelling program used to analyse the data. Numerous modelling programs are available from different companies, and the statistics and modelling parameters applied can differ among these programs (Rabinovitch, 1994). Thus different programs may require different minimum cell numbers to achieve a precise fit and fraction estimation. Consequently it is important for each system analysed that the effects upon fitting and sensitivity of cell number be determined.

3.1.5 Data Analysis

Once the flow cytometric data has been collected, a modelling program can be employed to generate DNA frequency histograms and analyse the fractions of cells in the different phases of the cell cycle. The aim is to analyse only the target cell population, in this case, larval brain cells. Other extraneous cell types or “events” that might be within a sample should preferably be removed (or gated) from the analysis. These “events” might include circulatory cells, which are associated with most tissues, as well as aggregates and debris. The extent and occurrence of these events, and their affect upon analysis of larval brain cells requires further investigation. Given the
potential presence of non-target cell populations and debris in sample data, it is important to establish rigorous gating protocols that exclude these from influencing modelling analysis.

### 3.1.6 Theoretical considerations: Apoptosis and net tissue growth

The indices proposed by Theilacker and Shen (1993b, 2001) and Bromhead et al. (2000) are based on the measurement of cell proliferation and how this relates to overall larval growth. However, as noted in Chapter 1 (Section 1.3.2.5), cell proliferation serves not only to add new cells to a population (growth) but also to replace dead cells that have been removed from the population (Baserga, 1985; Maclean and Hall, 1987). Overall growth is achieved only when the fraction of new cells being added to a population exceeds the fraction dying (apoptotic cells) (Baserga, 1985). Apoptosis is an important process in the remodelling and development of early stage vertebrate tissues and organs, including brain (Krueger et al., 1995; Blaschke et al., 1998; Clarke et al., 1998) and muscle (Nishikawa and Hayashi, 1995; Sachs et al., 1997) but also serves to remove critically stressed or abnormal cells (Maclean and Hall, 1987; Darzynkiewicz et al., 1992; Darzynkiewicz and Li, 1996; Hashimoto et al., 1998; King and Cidlowski, 1998). Starvation has been demonstrated to induce apoptosis in certain tissues of vertebrates (Boza et al., 1999; Tessitore et al., 1999) and in cancer cell lines (Franek and ChladkovaSramkova, 1995). However, there is also evidence of suppression of apoptosis in response to starvation in intestinal cells (Luciano et al., 1995), though this may be related to the specific function of these cells and their normal high turnover. Therefore it is reasonable to suggest that starvation or other physiological stresses in larval fish might induce apoptosis. Given the role of apoptosis in removing cells from the population, an index that considers both cell proliferation and apoptosis might be more sensitive to treatment effects upon overall growth. A number of methods now exist for the identification of apoptotic cells (Migheli et al., 1994; Darzynkiewicz and Li, 1996; Martin et al., 1996; Rojo and Gonzalez, 1998; Daniel et al., 1999; Schmid et al., 2000). These rely on detecting changes in cell characteristics that characterise the onset and progression of apoptosis. One of these changes involves the externalisation of phosphotidylserine (PS), which is normally almost totally confined to the inner leaflet of the plasma membrane. A specific probe for PS now exists, annexin-V, which allows...
for flow cytometric identification of apoptotic cells when coupled with fluorescein isothiocyanate (FITC) (Martin et al., 1996). Simultaneous staining with propidium iodide (PI) is generally used to confirm that annexin-V binding is not simply the result of a loss of membrane integrity (Martin et al., 1996). Alternatively, the level of apoptosis can be determined by fixation of cells in ethanol, followed by washing and staining with propidium iodide, and analysed as for cell cycle (Darzynkiewicz and Li, 1996). Fixed cells used for cell cycle analysis are "dead", and accessed by the PI. Cells that were apoptotic at the time of sampling will have DNA fragmentation, with these small degraded fragments being lost from the cells as a result of the fixation and washing process. Apoptotic cells will consequently appear on a size-by-DNA plot as subdiploid events of similar to slightly smaller size than the G01 events (Darzynkiewicz and Li, 1996). The use of cross-linking fixatives or detergents that can lyse cells will not allow analysis of apoptotic cells. The former prevent fragments being washed out, while the latter releases nuclei, which are generally fragmented in apoptotic cells (Darzynkiewicz and Li, 1996).

### 3.1.7 Aims

The following research aims to optimise and refine the protocol for analysis of cell proliferation in the teleost brain, by addressing unresolved issues associated with flow cytometric analysis of cell cycle fractions. It aims to firstly determine the need for, and effect of, ethanol fixation. Secondly, to determine the temporal stability of the interaction between propidium iodide and DNA. Thirdly, to optimise the collection parameters such that maximal processing efficiency can be achieved without loss of precision or accuracy. Fourthly, to determine the ideal gating protocol, and finally, to determine if a significant apoptotic population can be confirmed within larval brain tissue, and if so, devise a modified analysis protocol, which allows for the simultaneous analysis of both cell proliferation and apoptosis.

### 3.2 METHODS

Most of the following experiments assessed the effect of a number of technical variations upon the overall ability of the methodology to provide an accurate cell
fraction estimate. These technical variations were assessed by comparing mean measures of precision and accuracy of data collection (mean coefficient of variation, CV, of G01 peak), and of model fitting (mean reduced chi-square value, RCS), and assessing variation produced in mean cell fraction estimates. Increased distribution of estimates about the mean implies reduced precision. Analysis of variance (ANOVA) has been used to compare means in all of the following experiments.

3.2.1 Sample preparation and staining

3.2.1.1 Ethanol fixation
To determine the effect of fixation upon sample staining and cell cycle modelling, 20 snapper larvae were taken from a single fully fed treatment and snap frozen in liquid nitrogen. After 1 week, these larvae were thawed and their brain cells dissociated into 400 µL PBS. Each sample was then split into two aliquots of 200 µL, with one being stained and analysed immediately, while the other was fixed in ethanol for four hours at 4°C, prior to washing and suspension in PBS, staining and analysis (Robinson, 1993).

3.2.1.2 Circulatory cells
A second smaller population of cells was identified in fixed samples. The effect of this population upon analysis and modelling was examined by comparing the mean cell fractions and CV for samples with this population included, with those having this population gated out. It was suspected that these cells might originate from the circulatory system. Therefore cells were isolated from the heart of snapper larvae and flowed separately and in conjunction with pure brain samples, to determine if they had the same staining characteristics as the secondary population.

3.2.1.3 Staining stability
To determine the temporal stability of the DNA-PI interaction in brain cells of larval fish, 10 snapper larvae were sampled from a single tank, their brains dissected and dissociated into a single cryotube with 1 mL cryopreservative and snap frozen in liquid nitrogen. The cells were later rapidly thawed at 37°C, and 100 µL aliquots distributed into each of 6 FACS tubes. These were then fixed in 70% ethanol for 4 hours, stained with PI and placed in a 4°C fridge for the duration of the experiment. One tube was
analysed at each time interval, these being 0.25, 0.75, 1.5, 3, 7, and 18 hours. At each time point, 6 samples of 7000 cells were taken to test for effects of time upon precision and PI-DNA stability. Modfit (Verity) was then used to analyse the mean CV and standard errors of cell cycle fractions.

3.2.2 Data collection

3.2.2.1 Sample size
To determine the effect of cell sample size upon the precision and fraction estimation abilities of the Modfit modelling program, 20 snapper larvae were sampled from a single tank, and brain cell suspensions preserved in a single cryotube with cryoprotectant in liquid nitrogen. One week later they were thawed quickly at 37°C, fixed in 70% ethanol for 4 hours, then washed and stained with propidium iodide. Six samples each of 250, 500, 1000, 2000, 4000, 6000, 8000, 10000, and 15000 cells were collected for modelling analyses. They were then compared for CV, RCS, G01, S and G2M cell fractions, as well as standard error as a measure of precision of modelling.

3.2.2.2 Flow rate
To determine the effect of flow rate on precision of data collection, one of the samples from Section 3.2.2.1 was then used to take 5 readings on high (60 µL/sec) flow setting and 5 on low (12 µL) flow setting. These were compared according to cell cycle fraction and CV.

3.2.2.3 Cell concentration
To determine the ideal cell analysis rate (cell concentration or cells passing through observation point per second), another suspension consisting of cells from 10 larvae was thawed, fixed and stained as above. This was then analysed by flow, the cell concentration adjusted by dilution to 650 cells/sec, and 5 samples taken. Further batches of 5 samples were taken after serial dilutions (using a PI solution of the same concentration) were used to achieve cell flow rates of 400, 250, 210, 170, 140 and 120 cells/sec. These data were then analysed for the effect upon modelling analysis parameters as above.
3.2.3 Data analysis

3.2.3.1 Apoptotic cells

The following experiment aimed to determine if a significant population of apoptotic cells might be present in the brains of larval teleosts, and whether the density of their distribution might vary between different parts of the brain. Fresh cell preparations, equivalent to 5 x 10^5 cells/mL in PBS, were made from the fore and hind brain of 3, 22-day old golden perch. 200 µL of cells were placed in a FACS tube and centrifuged at 300 g, the supernatant siphoned off and 200 µL of annexin V-FITC working solution (1 µg/mL: commercially available as APOPTEST) and propidium iodide (10µg/mL) added to the cells (Martin et al., 1996). The sample was incubated for 5 minutes at room temperature, before analysis by flow cytometry. The data was viewed and analysed using WinMDI analysis program.

3.2.3.2 Net tissue growth

After confirmation of a large apoptotic cell population in larval brain, gating of PI analysed cell cycle fractions was used (see Figure 2.6, section 2.3.5) to indicate the extent of brain cell apoptosis in 9 day old larvae reared at salinities ranging from 5 ppt to 45 ppt. These data were collected by triggering on FL2-H, to prevent non-PI staining debris from contaminating the apoptotic region. This fraction of cells could then be used to formulate two parameters:

1. FOCD = (S+G2M)/(G01+S+G2M)
2. Net Tissue Growth = FOCD-FAp

The second equation provides an indication of the effect of treatment upon net tissue growth, by subtracting the fractions of cells that are apoptotic (FAp), from the FOCD.
3.3 RESULTS

3.3.1 Sample preparation and staining

Fixation significantly increased the fraction of S phase cells (ANOVA, p=0.0000) modelled by Modfit, along with a concurrent decrease in the G01 phase fraction (ANOVA, p=0.0001) (Figure 3.1). An increase in mean CV from 3.12 to 3.81 was not significant (ANOVA, p=0.1042). A secondary cell population, staining with the same fluorescence and size characteristics as circulatory cells isolated directly from the heart of snapper larvae (Figure 3.2), was apparent in fixed samples. This secondary population exhibited slightly higher fluorescence than G01 brain cells, and consequently was modelled as S phase within the brain cell population. Gating removal of this population increased the fraction of G01 cells modelled to levels not significantly different to unfixed and ungated samples (ANOVA, p=0.4355), while significantly decreasing the fraction S cells (ANOVA, p=0.0197). CV was reduced to a level not significantly different from unfixed samples (ANOVA, p=0.1153). Observations suggested that fixation increased the amount of clumping of cells (as distinguished by fluorescence pulse area by fluorescence pulse width analysis). Gentle trituration of samples prior to analysis was found to reduce the level of aggregation.

The PI-DNA staining interaction appeared to be reasonably stable over the time period (18 hours) examined (Figure 3.3), with no significant difference in the mean FOCD estimate (ANOVA, p=0.2910) or in the mean CV (ANOVA, p=0.1708). The standard error for FOCD did not change markedly over the experimental period.

3.3.2 Data Collection

There was a significant increase in the mean FOCD estimate as the concentration of cells passing through the observation point per second increases (ANOVA, p=0.0031)(Figure 3.4A). This increase seemed mostly apparent for samples flowed at above 210 cells per second. The mean CV and mean RCS values also differed significantly (ANOVA, p=0.0000, and p=0.0030 respectively) depending on cell concentration, however the relationship was not linear (Figure 3.4B).
Figure 3.1 - A comparison of the fraction of cells in A) G01, B) G2M, C) S phase or D) the mean CV, for larval brain cell samples split from a single original sample and either fixed (F) or not fixed (UF) in 70% ethanol prior to staining and flow cytometric analysis of DNA content. These two groups were then compared to a third, CGO, which comprise the same fixed samples which had the circulatory population gated out prior to modelling analysis. Lower case “a” and “b” represent means that are significantly different at the 0.05 level (as determined by ANOVA). Error bars represent ±2SE.
Figure 3.2 – Fixation of brain cells in ethanol allowed differential staining of two different cell populations, such that the second population (dashed arrows) appeared as a shoulder on the first (A) and could be more fully distinguished by fluorescence intensity against size plot (B). Latter isolation, fixation and staining of circulatory cells from the heart (C) strongly suggested the second population to be circulatory. Consequently, they were gated out of files (D) prior to modelling analysis.
Figure 3.3 – Effect of staining time upon: A) fraction of cells dividing; and B) coefficient of variation (CV) and reduced chi-square (RCS) for modelling fits of golden perch brain cells stained with propidium iodide (PI). The CV is based on the width of the G01 peak. Thin peaks have small CVs, denoting uniform fluorochrome binding and precise signal collection. Error bars denote ±2SE.

Figure 3.4 – Relationship between the number of larval brain cells passing through the observation point per second and: A) fraction of cells dividing (FOCD) estimate; and B) mean CV and RCS. Error bars represent ±2SE.
Mean FOCD differed significantly depending on the number of cells analysed per sample (ANOVA, \( p=0.0449 \)) (Figure 3.5). Fluctuations in mean G2M due to cell number were more significant (ANOVA, \( p=0.0009 \)) but there was no significant difference in mean S phase estimates (ANOVA, \( p=0.1496 \)). There was no significant difference in mean FOCD, S or G2M when comparing samples analysed with 10000 or 15000 cells. Standard error for mean cell fractions becomes smaller as cell number per sample increased. CV increased as sample size increased (ANOVA, \( p=0.0003 \)), however this increase equated to only 0.3.

Mean RCS differed significantly with the number of cells analysed per sample (ANOVA, \( p=0.0000 \)), increasing as cell number increased, but starting to level off between 10000 and 15000 cells. Only samples with 4000 cells or more had above the recommended lower RCS limit of 1.0.

The rate at which cells are passed through the observation point had a significant effect on both cell fraction estimation and sample CV (Figure 3.6). High flow setting of 60 \( \mu \text{L/sec} \) resulted in a significant decrease in the estimate of the fraction of cells dividing (ANOVA, \( p=0.0036 \)) and in S phase (ANOVA, \( p=0.0034 \)) but not in G2M phases (ANOVA, \( p=0.1165 \)). The CV was also significantly higher in samples analysed using a high flow rate (ANOVA, \( p=0.0001 \)) denoting a reduced precision of signal collection.

### 3.3.3 Data Analysis and Modelling

To assess both cell proliferation and apoptosis in the same cell population required further modifying the existing data analysis and modelling protocols used by Bromhead \textit{et al.} (2000). The first experiment identified a substantial population of cells exhibiting increased externalisation of phosphatidyl serine (PS) (Figure 3.7), which is indicative of apoptosis, within the brain of larval golden perch, and indicated that this population may not be evenly distributed throughout the larval brain. Apoptotic cells accounted for approximately 5.3\% of cells in forebrain and 42.3\% of cells in hindbrain sections. There was also some evidence for necrosis or damaged cells (PI +ve cells).
Figure 3.5 – The relationship between total number of larval brain cells analysed per sample and the modelling estimates of: A) fraction of cells dividing (FOCD) or in S and G2M phases; and B) corresponding coefficient of variation (CV) and reduced chi-square (RCS) levels. Error bars denote ±2SE.

Figure 3.6 – Relationship between sheath fluid flow rate (High = 60 μL/sec; Low = 12 μL/sec) and the modelled estimates of: A) cell cycle fractions (S, G2M and FOCD); and B) corresponding CV levels. Error bars denote ±2SE.
Figure 3.7 – Simultaneous staining of golden perch *Macquaria ambigua*, larva brain cells, with propidium iodide (PI) (FL2-H axis) and annexin-V (FL1-H axis) in samples divided into fore (A) and hind brain (B). Normal healthy cells will be both PI and annexin-V negative (R1). Apoptotic cells are PI negative but annexin-V positive (R2) due to binding of annexin-V to phosphatidyl serine, an inner membrane molecule that is externalised in apoptotic cells. Cells with damaged membranes (due to processing or necrosis) will be both PI and Annexin V positive (R3).
The second investigation into apoptosis analysed a subset of data for snapper larvae reared at different salinities (Figure 3.8). This analysis demonstrated that although there was no significant difference in the FOCD of larvae reared at different salinities (ANOVA, \(p=0.585\)), there were significant differences in net tissue growth index (ANOVA, \(p=0.0003\)). NTGI in brain of larvae reared at 10 ppt was significantly lower than that in most of the other treatments, due to a higher fraction of events in the apoptotic gate for larvae reared at this salinity (ANOVA, \(p=0.0000\)).

The gating protocol described in Chapter 2 is based upon the above investigations and avoids complicating modelling by exclusion of debris, aggregates and the secondary cell population (likely to be circulatory cells). The level of apoptosis can be estimated using a separate gate based on size against fluorescence height plots (see Section 2.3.5).

### 3.4 DISCUSSION

The results presented here demonstrated that the methods by which larval brain cells are processed and analysed can have important implications for subsequent data interpretation. To start with, fixation was used to ensure that all cells in the samples are permeabilised and have standardised fluorochrome uptake. Fixation also, however, appears to permeabilise circulatory cells that are present in the brain tissue of the larva. These cells have a slightly higher fluorescence emission level and are smaller than the brain cells, characteristics which can be used to gate these cells out of the analysis and thus prevent them being modelled as S phase brain cells.

Fixation followed by thorough cell washing in PBS also allows for the extraction of degraded low molecular weight DNA from apoptotic cells (Darzynkiewicz and Li, 1996), and thereby the distinction of these cells as being apoptotic by virtue of their sub-diploid DNA content (and fluorescence). This type of analysis is not the most specific for apoptotic cells, so annexin-V was used to show that there is definitely a significant natural occurrence of apoptosis in brain of larval fish. The preliminary analysis of 6 day old *Pagrus auratus* larvae reared at salinities ranging from 5 ppt to 45 ppt suggested that the effect of salinity upon larval brain cell proliferation may differ from its effect on apoptosis. Therefore an index which takes into account the relative
A.

\[ \text{FOCD} - \text{FAp} \]

B.

\[ \text{FOCD-FAp} \]

Figure 3.8 – A preliminary comparison of FOCD with an index of net tissue growth which subtracted the fraction of cells within the apoptotic gate (FAp) from the fraction of dividing cells (FOCD) in brain tissue from larval snapper *Pagrus auratus*. Error bars denote ±2SE.
levels of both cell proliferation and apoptosis might provide additional information regarding the effect of certain factors on overall growth (Bromhead, 1996).

Consideration of apoptosis may be particularly relevant to the current approach for two reasons. Firstly, apoptosis is an important mechanism involved in the development and remodelling of early stage vertebrate tissues (King and Cidlowski, 1998). It has been shown to occur at prolific levels in the developing CNS (Blaschke et al., 1998; Clarke et al., 1998) and developing muscle (Nishikawa and Hayashi, 1995; Sachs et al., 1997). Secondly it is a process which can be induced by environmental stress including starvation (Tessitore et al., 1999). This is relevant given that the index being developed is being applied to the assessment of starvation in larval fishes (Theilacker and Shen, 1993; Bromhead, 1996; Bromhead et al., 2000; Theilacker and Shen, 2001).

This chapter also investigated the relative importance of cell concentration, sample size (cell number), staining time and flow rate, to the end analysis of cell cycle fraction estimates. Quality control of such factors prevents serious errors being introduced into this type of analysis (Dressler and Seamer, 1994). Consequently, the main consideration was the maximisation of processing speed and efficiency without loss of precision or sensitivity, in line with the requirement for a fisheries based tool. The stability of the PI-DNA interaction is best described by any change in sample CV over time. This should increase if the interaction starts to break down (for whatever reason, i.e. DNA degradation) or variance in excitation emission starts to increase. However, despite some significant variation within a limited range, the CV of propidium iodide stained larval brain cells appeared to be reasonably stable over 18 hours. Overall variation in RCS was within the idealised limits recommended by Modfit Manual (Verity Software). On basis of these results, one could analyse a large batch of samples over the course of a number of hours without concern for any effect of changes in the staining interaction. This allows increased processing efficiency and overall speed.

In samples with less than 4000 cells, there appeared to be some variation and a large degree of error in the modelling precision of cell cycle fractions. The highest degree of modelling precision occurred in samples of 10000 cells or more. These results agree closely with those published for other cell types (Rabinovitch, 1994). If fewer cells are available for analysis than these idealised limits, the lower precision of the modelling
capabilities should be noted. Furthermore, given that cell fraction estimation can vary depending on number of cells in the sample, the number of cells collected for each larva sampled within an experiment should be kept as close to constant as possible (Shankey et al., 1993). While 10000 cells is ideal for a sample size, the ability of the program to fit DNA histogram only becomes inadequate below 4000 cells (i.e. RCS<1.0). If sample number is kept constant, sample sizes above 4000 may be acceptable, however the investigator should be aware of decreased precision at these levels. If a bias is recognised in cell fraction estimates which is due to inconsistent cell numbers, it may be possible to formulate an equation which describes the relationship and hence correct for this effect. However, clearly this situation is best avoided, if possible.

Flow cytometers generally have adjustable sheath fluid flow rates. The model of cytometer used in this study, a Becton Dickinson FACScan, had a high (60 µL/sec) and low (12 µL/sec) setting. The higher setting pushes more cells through the observation point per unit time, with each cell occupying the observation point for a relatively shorter period of time (Melamed et al., 1990). The higher speed can, depending on the cell system and parameters being investigated, increase the chance of signal misclassification (Melamed et al., 1990). Not surprisingly perhaps, the lower speed generated samples with a significantly lower CV, indicating a higher precision of signal collection. However, CV at both speeds was well within acceptable limits. The speed used may depend on the requirements of the investigator. Low flow is clearly recommended.

Cell concentration is also considered an important factor in cell cycle analysis (Rabinovitch 1994). As the concentration of cells passing through the observation point per second increases (under steady flow setting), the number of signals registered by the detectors increases, and the chance that signals may be misclassified increases also. The current analysis demonstrated a significant increase in the FOCD modelled for samples with cell concentrations equating to between 210 and 650 cells/sec. The CV and RCS also varied significantly, but again only within a narrow range and within acceptable limits. The increase in FOCD modelled for samples collected by analysis of more than 200 cells per second may indicate that as cell number in the focal point increased, there may have been an increased incidence by which two cells generating simultaneous
signals are classified as a single cell with twice the normal fluorescence of a single diploid (G01) cell. These cells would then be classified as G2M cells, and result in increased FOCD being modelled. Therefore the recommended cell analysis rate should not exceed 200 cells per second to ensure accurate fraction estimation. Analysis rate should be kept constant among samples. It should be noted that brain cell samples obtained from golden perch larvae had low levels of dividing cells, contrasting with samples taken from snapper which had much higher fractions of brain cells dividing. It would have been preferable to run all optimisation tests on faster growing brain cell populations from a single species. Some of the variation in cell fraction estimates which are due to different processing methods, might be lost due to the already low level of cell proliferation in these golden perch larvae.

In summary, the following modifications to the protocol used by Bromhead (1996) will be implemented for analysis of experimental samples in this thesis (see Chapter 2 for full protocol). When possible, pipette or needle dissociation, and cryopreservation of cells in cryoprotectant at –20°C is recommended (as in Theilacker and Shen, 1993b). Ethanol based fixation assures maximal stain uptake by all brain cells, as well as allowing for the assessment of both apoptosis and cell proliferation, and gating out of circulatory cells. All flow settings were kept constant between samples. This included recommended low flow rates (12 µL/sec) to maximise precision of signal collection, staining time of at least 45 minutes and anywhere up to 18 hours, during which CV is stable. Ideally, 10000 cells are analysed per brain sample, at a rate of not more than 200 cells per second. With these settings, an analysis rate of 50-60 samples per hour was possible. Subsequent data files should have circulatory, apoptotic, debris and aggregates gated out prior to DNA histogram analysis. An indication of the level of apoptosis can be gained by gating these events based on their sub-diploid DNA content. The full protocol is described in Chapter 2.
CHAPTER 4

4.1 INTRODUCTION

Adult snapper, *Pagrus auratus*, migrate into shallow coastal waters to spawn and the larvae and juveniles of many species tend to inhabit near-shore and estuarine environments until they mature. As such, they may be subject to the fluctuating salinity associated with these environments. Salinity is the main abiotic factor that will challenge the osmoregulatory capacity of salinity and has been demonstrated to influence larval growth and survival (Cassell et al., 1989; Winger and Latter, 1994; Han et al., 1995; Dobson et al., 1996; Ottone and Botta, 1996; Gelder, 1997), and is thought to play a role in the osmoregulatory capacity of some species (Ridgway and Nell, 1999; Miller et al., 1999; Presson et al., 1999). Consequently, the following chapter investigates the effect of environmental salinity upon the level of both brain cell proliferation and overall brain development of one species in snapper larvae.

4.1.1 Salinity, larval growth and survival

Based on an effect of salinity upon larval growth rates from both environmental and behavioral studies have shown that differences in larval growth rates can result in the disruption of larval survival and reduced post-larval survival. The larval period may act as a critical period for survival, and this highlights the potential for future research in this area.

The effect of salinity upon larval growth has also been investigated in many species, and is often dependent on developmental stage. This is likely in part due to the differences in osmoregulatory capacity that are available to a fish at different stages in development. Larval fish do not readily possess the highly functional gills, gut, and renal system that adult fish have to cope with an external environment (Holland, 1999; Hoar and Randall, 1983) nor the developed adaptive system which is also crucial to efficient osmoregulatory adaptation in adults (Maloney et al., 1999).
4.1 INTRODUCTION

Adult snapper, *Pagrus auratus*, migrate into shallow coastal waters to spawn and the larvae and juveniles of this species tend to inhabit near-coastal and estuarine environments until they mature. As such, they may be subject to the fluctuating salinities associated with these environments. Salinity is the main abiotic factor that will challenge the osmoregulatory capacity of teleosts, and has been demonstrated to influence larval growth and survival (Oozeki et al., 1989; Winger and Lasier, 1994; Han et al., 1995; Deboeck et al., 1996; Ottesen and Bolla, 1998; Bohlen, 1999), and is thought to play a role in the recruitment capacity of some species (Ponwith and Neill, 1995; Malloy et al., 1996; Peterson et al., 1999). Consequently, the following chapter investigates the effect of environmental salinity upon the level of both brain cell proliferation and overall growth, and on how these relate to one another in snapper larvae.

4.1.1 Salinity, larval growth and survival

Evidence for an effect of salinity upon teleost growth comes from both cellular and morphological investigations. Studies which have subjected fish cell lines to varying salinity levels have demonstrated significant salinity-dependant effects on cell growth and lipid composition (Tocher et al., 1994; Tocher et al., 1995) and the extent of apoptosis (Hashimoto et al., 1998; Hashimoto et al., 1999). *In vivo* studies have shown that a failure to regulate plasma ion concentrations relative to intracellular concentrations can result in the disruption of membrane potential and reduced or lost cellular function and eventual cell death (Holmes and Donaldson, 1969).

The effect of salinity upon overall growth has also been investigated in many species, and is often dependent on developmental status. This is likely to be due to the differences in osmoregulatory mechanisms that are available to a fish at different stages in its development. Larval fish do not initially possess the highly functional gills, gut and renal system that adult teleosts rely upon to maintain internal homeostasis (Holliday, 1969; Hwang and Hirano, 1985) nor the developed endocrine system which is also crucial to efficient osmoregulatory adaptation in adults (Mancera et al., 1995;
Shrimpton et al., 1995; McCormick, 1996; Nakano et al., 1997; Mancera and McCormick, 1998; Claireaux and Audet, 2000). Larvae rely upon chloride cells located in the integument around the trunk and yolk sac regions, as well as in the gills (Li et al., 1995; Wales, 1997). However, while the development of more mature osmoregulatory features can occur quite rapidly in larvae (Tytler and Ireland, 2000), the relative cost of osmoregulation is thought to be higher than that in adults. As larvae at higher salinities are required to increase water and therefore salt intake, the cost of active transport of salt out of the larvae could be relatively high (Tytler and Ireland, 1994), and may have implications for growth. In addition to effects upon osmotic and ion concentrations, the effects of salinity upon larvae extend to changes in availability of oxygen and in specific gravity. The former tends to decrease as salinity increases, while the latter affects larval buoyancy and thus has implications for transport, energy usage and maintenance of ideal position in the water column (Holliday, 1969).

Given the numerous physical and physiological affects of salinity on a larval fish, it is not surprising that it may play a large role in their growth and survival, as well as influencing other behavioural and ecological aspects such as habitat selection (Baltz et al., 1998; Peterson et al., 1999; Cardona, 2000). The growth and survival probability of larvae will generally decrease as the salinity deviates further from that to which they are adapted in their natural environment (Winger and Lasier, 1994; Kibria et al., 1999). Survival and growth of teleost larvae in response to differing salinity can change as the larvae develop and mature (e.g. Oozeki et al., 1989; Winger and Lasier, 1994; Han et al., 1995; Deboeck et al., 1996; Ottesen and Bolla, 1998; Bohlen, 1999). However, salinity tolerance measured as survival does not always show the same trends as growth. For example, growth as measured by length did not differ for stone flounder reared over a wide range of salinities, however survival (tolerance) was seen to differ significantly (Oozeki et al., 1989). In addition to the effect upon growth and survival, salinity has also been shown to affect other aspects that relate to the nutritional condition of teleosts, including food consumption (Jurss et al., 1987; Arnesen et al., 1993; Buckel et al., 1995), food conversion efficiency (Alava, 1998) and the ability to tolerate and survive starvation (Woo and Murat, 1981). There is some evidence that starvation can reduce the tolerance of fish to changes in salinity (Vijayan et al., 1996).
4.1.2 Flow cytometric approach

Previous uses of flow cytometry to assess cell proliferation in fish larvae have concentrated on the effects of starvation. This is a reasonable starting point given the aims of developing an index to assess the condition and growth of larvae at sea, which it is thought will rely predominantly on their nutritional intake (Theilacker and Shen, 1993; Bromhead et al., 2000; Theilacker and Shen, 2001). Aside from one investigation into the effects of temperature upon cell proliferation (Bromhead et al., 2000), scant consideration has been given to abiotic factors and their effects upon such an index. As described above, salinity can clearly affect the growth and condition of larval fish, yet little investigation has been made into how this may relate to biochemical indices of growth such as RNA/DNA or cell proliferation. A few studies have investigated the relationship between RNA/DNA and salinity, but have used older juvenile or adult fish. Older stages generally have increased tolerance of salinity, hence these studies have shown little effect of salinity upon RNA/DNA (Jurss et al., 1986; Chung et al., 1993).

No investigation could be found in the literature to date of the relationship between salinity, growth and a biochemical or cellular index of growth in larval teleosts, despite salinity clearly being an important factor in their growth. Nothing is known of the effect of salinity upon cell proliferation in the brains of larval fish.

4.1.3 Aims

Salinity clearly plays a large role in the physiological wellbeing of teleost larvae. It has the capacity to influence food consumption, growth and survival, and the nature of its interaction with these factors can change as the larvae develop. However, the effect of salinity upon brain cell proliferation, and larval growth and the relationship between...
these in teleost larvae is unknown. Consequently, the aims of the research presented in this chapter are to assess:

a) The effect of environmental salinity upon cell proliferation in brain tissue of snapper larvae, and thus determine the salinity at which maximal tissue growth is achieved;

b) The effects of salinity upon a morphological measure of growth (length) and determine if it divulges similar conclusions regarding the salinity-growth relationship. Which measure is more sensitive to the effects of salinity?

c) The relationship between larval age and brain cell proliferation at constant salinity;

d) The effect of larval age and development upon the relationship between salinity and brain cell proliferation; and

e) Whether consideration of apoptosis and net tissue growth changes the interpretation or conclusions that can be gained from the investigation regarding the effect of salinity upon growth.

4.2 MATERIALS AND METHODS

4.2.1 Spawning, Stocking and Design

Spawning induction of mature Pagrus auratus females, egg collection and initial egg and larval incubation methods are described in Chapter 2 (section 2.2.1.1). Larvae were stocked at 1500±100 larvae per tank in 32, conical bottom fibreglass tanks (100 L), which were initially filled with filtered and de-chlorinated seawater (salinity at 35 ppt). Each tank operated independently, as described in section 2.2.1.2. Salinity was gradually ramped over the next 27 hours, to produce four replicates of 8 salinity levels (5, 10, 15, 20, 25, 30, 35 and 45 ppt ± 0.5 ppt) arranged in a random block design (Figure 4.1). Salinity, water temperature, dissolved oxygen, pH and water levels were checked daily. Water temperature was maintained at 21.5 ± 0.5°C, dissolved oxygen (DO) at 7.1 ± 0.65 ppm and pH range 7.7-7.9, for the duration of the experiment. A 10% water exchange was made each day. On day 18, the presence of a single-celled parasite, Amyloodinium ocellatum, was noted as having infected larvae in a number of
tanks. All tanks were subsequently treated with chloroquine over the following 24 hours and larvae appeared to be parasite free by day 21.

4.2.2 Feeding

Snapper larvae were first fed on day 4 after hatching. Food consisted of live rotifers fed once daily, between 10:00 and 11:00, at a rotifer density of 1/mL in each tank, for the duration of the experiment. Prior to feeding, rotifers were acclimatised to either 15 ppt salinity (to be fed to 10 and 15 ppt larvae) or 25 ppt (to be fed to 20, 25, 30, 35 and 45 ppt treatment tanks) to prevent shock and dropout of rotifers after they were transferred to snapper larval tanks.

4.2.3 Sampling

An initial sample of 3 larvae was removed from each 5 ppt treatment tank on day 5. This treatment group suffered high mortality and no larvae were alive by day 6. A random sample of 3 larvae was then taken from each treatment tank on day 6, and then every third day thereafter. Samples were placed in 1 mL of tank water in a cryotube, which was sealed and stored in liquid nitrogen until analysis. The trial was terminated 22 days after hatching. In total, 550 larvae were collected for analysis.

4.2.4 Tissue preparation, data collection and analysis

Larvae were thawed in a 37°C water bath and details of morphology and size recorded (see section 2.3.1 for details). Brain tissue was then excised and dissociated in PBS prior to fixation in 70% ethanol at 4°C for 4 hours. These samples were then washed and stained with propidium iodide prior to analysis by flow cytometry (see section 2.3.3). The flow cytometric settings for collection of brain cell data are described in section 2.3.4. Brain cell proliferation data, as well as apoptosis data, were collected and analysed as described in section 2.3.5. It should be noted that the collection gate for flow cytometric analysis of day 18 samples was incorrectly set, resulting in the
Figure 4.1 – Salinity treatments were arranged in a random block design based on 4 replicates of 8 salinity levels (A), with larvae initially stocked on day 2 after hatching. Ramping, first feed and sampling days are outlined on the timeline (B).
exclusion of data from smaller G01 events from the overall samples. While this should not affect relative changes in cell fractions, it will lead to a consistent underestimation of the G01 fraction (and subsequent overestimation of S and G2M). Data from this age group were not included in the overall statistical modelling, but are presented graphically as a comparison for treatment effects.

4.2.5 Statistics

The current analysis looks at 5 response variates to determine which factors contribute to their variation. The two main response variates of interest are the fraction of cells dividing (FOCD) and standard length (SL), as the appropriateness of FOCD as a growth measure will be assessed by how it compares with SL. FOCD is the sum of the fraction of cells in S and in G2M phases. These two sub-fractions are also analysed separately, to determine if either conforms more closely to growth as estimated by length. This was prompted in part by some uncertainty as to which fraction, FOCD, S or G2M, is the more appropriate growth indicator (Bromhead, 1996). Finally, the fraction of apoptotic cells (FAp) is also analysed to determine if cell death is influenced by the same factors that produce variation in cell proliferation, and whether a measure of net tissue growth (FOCD-FAp) might correlate more closely to growth than FOCD. Daily growth rates (GR) are calculated for each sample group (i.e. sampled from a particular treatment on a particular day) using the following equation:

$$GR = \frac{(SL_c - SL_p)}{d}$$

Where $SL_p$ is mean length recorded for a sampling group at previous sampling, $SL_c$ is the current mean length of that group, and $d$ is the number of days in between the two samplings. The relationships between salinity and developmental features such as tail flexion are noted, but not analysed with statistics, as such basic developmental investigations have already been done on this species by other authors (e.g. Battaglene and Talbot, 1992).

The overall statistical approach to modelling the data sets in this thesis is described in detail in the general methods (section 2.3.6). A brief overview of the analysis of the
The current data follows here. The current data set is unbalanced, hence Genstat 5 (Genstat 5 Committee, 1993) was used to apply a restricted maximum likelihood (REML) based mixed model analysis, so as to determine those factors associated with variance in the response variates. Ultimately, a model was generated to describe the variance in each response variate, and these models used to generate predicted means and determine the least significant difference (LSD) between the means. Both actual means and predicted means are presented graphically for comparative purposes. An LSD error bar is included on predicted means graphs to allow an assessment of the significance of changes in response variates to different factors. The final models for each response variate are presented in Table 4.1.

Table 4.1 – Description of final models describing the variance in brain cell proliferation of snapper (*P. auratus*) larvae reared at 7 different salinities over 21 days and sampled every three days. Models were determined using mixed model analysis, by which variance due to individual tank effect was accounted for before that attributable to the main factors of interest was assessed. The response variates modelled are the fraction of cells dividing (FOCD), the fraction of cells in S phase and in G2M phase, the fraction of apoptotic cells (FAp), and the net tissue growth index (NTGI).

<table>
<thead>
<tr>
<th>Response variate</th>
<th>Mixed model component</th>
<th>Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOCD</td>
<td>Fixed</td>
<td>Salinity + age + (salinity×age).</td>
</tr>
<tr>
<td>S</td>
<td>Fixed</td>
<td>Salinity + length + age + (salinity×length) + (salinity×age) + (length×age) + (salinity×length×age)</td>
</tr>
<tr>
<td>G2M</td>
<td>Fixed</td>
<td>Salinity + age + (salinity×age).</td>
</tr>
<tr>
<td>FAp</td>
<td>Fixed</td>
<td>Salinity + age + (salinity×age).</td>
</tr>
<tr>
<td>NTGI</td>
<td>Fixed</td>
<td>Salinity + age + length + (salinity×age)</td>
</tr>
<tr>
<td>SL</td>
<td>Fixed</td>
<td>Salinity + age + (salinity×age).</td>
</tr>
</tbody>
</table>
4.3 RESULTS

So as to view the cell cycle results in a biologically relevant light with respect to fish larvae, analysis of data concerning the development and growth of the snapper larvae subjected to different rearing salinities will be presented first. This will be followed by data pertaining to the effect of these treatments upon cell proliferation and tissue growth.

4.3.1 Morphological growth and development

4.3.1.1 Length and growth rate

Length increased significantly over the course of the experiment (CID=1079.2, df=5, p=0.0000), however mean length did not differ among salinity treatments until the larvae were older (Figure 4.2). Growth rate (mm/d) increased between 6 and 9 days, then slowed as larvae became older (Figure 4.3), with no treatment showing a significant increase in length between day 18 and 21. During the period 18 to 21 days, mean standard length of larvae reared at 45ppt was significantly less than that of larvae reared at lower salinities. However, there was little significant difference between mean lengths for larvae reared at other salinities, at any age (Figure 4.2).

4.3.1.2 Feeding, swim bladder inflation and flexion notes

Feeding, swim bladder inflation and flexion all varied with salinity level and age (Figure 4.4). In general, fewer larvae reared at 10 ppt were feeding (defined by presence of food in their guts at the time of sampling) when compared to larvae reared at higher salinities. The percentage of feeding larvae was generally lower in larvae aged 6 days and then increased as larvae aged. The percentage of larvae achieving swim bladder inflation was highest at 30 and 35 ppt and lowest at 10 and 45 ppt, and with the exception of larvae aged 15 days, increased as larval age increased. Flexion was only apparent in larvae aged 12 days or older, at any salinity. The fraction of larvae with flexion varied between sampling days. The fraction of larvae sampled that had achieved flexion appeared to be less in the day 18 sample when compared to age 12 and 21 day
Figure 4.2 - Mean standard length (SL) of *Pagrus auratus* larvae reared at 7 different salinities ranging from 10 ppt to 45 ppt, and sampled every 3 days for a period of 21 days. Vertical bar represents the least significant difference (LSD), which can be used to determine which predicted means are significantly different. The cross “+” represents mean for larvae reared at 5ppt, which were only sampled on day 5.
Figure 4.3 – Mean growth rate (mm/d) of larval *Pagrus auratus* reared at 7 different salinities for a period of 21 days. Growth rate is calculated as the mean daily increase in length as occurs over the three days in between each sampling event. Given that larvae are sacrificed upon sampling, it was not possible to estimate means using increases in individual larval lengths.
Figure 4.4 – Percentage of larval *Pagrus auratus*, reared at 7 different salinities, which were identified as feeding, having swim bladder inflation or having tail flexion, when aged A) 6 days, B) 9 days, C) 12 days, D) 15 days, E) 18 days, or F) 21 days.
samples. The number of larvae achieving flexion was highest at salinities from 20 to 35 ppt.

4.3.2 Cell proliferation

4.3.2.1 Brain cell proliferation

Variation in FOCD was influenced by a highly significant interaction between larval age and the salinity at which the larvae were reared (CID 56.34, df=24, p=0.0002). Up to 12 days after hatching, few of the treatments differed significantly in mean FOCD (Figure 4.5A). In older larvae reared at 30 and 35 ppt, mean FOCD was significantly higher than for larvae reared at 10 and 15 ppt (day 15) as well as for 20 and 45 ppt after 21 days. This trend of higher mean FOCD for larvae reared at 30 and 35 ppt was evident from day 9 onwards. Variation in mean FOCD between treatments was clearly greater in age 15, 18 and 21 day larvae than in younger larvae (Figure 4.6). With the exception of larvae in 15 ppt, overall FOCD tended to fluctuate throughout the experiment regardless of treatment (Figure 4.5A). Initially, there was a general increase from day 6 to 9. From days 9 to 15, larvae reared at lower salinities (10, 15 and 20 ppt) had little difference in mean FOCD, while in larvae reared at higher salinities it fluctuated (decreasing to day 12 and increasing to day 15). Mean FOCD for all larvae was significantly lower on day 21 when compared to day 15.

When components of FOCD were analysed separately, two different trends were apparent. Variation in S phase fraction was influenced by a three way interaction between salinity level, age and larval length (CID 62.34, df=30, p=0.002) (Figures 4.6 and 4.7). Variation in G2M cell fraction was influenced by a weak interaction between salinity and age (CID=39.91, df=24, p=0.0218) (Figure 4.5B). However while S phase varied with age in a manner similar to mean FOCD (Figure 4.6), mean G2M increased significantly as larvae got older, and this was most apparent for larvae reared at 30ppt (Figure 4.5B). In general, the fraction of cells in S phase was greater than 0.05, which was greater than the fraction of cells in G2M (generally lower than 0.05). This trend was reversed in 21 day larvae (Figure 4.6).
Figure 4.5 – REML based predicted mean fractions of: A) cells dividing (FOCD); and B) G2M cells in brain tissue of *Pagrus auratus* larvae reared at 7 different salinity levels and sampled every 3 days after hatching (from day 6). Vertical bars represent the least significant differences (LSD), which can be used to determine which predicted means are significantly different. Cross “+” represents means for larvae reared at 5ppt, which were only sampled on day 5.
Figure 4.6 – Comparison of cell cycle fractions of brain cells isolated from larval *Pagrus auratus* which had been reared at salinities ranging from 10 to 45 ppt and sampled at days 6, 9, 12, 15, 18 and 21. Note that day 18 samples were not included in overall analysis (see section 4.2.4 for explanation). Error bars denote ±2SE.
Figure 4.7 – Comparison of *Pagrus auratus* larval size (standard length) with the fraction of larval brain cells in S, G2M or both (FOCD) for larvae reared at different salinities. Lines represent linear fits for each cell fraction (S, G2M or FOCD).
Figure 4.8 – Comparison of mean growth rates with mean cell cycle fractions for brain cells isolated from Pagrus auratus larvae. Each point represents the mean for a single sampling of larvae from a particular age and salinity treatment. R² values are placed at end of each regression line.
Mean growth rate (mm/d) was compared with FOCD, S and G2M in brain cells, and three main trends were evident (Figure 4.8). Mean FOCD (ANOVA, F=23.45, p=0.0000, r²=0.4155) and S (ANOVA, F=58.037, p=0.0000, r²=0.6375) increased as larval growth rate increased. The mean fraction of cells in G2M showed no relationship with mean growth rate (r²=0.0338).

### 4.3.2.2 Apoptosis and net tissue growth

REML analysis of apoptotic cells (defined by gating on the sub-diploid population) determined that variation in mean fraction of brain cells that were apoptotic (FAp) was significantly influenced by a larval age and salinity interaction (CID=49.25, df=24, p=0.0017) (Figure 4.9A). In general mean FAp was higher in younger larvae (aged 6, 9, or 12 days) and decreased as salinity increased for these age groups. Older larvae (particularly 15 and 21 day old larvae) had lower mean FAp, but with the exception of the 10 ppt treatment, there was little significant difference in FAp for larvae reared at different salinities at these ages.

REML modelling determined that the net tissue growth index (NTGI) for larval brain tissue was significantly related to salinity, larval age and length, and that the response of the NTGI to salinity varied with the age of the larvae (CID=45.8, df=24, p=0.0121) (Figure 4.9B). The overall trend with age was an increase in NTGI and concurrent reduction in the differences in mean NTGI between salinities as larvae got older. In younger larvae, NTGI tended to increase as the rearing salinity increased, from 10ppt to 45ppt. In larvae 15 days or older, NTGI followed a similar pattern to that of mean FOCD for these age groups, with NTGI peaking in larvae reared at 30ppt, and being least for larvae reared at 10ppt. However the differences in mean NTGI between salinities were less significant than for FOCD in these age groups. Mean NTGI had no significant relationship to mean growth rate (ANOVA, F=1.173, p=0.286, r²=0.0343) (Figure 4.10).

### 4.3.2.3 Comparison of the fraction of cells in different phases

A comparison of the fraction of brain cells in different phases of the cell cycle and which were apoptotic revealed 4 main trends (Figure 4.11). Firstly, both the fraction of
Figure 4.9 – A) The mean fraction of apoptotic cells (FAp) recorded from the brain tissue of *Pagrus auratus* larvae reared at 7 salinities and sampled every three days from day 6 after hatching. Apoptotic fractions were then subtracted from each larva’s FOCD to create B) a net tissue growth index (NTGI, actual values shown). Error bars for NTGI are –2SE.
Figure 4.10 – Comparison of mean growth rate (mm/d) with mean net brain tissue growth (NTGI) for Pagrus auratus larvae sampled by salinity (10, 15, 20, 25, 30, 35, 45 ppt) at ages 6, 9, 12, 15 and 21 days. The relationship has a very low correlation coefficient ($r^2 = 0.0343$).
Figure 4.11 – Comparison of the fraction of \textit{Pagrus auratus} brain cells in different stages of the cell cycle or apoptotic. A) S and G01 ($r^2 = 0.318$). B) G2M and G01 ($r^2 = 0.092$). C) S and G2M ($r^2 = 0.000$). D) G01 and FAp ($r^2 = 0.686$). E) S and FAp ($r^2 = 0.007$). F) G2M and FAp ($r^2 = 0.537$).
cells in S (ANOVA, F=158.91, p=0.0000, \( r^2 = 0.318 \)) or apoptotic (ANOVA, F=743.70, p=0.0000, \( r^2 = 0.686 \)) increased as the fraction in GO1 decreased. A positive correlation existed between the fraction of cells in G2M and GO1 (ANOVA, F=34.554, p=0.0000, \( r^2 = 0.092 \)), although the correlation only explained 9.2% of variation. The G2M fraction also decreased exponentially as the apoptotic fraction increased (ANOVA, F=207.82, p=0.0000, \( r=0.537 \)). There was no significant correlation between the fraction S and G2M (ANOVA, F=0.12, p=0.7281, \( r^2 = 0.000 \)), or the fractions S and apoptosis (ANOVA, F=2.62, p=0.1064, \( r=0.007 \)). Thus the following overall pattern emerged. As the fraction of cells in GO1 decreased, the fraction of cells in S and FAp increased. The G2M fraction appeared relatively stable, with perhaps a slight decrease, while fraction of apoptotic cells increased. FAp also appeared to decrease in larvae with a higher fraction of brain cells in G2M. The fraction of cells in S was not related to FAp or the fraction of cells in G2M.

4.4 DISCUSSION

The results demonstrated that, while environmental salinity can significantly effect the proportion of dividing brain cells in larval *P. auratus*, the effect depended upon the age of the larvae. Mean brain FOCD in younger larvae (≤12 days) did not differ significantly between salinity treatments, suggesting a wide tolerance to differing salinities in post-hatch larvae. However, in older larvae (>12 days) salinity significantly affected mean brain FOCD, which was highest in larvae reared at 30 ppt. Mean FOCD and S can significantly decrease or increase within a three day period, and this was evident in all treatment groups over the course of the experiment, suggesting that growth in brain tissue, whilst a constant process, may increase periodically in “bursts”. Further evidence for this will be presented in Chapter 5. RNA/DNA studies of condition have shown that if a biochemical or cellular parameter of growth varies naturally with ontogeny then it is best that comparisons between larval samples only be made on samples sharing the same developmental or age status (Richard *et al.*, 1991; Clemmesen, 1994; Bisbal and Bengtson, 1995; Suneetha *et al.*, 1999). On the evidence presented here, the same guidelines should be followed during brain cell proliferation studies.
The developmental and length data analyses also support the conclusions gained from the brain FOCD analysis. When assessed over the entire course of the experiment, the proportion of larvae achieving flexion and swim bladder inflation were generally highest in the 30 ppt treatment. Furthermore, while length measures did not differ significantly between salinity levels (excepting 45 ppt) on most days, it was clear from day 12 onwards that mean length was consistently higher for larvae reared at 30 ppt. Despite having adjusted length measures using the calibration described in chapter 2 (section 2.3.1), one would still expect a loss of sensitivity in the length measures as a result of freezing related shrinkage. This may partly explain the lack of significant differences in length measures over the course of the experiment. In a recent comparison of the simultaneous effects of both salinity and temperature on the growth of larval *P. auratus*, Fielder *et al.* (in prep.) found that dry weight was a more sensitive indicator of growth in larval snapper than length. Unfortunately, cell proliferation studies require wet tissue, which precluded the use of whole larval dry weight measurement. This aspect is one hindrance to the comparison of flow cytometer determined cell proliferation levels with overall morphological growth measures (Bromhead, 1996). The developmental, length and cell proliferation data together suggested that the optimum rearing salinity for snapper larvae was 30 ppt, particularly for older larvae. There have already been numerous documented examples of changing tolerance to salinity as fish develop (Oozeki *et al.*, 1989; Winger and Lasier, 1994; Han *et al.*, 1995; Deboeck *et al.*, 1996; Ottesen and Bolla, 1998; Bohlen, 1999). Often these have been associated with changes in habitat selection that occur as the fish mature. Adult snapper come into shallow coastal waters to spawn, and larval and juvenile snapper may be subject to ranging salinities associated with near coastal environs. Therefore a tolerance to a wide range of salinities is not surprising.

Mean FOCD, S and G2M for brain cells were compared to mean growth rate. The growth rate estimates may have lost some sensitivity and precision due to the fact that they are based on non-continuous samples (i.e. on a different samples of larvae). Despite this there was excellent correlation between the mean fraction of brain cells in S phase and mean growth rate (Figure 4.8), giving strong support to the use of a measure of brain cell proliferation as a growth indicator in larval fish. Our previous research (Bromhead *et al.* 2000) was not able to make comparisons to whole larval growth rates. When variation in mean length and in the mean fraction of cells synthesising DNA in
response to treatment levels are compared, it would appear that mean fraction of cells in S phase might be more sensitive to the effects of environmental salinity than mean length.

The current investigation initially intended to use S+G2M (=FOCD) as a growth indicator, based on the argument presented by Theilacker and Shen (1993b, 2001). They proposed that a measure of the combined fraction of cells in S and G2M represented an appropriate measure of cell proliferation, based on cell culture evidence that cells having entered S phase are committed to progressing through the cell cycle to physical division, independent of the external conditions (Hartwell and Weinert, 1989; Murray and Kirschner, 1989). However, these papers did not deal specifically with larval teleost cells, and one cannot assume that their findings are necessarily relevant to all taxa. To date, little analysis has examined the relationships between the proportions of larval brain cells in different phases and how these might change in response to different growth conditions. This seems particularly important when one considers that cells do not simply flow continuously through the cell cycle, but may leave the cycle at various points, either to die or to arrest, temporarily or permanently. (Soprano and Cosenza, 1992; Murray and Hunt, 1993). The FOCD index to some extent assumes a proportional and uninterrupted flow of cycling cells through S and G2M. In order to decipher these relationships and test the assumptions underlying the use of this index, comparisons were made between fractions of cells in various stages of the cycle, in the first instance regardless of treatment or larval age, and in the second instance, following how these fractions changed relative to one another in response to salinity and age. A third factor, disease, may prove fortuitous in helping to decipher these relationships.

A comparison of phase fractions revealed that a reduced G01 fraction might result not only from cells moving into S phase, but also through G01 cells becoming apoptotic. The G2M fraction was also related negatively to the apoptotic fraction, suggesting these cells may have contributed to the apoptotic population. It may be that G2M cells become apoptotic upon division. This might explain why a unit drop in the G2M fraction resulted in a disproportionately larger increase in the fraction of apoptotic cells (Figure 4.11). Perhaps the most interesting result was the lack of correlation between the fraction of cells in G2M and S. One might expect that if growth conditions are good that the proportion of S cells would increase and be followed by a proportional increase
in G2M. However the number of cells in each appeared to be independent of one another (Figure 4.11C). The advent of disease between day 17 and 21 may help to explain this phenomena. If one compares growth indices in larvae prior to day 21 to those on day 21 (after infestation by A. ocellatum and subsequent treatment) three major trends are apparent which suggest that, under sub-optimal growth conditions, brain cells might arrest in G2M. Firstly, overall FOCD was markedly lower at all salinites on day 21 (Figure 4.6). So too was mean growth rate (mm/d) (Figure 4.3). But perhaps most significantly, the mean fraction of cells synthesising DNA (fraction S) was lower, at all salinity levels, than in any earlier age group. In contrast, mean G2M was higher than fraction S for the first time, and generally higher than it had been at any previous age. These trends in fractions of S and G2M on day 21 were apparent also in an analysis of the effects of salinity upon proliferation of muscle nuclei (Appendix II). The most likely explanation for these trends is that the advent of disease and subsequent treatment led to a drastic reduction in growth, as reflected in both growth rate and FOCD and S fractions, and that cells which had been progressing through S at the physiological onset of the disease, arrested in G2M. The subsequent effect would be a reduced level of S and increased fraction of cells in G2M, as evident in the current data.

The presence of a cell cycle checkpoint in G2 phase is already well established (Norbury and Nurse, 1992; Dirks and Rutka, 1997; King and Cidlowski, 1998), and the arrest of cells in G2 phase in response to suboptimal growth conditions has been noted in both normal and modified cells. Prochlorococcus strains starved of phosphorous (Parpais et al., 1996) and smooth muscle cell lines starved of cholesterol (Martinez-Botas et al., 1999) exhibited substantial G2 arrest. This occurrence is also supported by evidence for G2 arrest in other modified cell lines (Stewart et al., 1995) and organisms (Kominami et al., 1998). However, it should also be noted that numerous chemical agents have been proven to arrest cells at various points in the cell cycle (Augustin et al., 1996; Sugai et al., 1998; Cortez-Bratti et al., 1999; De Rycke et al., 2000), and it is possible that the arrest observed here was as a result of the quinine treatment, not the disease itself. However, no mention of such a quinine-related effect could be found in cell cycle literature. Additionally, one needs to be aware that certain cell types, such as epidermal cells, will naturally arrest in G2 for long periods (Murray and Hunt, 1993). The advent of such a population in the developing larval brain should not be discounted. Regardless of whether sub-optimal growth conditions or a natural developmental
progression are responsible for G2M arrest, the results suggest that G2M may not be an appropriate component to include in a cell cycle-based growth index. The FOCD, originally proposed by Theilacker and Shen (2001), correlates well with overall growth rate, but not to the same extent as fraction S, as FOCD incorporates the G2M fraction. The current findings suggest that the most appropriate index simply comprises the fraction of cells in S phase. However, further evidence for G2M arrest, perhaps in association with other sub-optimal growth conditions, is required to ensure that it was not simply due to the action of quinine.

Another aspect not previously investigated is the effect of apoptosis on overall tissue growth in larval fish. Apoptosis was clearly highest in brain tissue of larvae reared at 10 ppt, regardless of age. Younger larvae showed increased apoptosis in lower salinities (10 to 25 ppt) while in older larvae there was little difference in apoptotic fraction at salinities above 10 ppt. When compared with the cell proliferation results, the data suggest that while cell proliferation was not significantly affected by salinity level in younger larvae, the lower salinities were associated with an increased level of apoptosis. Subsequently, the net tissue growth index (NTGI) data suggest that there was a net loss of brain tissue in younger larvae reared at lower salinities. As with FOCD, the NTGI was significantly related to both larval age and salinity. In contrast to the brain FOCD, the NTGI indicated differences in overall brain tissue growth in larvae younger than 12 days, with mean NTGI being highest at higher salinities (around 30-45 ppt). As with brain FOCD, the mean NTGI for larvae older than 12 days suggested that 30 ppt was the optimum salinity for tissue growth in this age group. However, unlike FOCD or S data, NTGI data did not relate closely to overall growth as based on length measures. This suggests that the NTGI index was not an appropriate indicator of the overall growth status of the larvae. There were a number of reasons this may have been so. While apoptosis can be induced by suboptimal growth conditions (Boza et al., 1999; Tessitore et al., 1999), it has also been shown to be a natural event in the early development and modelling of vertebrate tissues (Krueger et al., 1995; Nishikawa and Hayashi, 1995; Sachs et al., 1997; Blaschke et al., 1998). It is possible that if conditions for growth are not ideal, then remodelling (and therefore apoptosis) would be reduced also. In other words, apoptosis in developing organisms may respond to environmental conditions in a similar manner to cell division. Hence, incorporating a measure of apoptosis could potentially reduce the sensitivity of an index to growth responses to
environmental conditions rather than increase it. This may explain the loss of sensitivity of NTGI to overall growth when compared to the FOCD or fractions of cells synthesising DNA (S phase).

In summary, this investigation into the effects of salinity upon cell proliferation and death in brain tissue of larval snapper has four key findings:

a) Environmental salinity affected both brain cell proliferation and overall growth and development of snapper larvae, and the relationship changed with larval age. Both length and S phase fractions indicated growth was highest at around 30 ppt;

b) Disease (or its treatment) may have caused cells to arrest in G2M, resulting in an increased G2M fraction relative to S phase fraction. This, and a lack of correlation between G2M and overall growth, suggested that G2M cells should not be included in an index of larval growth;

c) The fraction of brain cells synthesising DNA was a sensitive and appropriate index of overall larval growth; and

d) Consideration of apoptosis and net tissue growth in the larval brain did not aid in the indication nor interpretation of overall larval growth responses.
CHAPTER 5

THE EFFECT OF TEMPERATURE UPON BRAIN CELL PROLIFERATION IN SNAPPER LARVAE

5.1 INTRODUCTION

Temperature plays a critical role in the survival and growth of early life history stages of fish and as such may be an important factor in the recruitment of fish species (Buckley et al., 1999; Wassenberg and Jones, 1999; Darling and Case, 1996; Vonesh and Baskett, 1996; Vonesh and Baskett, 1996; Bouchet and Ohnsorge, 1998; and Ohnsorge, 1999). Consideration of temperature is therefore important when estimating larval growth, by what is referred to as ‘growth rate’ (Buckley et al., 1999). Both cell cycle oscillations and the rate of migration through the cycle have been shown to be temperature dependent in different vertebrate organisms (Miyata and Hess, 1993). Brenchley et al. (2000) demonstrated that temperature influenced the level of brain cell proliferation of larvae of the bony fish larvae, but did not investigate this in relation to overall growth. The proliferation index for larval growth requires an understanding of the effects of temperature upon both cell proliferation and overall growth, and the interrelationship of these two processes. This validation is currently restricted when one considers that other factors, such as food availability, may influence the overall growth of the larva. Temperature, food availability, and other environmental factors have been shown to affect the growth of larval fish (Jennings et al., 1984; Jennings and Kenchington, 1984; and Kenchington, 1984; Kenchington and Jennings, 1984; and Jennings and Kenchington, 1984). The proliferation and growth of the larval brain cells are known to be more sensitive to the effects of temperature than are larval development stages (Grizzle et al., 1999). Larvae may grow more efficiently when reared at the same temperature to which they are exposed during gonad formation (Buckley et al., 1999). After fertilization, the development of which embryos developing has been also known to affect the larval growth (Janick and Fiffick, 1998; Baskett and Baskett, 1999). The development of the phenotype of muscle tissue (Fifield, 1998; Warren and Johnston, 1998; Johnston et al., 1999; Johnston et al., 1999; and Johnston et al., 1999) and subsequent larval growth. Within the marine environment, some of the larvae, growth generally increases as temperature increases (Kemp, 1991). Increasing temperatures above the natural range generally results in a growth rate reaching an asymptote, but in adverse growth rates at decline above the optimal (Kemp, 1991).
5.1 INTRODUCTION

Temperature plays a critical role in the survival and growth of early life history stages of fish and as such may be an important factor in the recruitment of fish species (Buckley et al., 1990; Rutherford and Houde, 1995; Gadomski and Caddell, 1996; Vonherbing and Boutilier, 1996; Sogard and Olla, 2000). Consideration of temperature is therefore important when assessing larval growth, by whatever means (Buckley et al., 1999). Both cell cycle initiation and the rate of progression through the cycle have been shown to be temperature dependant in ectothermic organisms (Murray and Hunt, 1993). Bromhead et al. (2000) demonstrated that temperature influences the level of brain cell proliferation in larva of the mountain minnow, *Galaxias olidus*, but did not investigate this in relation to overall growth. The validation of a cell proliferation index for larval growth requires an understanding of the effects of temperature upon both cell proliferation and overall growth and upon the inter-relationship of these two processes. This validation is particularly important when one considers that other nucleic acid based indices, such as the ratio of RNA to DNA, have been shown to become uncoupled from morphological growth and condition under certain thermal conditions (Goolish et al., 1984; Jurss et al., 1987).

5.1.1 Temperature and growth of fish larvae

The metabolism and growth of early life history stages of fishes are known to be more sensitive to the effects of temperature than are later developmental stages (Rombough, 1996). Larvae may grow more efficiently when reared at the same temperature as experienced by parents during gamete formation (Buckley et al., 1990). After fertilisation, the temperature at which embryos develop has been also shown to affect the size at hatch (Canino, 1994; Baynes and Howell, 1996), the development and phenotype of muscle tissue (Hanel et al., 1996; Vieira and Johnston, 1996; Johnston et al., 1997; Johnston et al., 1998; Matschak et al., 1998), endogenous fuel (yolk) reserves and subsequent larval growth. Within the normal thermal tolerance zone of a larva, growth generally increases as temperature increases (Rombough, 1996). Increasing temperatures above the normal range generally results in a growth rate reaching an asymptote, but in adults growth tends to decline above the optima (Rombough, 1996).
Overall developmental time generally decreases with increasing temperature, however, temperature can also differentially effect the developmental sequence in post-hatch larva (Fukuhara, 1990; Pepin, 1991; Haylor and Mollah, 1995; Johnston et al., 1995; Brightman et al., 1997). In general, the zone of thermal tolerance expands as fish develop, while the thermal optima for growth generally decreases with increasing size (Rombough, 1996; Aune et al., 1997). Thus larvae generally have a reduced range but higher optimum temperature for growth than later stages.

### 5.1.2 Temperature and larval survival

Numerous studies have shown that the optimal temperature for growth is rarely the same as that for larval survival (Bestgen, 1996; Hart et al., 1996). Optimal survival temperatures are generally lower than those at which maximal growth is achieved. Mortality attributable to temperature effects alone is reduced at lower bounds of a species natural temperature range. This may complicate interpretation of growth data as relates to survival and recruitment. Furthermore, this relationship is likely to be age-, species- and stage-specific (Oozeki et al., 1989; Oozeki and Watanabe, 2000).

Temperature influences larval survival probability in a number of ways. In yolk-sac larvae, yolk absorption and utilisation efficiency increase with temperature (Fukuhara, 1990; Haylor and Mollah, 1995; Baynes and Howell, 1996; Conceicao et al., 1998). Although overall growth is faster at higher temperatures, the time in which larvae have to find exogenous nutrition sources is reduced and the risk of starvation increases (Fukuhara, 1990; Brightman et al., 1997). The same risk applies to feeding larvae, for which the required maintenance ration increases as temperature increases (Johnston and Mathias, 1996). When the prey source becomes limited, larvae become reliant on their endogenous tissue energy stores. Thus, while growth in starved larvae is minimal, and not differentially affected by temperature (Fukuhara, 1990; Mathers et al., 1993; Malloy et al., 1996) temperature does have a large influence upon survival (Oozeki et al., 1989), by determining the rate at which endogenous energy reserves are exhausted.
5.1.3 Growth indices and temperature effects

The most popular method for assessing instantaneous larval growth and condition in the field is the RNA/DNA index. RNA levels have been shown in laboratory (Buckley, 1984), mesocosm (Folkevord et al., 1996) and field studies (Hovenkamp and Witte, 1991; Bailey et al., 1995; Rooker et al., 1997; Garcia et al., 1998) to be particularly responsive to temperature. However, as described in Chapter 1 (section 1.2.4.3) the incorporation of temperature terms into growth equations for RNA/DNA relationship is complicated by the occurrence of compensatory responses at low temperatures, that result in RNA levels and growth being uncoupled. Whether this occurs in larval tissues has been questioned (Mathers et al., 1993), but highlights the importance of determining the relationship between a particular biochemical parameter of growth, temperature and overall growth.

5.1.4 Aims

In order to overcome the shortcomings of previous investigations, and to more thoroughly investigate the relationship between temperature, brain cell proliferation and overall larval growth, the following experiment aimed to assess:

1) The effect of environmental temperature upon cell proliferation in brain tissue, and thus determine the temperature at which maximal brain tissue growth is achieved;
2) The effect of temperature upon morphological measures of growth (length) and determine if firstly, they divulge similar conclusions regarding the temperature growth relationship and secondly, which measure is more sensitive to the effects of temperature upon growth;

3) Whether, at constant temperature, cell proliferation varies dependant on larval age;

4) Whether the relationship between temperature and cell proliferation change as the larvae develop and grow older; and

5) Whether consideration of apoptosis and net tissue growth changes the interpretation or conclusions that can be gained from the investigation regarding the effect of temperature upon growth.

5.2 MATERIALS AND METHODS

5.2.1 Spawning, stocking and design

The larvae used in this analysis were generously reared and supplied by Stewart Fielder and colleagues at the NSW Fisheries Port Stephens Research Centre. Methods used to achieve adult spawning, egg collection, egg and larval incubation are described in Chapter 2 (section 2.2.1.1). Larvae were stocked at 627 ± 31 larvae per tank in 24 (100 L) conical bottom fibreglass tanks, on the second day after hatching. Tank design and operation is also as described in section 2.2.1.2. Temperature in all tanks was initially 21°C, and was then ramped over the next 24 hours, to produce four replicates of 6 temperature levels. These were 15°C, 18°C, 21°C, 24°C, 27°C and 30°C, arranged in a random block design similar to that described for the salinity trial (Chapter 4, Figure 4.2). However, larvae reared at 30°C all died within the next 48 hours and this treatment was consequently removed from the experiment. Salinity, water temperature, dissolved oxygen, pH and water levels were all checked daily. Salinity was maintained at 35 ± 0.4 ppt, dissolved oxygen (DO) within the range 7.6-7.8 ppm, pH range 7.7-8.1, for the duration of the experiment. A water exchange of 10% was made each day. Notes on survival were also taken at day 14. Actual survival data taken from a parallel sampling experiment (Fielder et al., in prep) will be quoted in the discussion.
5.2.2 Feeding and sampling

As with the salinity trial, larvae were first fed live rotifers on day 4 after hatching. Feeding occurred once daily, between 10am and 11am, at a rotifer density of 1/mL in each tank, for the duration of the experiment. Rotifers were acclimatised to the ambient temperature into which they would be transferred at feeding, so as to prevent rotifer dropout and allow a consistent feed level between treatments. A random sample of 3 larvae was taken from each rearing tank on day 6 after hatching. On days 8 and 11, sample numbers were increased to between 6 and 9 larvae per tank, and then back to 3 per tank on the last sampling, day 14. Samples could not be obtained on day 14 for larvae reared at 27°C, as all the larvae in these treatment tanks had died. The trial was terminated 15 days after hatching. All samples were stored in cryotubes in liquid nitrogen until analysis. In total, 430 larvae were collected for analysis.

5.2.3 Tissue Preparation and data collection

Brain tissue was excised, prepared and analysed from each larva sampled, as described in section 2.3. The flow cytometric settings for collection of brain cell data, as well as methods describing subsequent analysis of this data, are described in section 2.3.3.

5.2.4 Statistics

As with the salinity analysis, the current experiment analyses 5 separate response variates. These are the fraction of (brain) cells dividing (FOCD), the fractions of cells in S and G2M phases, the fraction of apoptotic cells (FAp), and the net tissue growth index (NTGI). Mixed model analysis is used to determine the extent that treatment level, age or developmental factors explained variation in each response variate. The final models are presented in Table 5.1. All response variates are also compared to morphological growth as measured by standard length (SL) and growth rate (GR), to determine which response variate correlates most closely to overall larval growth, and therefore acts as an appropriate recent or instantaneous growth indicator. The models are used to generate predicted means, which will occasionally be used to represent the models graphically.
Table 5.1 – Response variates relating to brain cell proliferation (FOCD, S, G2M, FAp, NTGI) and overall morphological growth (standard length - SL) of Pagrus auratus larvae reared at various temperatures. Restricted maximum likelihood (REML) models were used to determine those factors which best explain variation in each response variate. Below is a summary of models for each response variate.

<table>
<thead>
<tr>
<th>Response variate</th>
<th>Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOCD</td>
<td>Temperature + Age + (Temperature×Age) + SL</td>
</tr>
<tr>
<td>Fraction S</td>
<td>Temperature + Age + SL + (Temperature×Age) + (Temperature×Age×SL)</td>
</tr>
<tr>
<td>Fraction G2M</td>
<td>Age + (Temperature×Age)</td>
</tr>
<tr>
<td>Fraction apoptosis</td>
<td>Age + (Temperature×Age)</td>
</tr>
<tr>
<td>NTGI</td>
<td>Age + (Temperature×Age)</td>
</tr>
<tr>
<td>Standard length</td>
<td>Temperature + Age + Flexion + Swim-bladder inflation</td>
</tr>
</tbody>
</table>

An error bar representing the least significant difference (LSD) aids in visual analysis of graphs, allowing the discrimination between data points that are significantly different. A more detailed explanation of this statistical approach is provided in Chapter 2 (Section 2.3.6).

5.3 RESULTS

5.3.1 Morphological growth, development and survival

Mean length of Pagrus auratus larvae (at each temperature) increased significantly between hatching and day 14 (CID=286.4, df=3, p=0.0000) (Figure 5.1). There were significant differences between the mean lengths of larvae reared at different temperatures, which were apparent from day 6. By day 14 after hatching, mean length differed significantly between all temperature treatments, with mean length increasing.
Figure 5.1 - Mean standard length of *Pagrus auratus* larvae reared at 5 different temperatures ranging from 15°C to 27°C, and sampled on days 6, 8, 11 and 14 after hatching. Error bars are ±2SE.
Figure 5.2 - Mean growth rate (mm/d) of larval *Pagrus auratus* reared at 5 different temperatures for a period of 14 days. Growth rate was calculated as the mean daily increase in length as occurred over the period between each sampling. Thus, the day 6 estimate was calculated by subtracting mean length on day 1 from mean length at day 6 and dividing by 6 to obtain mean daily growth rate for that period. Given that larvae were sacrificed upon sampling, it was not possible to estimate means using increases in individual larval lengths.
with temperature (Figure 5.1). Consequently, growth rates were, on average, higher at higher temperatures (Figure 5.2). Growth rate appeared to decrease between days 6 and 8, regardless of temperature, and then increased again through to day 14. For all periods except between days 8 to 11, mean growth rate was lowest at 15°C. Growth rate at this temperature for days 6-8 was negative, but the difference in mean length between these two days (at 15°C) was not significant.

The percentage of larvae feeding was generally over 90%, with the exception of three instances: day 6 (15°C); day 8 (18°C); and day 11 (27°C) (Figure 5.3). Percentage swim bladder inflation appeared higher in 6-day-old when compared to 8-day-old larvae, and was highest at 24°C for each age group except age 11 larvae. Flexion only occurred in 11 day or older larvae, which had been reared at 21°C, 24°C and 27°C, and was highest in 14-day-old larvae reared at 24°C. While the specific number of mortalities was not recorded, it was noted that no larvae remained alive at 27°C by day 12, and only three 24°C tanks had larvae alive by day 14. All larvae had died in 30°C treatment by day 5. A parallel experiment run by Fielder *et al.* (in prep.) calculated end survival percentages and these data will be discussed later.

### 5.3.2 Brain cell proliferation

Variation in FOCD was influenced by a highly significant interaction between larval age and the temperature at which the larvae were reared (CID = 96.87, df = 11, p = 0.0000) (Figure 5.4), as well as a weaker dependence on the standard length of the larvae (CID = 8.068; df = 1; p = 0.0045). The temperature-age interaction manifested as the following trend. Mean FOCD tended to decrease with age, but the degree to which this occurred became less as the rearing temperature decreased (Figure 5.5). Thus for larvae reared at 27°C, FOCD was initially higher than that for larvae reared at 15°C and 18°C, but by day 11 was significantly lower than the mean FOCD of larvae reared at any other temperature. Similarly for larvae reared at 24°C, mean FOCD was highest on day 6, but lower than that for larvae reared at other temperatures by day 14 and significantly lower than FOCD of larvae reared at 18°C. Mean FOCD for larvae reared at 21°C also decreased significantly by day 14 but to a lesser degree than that seen at higher temperatures. There was no significant age related decrease in FOCD for larvae reared
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at 18°C or 19°C, and in fact larvae reared at 15°C showed a significant increase in mean F0CD by day 11 (Figure 5.3). The degree of variation between mean F0CD values at different temperatures was lower on day 14 than on day 6.

An analysis of F and C L as separate variables confirmed that temperature and age were significant variables, and that temperature and age interacted (ANOVA, p = 0.0001 and p = 0.0000) (Figure 5.3). There was no interaction between temperature and age (ANOVA, p = 0.5999) (Figure 5.3). However, there was a significant effect of temperature by itself (ANOVA, p = 0.0001). The day 6

larvae were significantly more likely to have swim bladder inflation than the day 11 larvae, and day 11 larvae were more likely to have tail flexion than day 6 larvae.

Figure 5.3 – Percentage of larval Pagrus auratus, reared at 5 different temperatures, which were identified as feeding, having swim bladder inflation or tail flexion, when aged: A) 6 days; B) 8 days; C) 11 days; and D) 14 days.

5.3.3 Growth rate and cell cycle

The growth rate and cell cycle data showed that larval growth rates increased significantly with temperature. The mean F0CD values were significantly higher at 15°C than at 18°C or 19°C, and the mean F0CD values were significantly lower at 15°C than at 18°C or 19°C. The mean F0CD values were also significantly higher at 18°C than at 19°C.
at 18°C or 15°C, and in fact larvae reared at 15°C showed a significant increase in mean FOCD by day 11 (Figure 5.5). The degree of variation between mean FOCD levels at different temperatures was less on day 14 than on day 6.

An analysis of S and G2M as separate components showed differing interactions with temperature and age. Variation in mean fraction of G2M cells was explained by a strong interaction between temperature and age (CID=53.84, df=11, p=0.0000) (Figure 5.5), but there was no effect of temperature by itself (CID=1.19, df=4, p=0.8797). On day 6, larvae reared at 21°C, 24°C and 27°C all had significantly higher mean fraction G2M than that of larvae reared at 15°C. But from day 8 onwards, there was little significant difference in mean G2M between temperatures. Mean G2M increased significantly between day 8 and 11 in larvae reared at 15°C and 18°C and dropped again by day 14. Mean G2M of larvae reared between 21°C and 27°C decreased with age, with most of this decrease apparent between days 6 and 8.

Variation in fraction of brain cells in S phase was predominantly explained by a three way interaction between temperature and age (Figure 5.4), and larval length (CID 40.10, df=11, p=0.0000) (Figure 5.6). Each factor also independently explained some of the variation in fraction S. The variation in the mean fraction S, when compared between temperatures within any single age group, closely followed the trends shown by FOCD. However, the magnitude of differences in fraction S between treatments was greater than that shown by mean FOCD. In particular, for all age groups in which a comparison was possible, mean fraction cells in S phase at 24°C was significantly greater than that at 27°C. As with FOCD, there was a decrease in the temperature at which highest fraction S occurs as larval age increased, such that by age 14 mean S was significantly higher at 18°C. The fraction of cells in S phase was generally equal to or higher than the fraction in G2M, excepting those larvae reared at 27°C, in which mean S was consistently lower.

### 5.3.3 Growth rate and cell cycle

Neither mean fraction S (ANOVA, F=1.59, p=0.2235, $r^2 = 0.0858$) nor mean FOCD (ANOVA, F=3.88, p=0.0650, $r^2 = 0.1859$) were significantly related to mean growth
Figure 5.4 – Comparison of cell cycle fractions of brain cells isolated from larval *Pagrus auratus* which had been reared at temperatures ranging from 15°C to 27°C and sampled at days: A) 6; B) 8; C) 11; and D) 14, after hatching. Error bars denote ±2SE.
Figure 5.5 – REML based predicted mean fraction of: A) cells dividing (FOCD); and B) G2M cells, in the brain tissue of *Pagrus auratus* larva reared at 5 different temperature levels and sampled on days 6, 8, 11 and 14 after hatching. Error bar represents the least significant difference (LSD).
Figure 5.6 – Comparison of *Pagrus auratus* larval size (standard length) with the fraction of larval brain cells in S, G2M or both (FOCD) for larvae reared at different temperatures (15°C, 18°C, 21°C, 24°C and 27°C).
rates of larvae reared at different temperatures and sampled on days 6, 8, 11 and 14 after hatching (Figure 5.7). Mean G2M held a weakly significant and positive relationship with mean GR (ANOVA, F=4.98, p=0.0393, $r^2=0.2267$) (Figure 5.7). Given some evidence for cell cycle arrest in G2M phase in larvae reared at 27°C and possibly 24°C (day 14) (Figure 5.4), the mean data points for the 27°C data and 24°C (day 14) were removed and the data re-analysed. Each of the mean cell fractions were then positively and significantly related to mean growth rate for each treatment/age sample (S: ANOVA, F=6.19, p=0.0270, $r^2=0.3221$; FOCD: ANOVA, F=7.57, p=0.016, $r^2=0.365$; G2M: ANOVA, F=5.26, p=0.03, $r^2=0.288$).

5.3.4 FAp and NTGI

Variation in the fraction of apoptotic cells was influenced by an interaction between larval age and rearing temperature (CID=30.90, df=11, p=0.0011) (Figure 5.8A). In addition, age but not temperature influenced apoptosis as an individual factor. On day 6, the mean FAp was significantly higher at 15°C than at 24°C or 27°C. On day 14, FAp was significantly higher at 18°C than at 15°C. Otherwise, there was no significant difference in FAp between temperatures on different days (Figure 5.8A). FAp also increased significantly between day 8 and 11 at 15°C, 24°C and 27°C and decreased significantly by day 14 at 24°C and 15°C. The apparent increase in mean FAp with age at 18°C and 21°C was not significant. Variation in mean NTGI for brain in larval *P. auratus* reared at different temperatures was driven by the interaction between age and temperature (but without any individual temperature effect) (CID=37.18, df=11, p=0.0001). There was no significant difference in mean NTGI between temperatures for any age group except 6 day old larvae, for which brain NTGI in larvae reared at 15°C and 18°C was significantly less than that at 24°C and 27°C (Figure 5.8B). With the exception of larvae reared at 15°C, mean NTGI was generally lower in older larvae. Mean FAp (ANOVA, $r^2=0.050$, F=0.900, p=0.356) and mean NTGI (ANOVA, $r^2=0.185$, F=3.88, p=0.065) were not significantly related to mean growth when all data points were included in analyses (Figure 5.9). When mean data points for 27°C and 24°C (Day 14) larvae were excluded (as in previous section for S, G2M and FOCD), the relationship between FAp and growth rate remained non-significant (ANOVA, F=0.422
Figure 5.7 - Comparison of mean growth rates with mean cell cycle fractions for brain cells isolated from Pagrus auratus larvae. Each point represents the means for a single sampling of larvae from a particular age and temperature treatment. Only fraction G2M had a significant and positive correlation with larval growth rate.
Figure 5.8 – A) The mean fraction of apoptotic cells recorded from the brain tissue of *Pagrus auratus* larvae reared at 5 temperatures and sampled on days 6, 8, 11, and 14 after hatching. These fractions were then subtracted from each larva’s FOCD to create B) an index of net tissue growth (NTGI) status. Vertical bars represent LSD.
Figure 5.9 – Comparison of mean growth rates with mean A) net tissue growth index (NTGI) and B) fraction apoptosis (FAp) for brain cells isolated from *Pagrus auratus* larvae. Each point represents the means for a single sampling of larvae from a particular age and temperature treatment.
p=0.5272, r^2=0.031). However, mean NTGI became significantly correlated to growth rate (ANOVA, F=6.0488, p=0.0287, r^2=0.3175).

### 5.4 DISCUSSION

Brain cell proliferation, as indicated by FOCD or S, did not appear to correspond to overall growth rate of snapper larvae reared at different temperatures, in contrast to the results presented in Chapter 4. Both FOCD and S were initially highest at 24°C, but the optimum temperature decreased as larvae grew older. Growth however, was generally optimal at 24°C and 27°C, regardless of age, as was developmental rate (indicated by fraction of larvae achieving flexion). The fraction of cells in G2M was the only proliferating fraction to show a significant and positive correlation with growth rate. This suggests that the fraction of cells in G2M is the more appropriate index of larval growth. However, the following section will discuss evidence which suggests that G2M increased at higher temperatures as a result of cell cycle arrest, and that the fraction of brain cells synthesising DNA (S phase) was significantly correlated to growth but became uncoupled at higher temperatures.

Mean G2M varied little with temperature in 8 or 14 day old larvae, and in contrast to fraction S, did not decrease between 24°C and 27°C on days 6 and 11. The significant decrease in S at 27°C (when compared to 24°C), and the absence of any concurrent decrease in G2M, may imply cell cycle arrest in G2M at this temperature. Fewer cells in S should imply fewer cells in G2M, providing cells do not arrest in G2M. Hence higher G2M levels at 27°C may have resulted from arrest rather than increased brain cell proliferation. Cell cycle arrest suggests that larval physiological condition was low, and indeed this notion is supported by evidence for reduced survivorship at higher temperatures as well as a lower incidence of swim bladder inflation at 27°C.

The negative relationship between temperature and survival was evident from the complete mortality of larvae reared at 30°C after only 48 hours, and of larvae reared at 27°C by the end of day 12, and the presence of larvae in only three of the four 24°C replicate tanks on day 14. Sampling for a separate but parallel experiment was conducted by another group of researchers (Fielder et al, in prep) who found that
snapper survival after 21 days was 6.1% at 15°C, 24.2% at 18°C, 3.4% at 21°C and 1.4% at 24°C, and zero at higher temperatures. The advent of higher larval mortality and increased growth rates at higher temperatures, as seen here with snapper larvae, has been commonly observed in numerous species of marine teleost (Pepin, 1991; Rombough, 1996), although not all (Leach and Houde, 1999). The reduced survival of snapper larvae at 27°C was associated with a very low level of swim bladder inflation. Lack of swim bladder inflation has been linked to increased mortality (Vonherbing et al., 1996; Goolish and Okutake, 1999) and reduced growth (Martinrobichaud and Peterson, 1998) due to dysfunctional buoyancy control and lowered prey capture efficiency (Vonherbing et al., 1996; Martinrobichaud and Peterson, 1998). The period in which swim bladder inflation is possible may be limited by the rate at which the gut develops (Marty et al., 1995) and at higher temperatures development was increased and the window for swim bladder inflation is likely to be smaller. There are two other factors that might explain why survival of larvae was so low at 27°C. Firstly, spawning of adult snapper was induced at 21°C, and it has been shown in cod larvae that the ability to grow and survive at a particular temperature is greater if gamete maturation in the parents occurred at that same temperature (Buckley et al., 1990). A second possibility was that larvae reared at 27°C did not have enough food to support their increased metabolic rate. While there is evidence that higher temperatures are associated with increased activity (Fukuhara, 1990) and higher feeding success (Morley and Batty, 1996), it has also been associated with increased metabolic rate (Pfeiler and Govoni, 1993) metabolic cost (Letcher and Bengtson, 1993) and required maintenance ration in larvae (Johnston and Mathias, 1996). Even though nearly all larvae at 27°C were feeding (and gut fullness was high), the inability to feed at night when temperature remained high might have been costly.

Physiological condition of larvae appeared to deteriorate at higher temperatures, particularly as larvae aged. This would explain why cell proliferation was initially high at 27°C and 24°C but decreased rapidly as larvae got older, with the rate of decrease greatest at 27°C, followed by 24°C then 21°C. At 18°C the rate of initial cell proliferation was comparatively low but did not drop or change significantly as larvae got older. A similar trend was evident in larvae reared at 15°C, although cell proliferation did increase on day 11. The pattern of cell proliferation at 15°C and 18°C
may be consistent with larvae which are not heat stressed and able to maintain a consistent level of tissue growth. An alternative hypothesis suggests that cell proliferation in the brain may slow once the brain achieves a certain developmental status. Larvae at higher temperatures would grow and develop more rapidly and reach this status sooner, and therefore the “drop-off” in cell proliferation in the brain would be achieved sooner. However, given evidence of G2M arrest, increased mortality and low swim bladder inflation at 27°C, it would seem much more likely that this decrease in cell proliferation was directly due to the sublethal effects of higher temperatures upon larval physiology.

The evidence for reduced physiological condition at 27°C contrasted with the growth data, which showed growth rate was highest at 27°C then 24°C and lowest at 15°C. Clearly somatic growth as measured by length (mm/d), and brain growth (fraction S) did not react in the same way to temperature. When data means for larvae which were deemed to be clearly stressed (i.e. those for 27°C and the last 24°C sample) were dropped from the analysis, then the fraction S became significantly and positively correlated with mean growth rate. This suggests that the relationship between brain cell proliferation and length based growth rate, became uncoupled at higher temperatures. It would be interesting to determine if cell proliferation in larval muscle tissue (see Theilacker and Shen, 2001) correlates more closely with length (somatic) based growth variations, under different temperature regimes, as increasing length should require an increase in muscle mass. A measure of larval mass would also determine if the increases in length at higher temperatures were associated with an increase in mass when compared to larvae reared at lower temperatures. It is possible that larvae at higher temperatures were longer but “skinnier”, than those reared at lower temperatures and that the relationship reflected body shape rather than mass (and growth).

The results suggest that growth in brain may have been more closely related to the physiological condition and survival likelihood than was recent growth as estimated by length. Given the importance of the central nervous system to the integrated functioning of motor coordination, development, behaviour and hormonal regulation, any adverse effect on brain development may adversely affect the whole larvae, and potentially have consequences for survival.
The NTGI and fraction of apoptotic cells were briefly analysed to provide evidence to further support or refute the conclusions gained from the previous chapter regarding the applicability and relevance of these indices to growth assessment in larval fish. In the current experiment, there were only a few significant differences in mean FAp attributable to temperature. Similarly, subtraction of FAp from FOCD to determine NTGI reduced the level of variation in growth attributable to temperature. Significant differences were only evident in 6 day old larvae, suggesting that overall growth in brain was only sensitive to temperature in younger post-hatch larvae. When mean NTGI was compared to mean growth rate (mm/d), there was no significant correlation. A significant relationship was only evident between these parameters if 27°C and 24°C data were excluded, further supporting the idea that growth in brain becomes uncoupled from length-based growth estimates at higher temperatures. However, the correlation between mean NTGI and mean growth rate (with higher temperature data excluded) was slightly less than that for fraction of cells in S phase or FOCD with growth rate. Consequently, assessing mean NTGI did not aid in the assessment of overall larval growth, even if it may have been a better indicator of mass gain in larval brain tissue. Potential reasons why this was so are discussed in Chapter 4.

The close correlation between fraction S and growth for larvae reared at different salinities (Chapter 4), suggests that the current lack of correlation between these two parameters specifically relates to a differential effect of temperature upon the relationship between growth (as measured by length) and brain cell proliferation. Just as RNA/DNA can dissociate from the morphological growth at low temperatures (Goolish et al., 1984; Jurss et al., 1987), it would appear that the fraction of S phase cells also becomes uncoupled from morphological growth, in this case at higher temperatures. However, it would also appear that higher temperatures uncouple growth (as measured by length) from physiological condition and survival capacity. The fraction of brain cells synthesising DNA may relate more closely to the physiological condition and survival probability than does recent growth rates as calculated from standard length. Future analyses of temperature effects on cell proliferation may be better served by using a volume- or mass-based morphological growth measure (possibly using parallel sampling), and more detailed recording of survival data.
6.1 INTRODUCTION

The physiology of most organisms is adapted to changes in environmental conditions that are associated with the cycling day/night of light and dark (Hastings and Schweger, 1975; Fatou, 1969). This suggests that behavioral or metabolically-based growth indices might vary in a diel basis. Evidence has already been found for circadian variation in RNA/DNA content of larval fish (Delo and Oko, 1991; Bover and Het, 1999). However, the specific response of larval cell proliferation requires further investigation (Sun et al., 2000). Determining whether variation is present, whether it is evident at different stages, what triggers such changes, and how these responses affect development, will show the immense potential interplay of cell proliferation rate and larval survival and condition of the larvae.

CHAPTER 6

DIEL VARIATION IN CELL PROLIFERATION OF BRAIN CELLS OF LARVAL TELEOSTS

6.1.1 Circadian rhythms and diel variation in the physiology of living systems

Circadian rhythms are biological clocks that govern daily cycles in metabolism and behavioral patterns. They are observed in both plants and animals and involve changes in their environment that occur at a scale of minutes and hours. Any repeated recurring cycle in metabolism and behavioral patterns, especially in organisms, are known as circadian rhythms (Hastings and Schweger, 1975). Circadian rhythms are rhythmically produced and synchronized and are generally tolerant and able to respond to changes in environmental conditions, such as light, food, and temperature but disappear when they are held constant (Cpackary, 1980). As a chain, circadian rhythms are driven by specialized biological clocks that are biological clocks, dedicated to coordinating the metabolism, physiology, and behavior of each species to the daily changes in its environment.

In most fish, the pineal gland acts as the circadian pacemaker, timing repeating
6.1 INTRODUCTION

The physiology of most organisms are adapted to changes in environmental parameters that are associated with the cycling diel phases of light and dark (Hastings and Schweiger, 1975; Falcon, 1999). This suggests that biochemical or metabolic-based growth indices might also vary on a diel basis. Evidence has already been found for circadian variation in RNA/DNA contents of larval fish (Mugiya and Oka, 1991; Rooker and Holt, 1996), but the diel response of larval cell proliferation requires further investigation (Bromhead et al., 2000). Determining whether variation is present, whether it is cyclical or “random”, what triggers such changes, and how these responses might vary with developmental status, will allow for more accurate interpretation of cell proliferation data and how such data relate to the growth and condition of the teleost larva.

6.1.1 Circadian rhythms and diel variation in the physiology of teleosts

The cyclic recurrence of day and night profoundly influences nearly every aspect of all living systems, from whole ecosystems to individual organisms (Hastings and Schweiger, 1975; Brady, 1979; Falcon, 1999). Many organisms have evolved so as to be able to anticipate and utilise changes in their environment that occur as a result of day and night, and exhibit recurring cycles in metabolism and behaviour which are known as circadian rhythms (Hastings and Schweiger, 1975). Truly endogenous circadian rhythms are internally produced and controlled (i.e., genetically inherent) and will persist at least for some time under constant environmental conditions, while exogenous rhythms occur as a response to external changes in environmental conditions such as light, food and temperature but disappear when these are held constant (Cloudsley-Thompson, 1980; Ali et al., 1991; Dunlap et al., 1996). Circadian rhythms are driven by specialised tissues or organs that act as biological clocks, dedicated to coordinating the metabolism, physiology and behaviour of each species to the daily changes in its environment.

In most fish, the pineal gland acts as the circadian pacemaker tissue, converting
environmental stimuli such as light levels, into nervous and hormonal signals. In doing so, they directly influence the physiology and behaviour of fish (Ali, 1991; Falcon, 1999). Hormones shown to exhibit a circadian rhythmicity in their production and release in fishes include melatonin (Zachmann et al., 1991; Bolliet et al., 1996; Mayer et al., 1998), plasma growth hormone (GH) (Leatherland et al., 1974; Bates et al., 1989), insulin and cortisol (Cerdareverter et al., 1998). The pineal is directly responsible for the production and release of melatonin, which acts as an internal zeitgeber to synchronise physiological functions and behaviours (Begay et al., 1994), but whose production is directly inhibited by light filtering through the cranial wall (Ali, 1991; Falcon, 1999). Hence, many fish species (and vertebrates in general) display high levels of melatonin at night and low levels during the day (Falcon et al., 1994; Bolliet et al., 1996; Gaildrat et al., 1998; Mayer et al., 1998; Falcon, 1999). A circadian rhythm in melatonin production has also been demonstrated in larvae of the sailfin molly, *Poecilia velifera* (Okimoto and Stetson, 1999). Melatonin and growth hormone are directly associated with the promotion of growth. Melatonin plays a role in the circadian production of other hormones, such as growth hormone (GH), whose synergistic action with other hormones promotes growth (Ball, 1969), regulates protein synthesis and stimulates appetite (Houlihan, 1991).

The hypothesis that cell proliferation in larval fish might vary on a diel basis is based on both direct evidence from preliminary investigations using flow cytometry (Bromhead et al., 2000) and mitosis promoting factor (MPF) assays (Westerman, personal communication), and indirect evidence for growth rhythms (Mugiya and Oka, 1991; Rooker and Holt, 1996; Okimoto and Stetson, 1999) as well as abundant evidence for the occurrence of such rhythms in other phyla, spanning single-celled organisms (Vandolah et al., 1995; Van Dolah and Leighfield, 1999) right through to whole tissues in higher vertebrates such as mice and rats (Rensing, 1976; Scheving, 1981; Scheving, 1984; Ohdo et al., 1997; Scheving and Gardner, 1998; Blumenthal et al., 1999; Thomson et al., 1999), humans, and adult teleosts (Chiu et al., 1995; Kwan et al., 1996).
6.1.2 Implications of diel variation for assessment of condition and growth.

Evidence for circadian fluctuations in physiological and growth-associated parameters in fish suggests that cell proliferation measures of instantaneous growth in fish larvae might also vary on a diel basis. Previous studies of condition and growth in fish larvae have shown the potential for circadian rhythms to confound the interpretation of data generated by biochemical or cell based indices. Rooker and Holt (1996) demonstrated a repeating daily cycle of RNA:DNA in white muscle tissue isolated from larval red drum (Sciaenops ocellatus), with peak values occurring between 1200 and 1600 hr, and marked decline during nocturnal periods. This rhythm was found to occur regardless of whether water temperature fluctuated on a diel basis or was held constant. Mugiya and Oka (1991) also found evidence for diel variation in RNA levels in their study of condition in rainbow trout. However, the occurrence of such variability appears likely to be species-specific given a lack of evidence for diel variation of RNA:DNA in other species (Buckley et al., 1999). Bromhead et al. (2000) have demonstrated that cell division rates in brain tissue of Galaxias olidus larvae captured from the wild also varied significantly over a single 24h period. In this case, variations were significantly linked to feeding and gut fullness, but it was not determined if they were circadian in nature. Chiu et al. (1995) demonstrated a clear endogenous rhythm in cell division of pre-neural retinal cells in adult of the teleost, Haplochromis burtoni, supporting the need to investigate the possibility of rhythmic cell division, particularly in neural tissues. However, in contrast to these results, a study by Theilacker and Shen (2001) did not demonstrate any diel variation in muscle cell proliferation for larval walleye pollock (Theragra chalcogramma) sampled every 8 hours in the field.

6.1.3 Aims

The above discussion has highlighted the fact that relatively little is known about the diel nature of cell proliferation in fish larvae. The current investigation uses two experiments to test a number of hypotheses regarding the occurrence and nature of diel variation in brain cell proliferation in larval fish. As such, the main aims are to:
1) Determine if there is any diel variation in brain cell proliferation, and if so, is it circadian (cyclical) or random in nature;
2) Determine if patterns in diel variation of cell proliferation are species- or stage-specific, by conducting diel sampling at different developmental stages and in unrelated species;
3) Determine if nutrition (feeding) or light might be linked to diel variation in cell proliferation by comparing variation in larvae exposed to extended photoperiod or shifted feeding time with that of control larvae; and
4) Determine if starvation dampens any diel variation, and if so, are starved larvae more easily distinguished from fed at time points when the fed cycle is peaking. In other words, does sampling time affect the ability of an index to distinguish between fed and starving larvae.

Overall, the experiments aim to determine what implications diel variation in cell proliferation may have for the assessment of larval condition and growth using flow cytometric cell cycle analysis.

6.2 MATERIALS AND METHODS

This chapter describes two experiments, each using a different species of fish. The first experiment describes the effect of prolonged photoperiod and changed feeding time upon diel variation of cell proliferation in the larval brain of a marine teleost, *Pagrus auratus*. This took place at the Port Stephens Research Centre, NSW. The second experiment investigates the effect of starvation upon diel variation in brain cell proliferation in larvae of a freshwater teleost, golden perch (*Macquaria ambigua*). This was conducted in the freshwater aquarium facility at the Department of Botany and Zoology, Australian National University in Canberra, ACT.
6.2.1 Experiment 1 – *Pagrus auratus*: effects of photoperiod and feed time

6.2.1.1 Spawning, Stocking and Design

The facilities and larvae used in this analysis were generously supplied by Stewart Fielder and colleagues at the Port Stephens Research Centre (NSW Fisheries). Methods used to achieve adult spawning, egg collection, egg and larval incubation are described in Chapter 2. The original design intended conducting diel sampling at three developmental points, pre-feeding (yolk-sac/oil), mixed feeding (yolk/oil and prey), and prey-dependant feeding. Approximately 400 larvae were transferred to 9 (40 L) tanks on the day of hatching using gradual acclimation in floating buckets and 10% water exchanges every 5 minutes. Tank design (Figure 6.1) was different to that described in Chapters 4 and 5, due to unavailability of previously used facilities. Water exchange was by constant flow through (28ml/min) from a 2000 litre supply tank filled with dechlorinated filtered seawater, approximating a 100% water exchange each day in each tank. Over the next 11 days, three diel samplings were conducted, during which larvae were sampled every 3 hours over 24 hr periods. Yolk-sac larvae were sampled during the period spanning 0800 (day 1) to 0500 (day 2). Mixed feeding larvae were sampled from 1100 hr (day 4) until 0800 hr (day 5). Feeding larvae were sampled over 3 days, from 0800 (day 8) until 0800 hr (day 11). Unfortunately, mass mortality of larvae occurred on days 3 and 4, most likely due to contaminated water supply lines. Consequently the mixed feeding sample (day 4-5) had to be taken from the original larval hatching tank. The feeding larvae (days 8-11) were sampled after the 9 tanks were stripped down, cleaned, supply lines replaced, and each restocked with 400 ± 10 larvae from the hatching tank (on day 5). Consequently, the experiment is not continuous.

Three treatments were applied to the 9 tanks from day 5 onwards, with three replicate tanks per treatment. The first treatment subjected larvae to an extended photoperiod of 16L:8D and a 0800 feeding time. The second treatment was a control, using 0800 feeding and normal 12L:12D photoperiod. The third treatment delayed feeding until 1300 hr, with a 12L:12D light cycle. Larvae were acclimatised to these treatments until day 8, by which time increasing mortalities were again noted. Due to decreasing
Figure 6.1 – Tank design used for experiment assessing effect of photo-period length and feeding time upon the diel variability of cell proliferation in brains of larval snapper *Pagrus auratus*. Tanks were 40 litre clear glass surrounded by black plastic and with fitted blackout lid, situated on white foam base, with a 400um outflow filter protecting the outflow pipe. The light source was fluorescent roof lighting (fixed) which was situated above and to side of tanks. Airstone and water in-let were situated towards light to prevent photo-tactic congregation of rotifers and larvae.
numbers, the acclimation time was reduced and diel sampling started at 0800 on day 8 and continued every three hours until 0800 on day 11. Three larvae were sampled from each tank at each sample time. Not all tanks had larvae surviving by day 11. Larvae were checked for disease, and water quality was closely monitored, with neither considered a likely causative agent in larval mortality.

6.2.1.2 Feeding, maintenance and sampling

Larvae were fed using the methods described in Chapter 2 (Section 2.2.1.3). The fixed light source for the tanks was at the opposite end to the outflow and rotifers exhibited photo-tactic congregation at this end of the tanks. Airstone position was adjusted to disperse rotifers throughout the water column but this was limited in its success, and the phototactic response of the rotifers may have nullified some of the effect of the delayed feeding treatment, as that relied on water flow to remove rotifers.

Water quality was monitored twice a day. Temperature was maintained at 22.05 ± 0.12°C, salinity at 35.0 – 35.6 ppt, pH at 7.7 – 7.9, and dissolved oxygen at 6.9 – 7.5 ppm. Due to insufficient storage space, larvae were initially frozen in tank water at 20°C and transported back to the university on dry ice and stored at -75°C.

6.2.1.3 Tissue Preparation

Brain tissue was excised, prepared and analysed from each larva sampled from the trial, as described in Chapter 2 (Section 2.3). However, after brain was removed, the gut was dissected and the number of ingested rotifers counted as an index of gut fullness. The width of oil droplet was measured to determine the diel usage of endogenous oil reserves and any possible relationship to diel variation in cell proliferation.

6.2.2 Experiment 2 – Macquaria ambigua: effect of starvation

6.2.2.1 Spawning, Stocking and Design

The supply, transport and initial acclimation of M.ambigua larvae used in this
experiment are described in Section 2.2.2.1, the experimental tank design and set-up described in Section 2.2.2.2, and the procedures used in maintenance and feeding of larvae explained in Section 2.2.2.3. After larvae were stocked into the 8 tanks on day 7, they were allowed 48 hours to acclimatise. A sample of three larvae was then collected from each tank, commencing on day 9 at 0800 hr, and repeated every three hours until 0800 hr on day 12. The experiment utilised a random block design, with four constant fed tanks acting as control tanks, with *Artemia* feed densities maintained at 3/mL for the entire experiment, days 9 to 12. The other four tanks were fed on day 9, then fasted days 10 to 12. Starting at 1200 on day 23, another diel sampling was commenced, with samples taken every 4 hours. These samples were only taken from the 4 tanks that had been constant fed. This experiment examined whether diel variability changed as larvae developed.

6.2.2.2 Sampling and tissue analysis

Larvae were sampled using a siphon into a bucket, and then pipetted into a cryo-tube which was sealed and snap frozen in liquid nitrogen. In total, 648 larvae were sampled for analysis. The procedures used in dissection, dissociation and analysis of larval brain cells are described in Chapter 2 (Section 2.3.3).

6.2.3 Experiments 1 & 2: Data collection, analysis and statistics

The flow cytometric settings for collection of brain cell data, as well as methods describing subsequent analysis of this data, are described in Chapter 2 (Section 2.3). The current experiments analysed 4 separate response variates (fraction of cells dividing, FOCD; fraction S, fraction G2M, and standard length) and determined whether time of day, treatment level, age or a range of developmental and morphological variables explained variation in each. Apoptosis and net tissue growth were not considered as part of these analyses, as they have been shown not to correlate any more closely to growth rate than do FOCD or S phase fractions (see Chapters 4 and 5). Restricted maximum likelihood (REML) analyses of logit transformed cell fraction data have been used to determine the most appropriate model for each response variate. A more detailed explanation for this statistical approach is provided in chapter 2.
(Section 2.3.5). There was some between sample variation in cell numbers (i.e. cells analysed per larva). For both experiments, there was found to be an effect of sample size (total cell number per sample) upon modelled estimates of S phase that was independent of larval size. The snapper analysis used the original unadjusted data, and incorporated “total cell number” as the first term in all models used. This ensured that variation in S phase estimates that was caused by sample size was not attributed to any other factor. Golden perch samples showed a much tighter linear relationship between sample size and estimated S phase. Linear regression based adjustment was used to standardise samples to 7000 cells per sample. This did not result in negative values, and was the preferable method given that, graphically, it presents a more meaningful result.

For both analyses, a new factor “Sampling Point” was created as the product of age with time of day. This allowed treatment effects to be assessed by the change in deviation attributable to treatment after time of day or age based variation was accounted for, e.g.:

Model: Total cells + Sampling Point + Treatment  
Sub-model: Total cells + Sampling Point

Those factors which were related to the variation attributable to “Sampling point” (i.e. those factors which caused diel variation in cell fractions) will have been confounded with sampling point and therefore could be assessed by dropping this term from the model.

6.3 RESULTS

6.3.1 Experiment 1 – *Pagrus auratus*: effects of photoperiod and feed time

The following results are summarised in Tables 6.1 and 6.2, which detail the full models, the effects of the factors within these models (indicated by the change in deviance, degrees of freedom, and the associated p values). The “slopes” of any significant interactions between response variates and explanatory factors are also occasionally presented in the main text.
Table 6.1 – Statistical summary of the effects of Time (of day), Oil (droplet size), standard length (SL) and the number of rotifers ingested, upon variation in both morphological (SL, oil droplet size) and cell proliferation parameters (FOCD, S, G2M) for <i>Pagrus auratus</i> larvae aged 1 and 4 days old and sampled over 24 hours. The “dropped factor” column lists those factors which were dropped from the full model to create a sub-model. The change in deviance (CID) between the full and sub-models approximates a chi-square distribution and can be used to assess the significance (p value) of the factor in explaining variation in the response variate being assessed. Asterisks (*) denote factors which explain a significant amount of variation in the response variate (at p<.05).

<table>
<thead>
<tr>
<th>Age</th>
<th>Response variate</th>
<th>Sub-Model</th>
<th>Dropped factor</th>
<th>CID</th>
<th>DF</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 SL Oil FOCD</td>
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<td>Time</td>
<td>51.95</td>
<td>7</td>
<td>0.0000*</td>
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<tr>
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<td>7</td>
<td>0.5286</td>
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<td></td>
</tr>
<tr>
<td>1 Total</td>
<td>FOCD Time</td>
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<td>7</td>
<td>0.9273</td>
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<td></td>
</tr>
<tr>
<td>1 Total</td>
<td>Total Oil</td>
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<td>1</td>
<td>0.4625</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Total</td>
<td>Total SL</td>
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<td>0.2058</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Total+Time+Oil</td>
<td>Time x Oil</td>
<td>16.86</td>
<td>7</td>
<td>0.0186*</td>
<td></td>
<td></td>
</tr>
<tr>
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Table 6.2 – Statistical summary of the effects of Time (of day), age, sampling point (Samp.) swim bladder (SB), oil (droplet size), standard length (SL), number of rotifers ingested and treatment (Treat), upon variation in both morphological (SL, rotifers) and cell proliferation parameters (FOCD, S, G2M) for Pagrus auratus larvae aged 8 to 11 days old and sampled every three hours. Term are change in deviance (CID), degrees of freedom (DF), and p value of CID (p). Statistics are explained in Table 6.1 (prev. pg)

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6.3.1.1 Morphological growth and development

The mean length of snapper larvae sampled on day 1 after hatching was significantly lower between 1700 and 2300 hr than at other time points during the 24 hour sampling (p=0.0000) (Figure 6.2A). This may have been due to a sampling bias (see discussion). However, time of day did not explain variation in oil droplet diameter, on day 1, nor variation in length or oil droplet diameter on day 4 (Table 6.1, Figure 6.2B). The number of ingested rotifers per larvae peaked at 1700hr on day 4 (Figure 6.2C), however not all larvae had commenced feeding at this stage.

All larvae had exhausted oil droplet reserves by day 8. After variation due to sampling time was accounted for, larvae reared under control (mean SL = 4.214 mm) and delayed feeding (SL = 4.337 mm) regimes were significantly longer than larvae reared under the extended photoperiod treatment (mean=4.084, p=0.0000; Figure 6.3A). Mean larval length increased significantly with age (p=0.0036) (Figure 6.3A, Table 6.2). The percentage of larvae with swim bladder inflation at each sampling fluctuated markedly with levels up to 80% at some time points and as low as 0% at others (Figure 6.3B), particularly between 1400hrs (day 9) and 2000 hr (day 10). Swim bladder inflation was lower in larvae reared with extended photoperiods prior to day 10 (CID=7.2, df=2, p=0.0273).

The mean number of rotifers ingested per larvae increased during the day and decreased at night (p=0.0000), and overall, was significantly higher in delayed fed larvae (p=0.0417). However, when larval size (length) is included in the model (Table 6.2), there is no longer any difference between rotifer content in larvae reared under different treatments (p=0.1716) (Figure 6.4).

6.3.1.2 Survival

No delayed fed larvae survived past 1700 hr on day 9, while there were less than 10 extended photoperiod larvae remaining at the end of the experiment, and no larvae remained in the control tanks after the second last sampling.
Figure 6.2 – Mean standard length (SL), oil droplet diameter and rotifer gut content of Pagrus auratus larvae sampled every three hours from 0800 hrs on days 1 (A) and 4 (B and C) after hatching. Day one larvae were sampled from 9 separate rearing tanks, and had not started feeding on rotifers. Day 4 larvae were sampled from the supply incubation tank, and many of these had started feeding on rotifers (C). Error bars are ±2SE
Figure 6.3 – Mean standard length (A) and percentage of larvae with swim bladder inflation (B) for Pagrus auratus larvae sampled every three hours from 1100 hr on day 8 after hatching to 1100 hr on day 11. Larvae were reared under three different treatments, these being a control treatment of 12:12 light:dark cycle and feeding at 0800 hr, an extended photoperiod treatment (16L:8D; 0800 hr feed) and a delayed feed treatment (12L:12D; 1300 hr feed). Error bars in A represent ±2SE.
Figure 6.4 - Mean number of ingested rotifers per larva, in larval Pagrus auratus reared under three treatments and sampled every three hours from 1100 hr on day 8 after hatching to 1100 hr on day 11.
### 6.3.1.3 Brain cell cycle fractions

On day 1 after hatching, mean FOCD did not vary with either time of day or oil droplet diameter (Table 6.1) but was explained partially by an interaction between these (p=0.0186). S phase varies by both time of day (p=0.0000) and the interaction between time of day and oil droplet size (p=0.0319) (Figure 6.5A). Time of day also explained variation in the fraction of cells in G2M (p=0.0000) (Figure 6.5A). For 4-day-old larvae (Figure 6.5B), FOCD did not vary according to time of day (p=0.7872), nor was it related to any other parameter measured, including oil or rotifer content (Table 6.1). There was no relationship between any of these explanatory variables with the fraction of cells in S phase. There was a weak effect of time of day upon the fraction of cells in G2M phase (p=0.0461). Mean G2M was higher at 2300 than at 1700 or 0200 and 0500 hr.

For snapper larvae sampled every three hours from day 8 to day 11 (Figures 6.6, actual data and: 6.7, predicted data), there was no effect of total cell number upon the end-estimates of FOCD (p=0.8428) (Table 6.2). FOCD did vary with sampling point over this period (p=0.0000), and once this variation due to sampling point was taken into account, there was no significant effect of treatment upon FOCD (p=0.1670) (Figure 6.7a). Significant variation in FOCD was explained by rotifer content once sampling was removed from the model or rotifers was included first (p=0.001), with mean FOCD increasing with increasing larval gut content (slope=0.01182) (Table 6.2). When considered by sampling point, there were periods of significant or close to significant decreases (followed by increases) early on day 9, from 2300 on day 9, from 0500 on day 10 and possibly from 2300 on day 11 in control larvae. Other factors such as light (CID=0.0), swim bladder inflation and larval length did not further explain variation in FOCD, however the age of larvae does (p=0.0194), with predicted mean FOCD significantly higher on day 9 and day 11 than on day 8 (Figure 6.7).

S phase estimates increased slightly as total cell number in the sample increased (slope=0.00102, p=0.0000)(Table 6.2). Once variation due to cell number was accounted for, there was a highly significant effect of sampling point on the mean fraction of cells in S phase (p=0.0000)(Figure 6.7B). Furthermore, the level of
Figure 6.5 – A comparison of the diel variation in the mean fraction of dividing brain cells (FOCD) with fraction of cells in S or G2M phases for *Pagrus auratus* larvae aged 1 day (A) and 4 days (B). Larvae were sampled every three hours starting from 0800 hrs. Errors bars represent ±2SE.
Figure 6.6 – Mean fraction of brain cells dividing (FOCD) in larval *Pagrus auratus* reared under three treatments and sampled every three hours from 1100 hr on day 8 after hatching to 1100 hr on day 11. Error bars in B denote ±2SE.
Figure 6.7 – Comparison of the predicted fraction of brain cells dividing (FOCD; A) with fraction of cells in S phase (B) and the fraction of cells in G2M phase (C) for larval *Pagrus auratus* reared under three different treatment regimes and sampled every three hours from 1100hr on day 8 after hatching to 1100hr on day 11. Error bar signifies the least significant difference (LSD). Values $\pm$ LSD are significantly different.
cells in S varied depending on the treatment (p=0.0000) (Table 6.2). Larvae reared with an extended photoperiod had significantly lower mean S than did those in control or delayed feeding treatments. In addition, the effect of treatments varied with sampling point (Treatment x sampling pt; p=0.044).

The fraction of cells in S phase increased significantly as the number of rotifers digested increased (slope of “Fraction S” by “Rotifer Number” = 0.05486; p=0.0000). Furthermore, larvae with swim bladder inflation had a higher mean fraction of cells in S than those which were lacking swim bladders (p=0.0085). The fraction of cells in S phase decreased as larvae aged (p=0.0000) but there was no significant variation in S attributable to time of day (p=0.3983) (Figure 6.7). The fraction of cells in S was higher in larger larvae (slope of “Fraction S” by “Length” = 0.6347), particularly after variation due to sampling point was included (p=0.0001) (Table 6.2).

Once variation due to, firstly, a slight negative relationship between G2M and total cell number (p=0.0000, slope=-0.0003) and secondly, sampling point (p=0.0000) were included in the model, there was no overall effect of treatment upon G2M (p=0.0983) (Figure 6.7). Neither swim bladder inflation, larval age, nor number of rotifers ingested were related to G2M (Table 6.2), however rotifer content was weakly related to G2M within sampling points (i.e. at a particular point in time, G2M was higher in larvae with fewer rotifers) (p=0.025, slope =-0.01003). There was an effect of time of day upon G2M (p=0.0000), with mean G2M significantly lower at 1100 hr. However, this is most likely due to a large single decrease in G2M at 1100 hr on day 9. There is little evidence for decreased G2M at 1100 hr on any other day (Figure 6.7C).

6.3.2 Experiment 2 – *Macquaria ambigu*a: effect of starvation

This experiment comprised two stages. The first, spanning days 9 to 12 after hatching, examined the effect of starvation upon diel variation in larval brain cell proliferation, and the second examined developmental effects by diel sampling 23-day-old, post-flexion larvae. Tables 6.3 and 6.4 contain summaries describing the significance of different factors in explaining variation in the response variates. These tables should be referred to throughout the results section, which in the text will only state p values.
Table 6.3 – Statistical summary of the effects of Time (of day), compressed cycle time (CCT), age, sampling point (Samp.) swim bladder (SB), oil (droplet size), standard length (SL), gut fullness (GF) and treatment (Treat), upon variation in both morphological (SL, rotifers) and cell proliferation parameters (FOCD, S, G2M) for *Macquaria ambigua* larvae aged 9 to 12 days old and sampled every three hours.

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<td>Samp x Treat</td>
<td>24.77</td>
<td>14</td>
<td>0.0369*</td>
</tr>
<tr>
<td>-</td>
<td></td>
<td></td>
<td>Samp x GF</td>
<td>27.11</td>
<td>23</td>
<td>0.2513</td>
</tr>
<tr>
<td>-</td>
<td></td>
<td></td>
<td>SB</td>
<td>9.678</td>
<td>1</td>
<td>0.0018*</td>
</tr>
<tr>
<td>-</td>
<td></td>
<td></td>
<td>Age</td>
<td>9.147</td>
<td>3</td>
<td>0.0274*</td>
</tr>
<tr>
<td>-</td>
<td></td>
<td></td>
<td>Time</td>
<td>11.07</td>
<td>7</td>
<td>0.1356</td>
</tr>
<tr>
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<td></td>
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<td>1</td>
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<tr>
<td>(fed)</td>
<td></td>
<td></td>
<td>CCT</td>
<td>10.65</td>
<td>4</td>
<td>0.0307*</td>
</tr>
<tr>
<td>(starved)</td>
<td></td>
<td></td>
<td>CCT</td>
<td>2.664</td>
<td>4</td>
<td>0.1026</td>
</tr>
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<td>23</td>
<td>0.0000*</td>
</tr>
<tr>
<td>-</td>
<td></td>
<td></td>
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<td>1</td>
<td>0.3928</td>
</tr>
<tr>
<td>-</td>
<td></td>
<td></td>
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<td>14</td>
<td>0.0168*</td>
</tr>
<tr>
<td>-</td>
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<td></td>
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</tr>
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<td>-</td>
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<td></td>
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<td>1</td>
<td>0.0717</td>
</tr>
<tr>
<td>-</td>
<td></td>
<td></td>
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<td>3</td>
<td>0.0008*</td>
</tr>
<tr>
<td>-</td>
<td></td>
<td></td>
<td>Time</td>
<td>5.141</td>
<td>7</td>
<td>0.6428</td>
</tr>
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<td>-</td>
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<td></td>
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<td>0.0585</td>
</tr>
<tr>
<td>(fed)</td>
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<td></td>
<td>CCT</td>
<td>27.65</td>
<td>4</td>
<td>0.0000*</td>
</tr>
<tr>
<td>(starved)</td>
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<td></td>
<td>CCT</td>
<td>7.504</td>
<td>4</td>
<td>0.1115</td>
</tr>
<tr>
<td>CCT + GF +</td>
<td></td>
<td></td>
<td>CTC x GF</td>
<td>21.69</td>
<td>4</td>
<td>0.0002*</td>
</tr>
</tbody>
</table>
Table 6.4 – Statistical summary of the effects of Time (of day), gut fullness (GF) and light upon variation in both standard length (SL) and cell proliferation parameters (FOCD, S, G2M) for *Macquaria ambigu*a larvae aged 23 days old and sampled every four hours.

<table>
<thead>
<tr>
<th>Response variate</th>
<th>Model</th>
<th>Final Component</th>
<th>CID</th>
<th>DF</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL</td>
<td>-</td>
<td>Time</td>
<td>32.40</td>
<td>6</td>
<td>0.0000*</td>
</tr>
<tr>
<td>FOCD</td>
<td>-</td>
<td>Time</td>
<td>10.5</td>
<td>6</td>
<td>0.1051</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GF</td>
<td>6.4</td>
<td>1</td>
<td>0.0114*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Light</td>
<td>6.5</td>
<td>1</td>
<td>0.0107*</td>
</tr>
<tr>
<td>S</td>
<td>-</td>
<td>Time</td>
<td>8.6</td>
<td>6</td>
<td>0.1974</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GF</td>
<td>4.9</td>
<td>1</td>
<td>0.0268*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Light</td>
<td>5.0</td>
<td>1</td>
<td>0.0253*</td>
</tr>
<tr>
<td>G2M</td>
<td>-</td>
<td>Time</td>
<td>13.5</td>
<td>6</td>
<td>0.0357</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GF</td>
<td>7.0</td>
<td>1</td>
<td>0.0081*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Light</td>
<td>7.1</td>
<td>1</td>
<td>0.0077*</td>
</tr>
</tbody>
</table>

For larvae aged 9 to 12 days, sampled every three hours, variation in the fraction of dividing fish cells (FOCD) was not explained by sampling point, time of day, age, gut fullness or by interaction (Table 6.3). There was no repeating cell cycle in starved or fed larvae (Figure 6.10). However, the acceleration of the data suggested the presence of a shorter compressed cycle, approximating 13 hours (Figure 6.10). Compressed cycle time point (CIP) explained variation in FOCD (p = 0.0798) of fed but not starved (p = 0.2779) larvae. Variation in FOCD was also explained by using bladder inflation with FOCD significantly higher in larvae with swim bladder inflation than those without (p = 0.0079).
6.3.2.1 Morphological and developmental effects: Days 9-12

From days 9 to 12 (Figure 6.8A), variation in larval length was explained by sampling point (p=0.0000) but not by treatment (p=0.2201). The percentage of larvae with swim bladder inflation in each sampling varied greatly between samples, ranging between 20 and 70% up to day 12 (Figure 6.8b). The percentage of larvae achieving flexion started increasing at the end of day 9, and in constant fed fish reached 100% by day 12 (Figure 6.8c). This level was not achieved in the starved group until day 14 (unpublished data).

Variation in oil droplet volume was explained by sampling point (p=0.0000) and by treatment (p=0.0028) with volume larger in starved larvae (Figure 6.9). The amount of variation explained by treatment depended on sampling point (p=0.0014). In both starved and fed groups, oil volume decreased with age (p=0.0000). Variation in oil volume was also explained partially by an interaction between sampling point and gut fullness (p=0.0065).

Gut fullness decreased to zero in starved larvae by day 12. Feeding over diel periods was not rhythmic in nature, and there was some evidence for feeding or retention of gut contents at night as well as during the day. Larvae were observed to sink to bottom at night and brine shrimp also did this. Larvae with swim bladder inflation tended to have smaller oil droplet volumes (mean = 0.1065 mm\(^3\)) than those lacking swim bladder inflation (mean=0.1127 mm\(^3\), p=0.0010).

6.3.2.2 Brain cell proliferation: Days 9-12

For larvae aged 9 to 12 days, sampled every three hours, variation in the fraction of dividing brain cells (FOCD) was not explained by sampling point, time of day, age, gut fullness or by treatment (Table 6.3). There was no repeating diel cycle in starved or fed larvae (Figures 6.10). However, the examination of the data suggested the possibility of a shorter compressed cycle, approximating 15 hours (Figure 6.10). Compressed cycle time point (CTP) explained variation in FOCD (p=0.0209) of fed but not starved (p=0.2377) larvae. Variation in FOCD was also explained by swim bladder inflation, with FOCD significantly higher in larvae with swim bladder inflation than those without (p=0.0073).
Figure 6.8 – Mean standard lengths (A), percentage swim bladder inflation (B) or flexion (C) for Macquaria ambigua larvae aged 9 to 12 days, sampled every three hours up to day 12, then every 12hrs. Larvae were split into two treatments from 0800 on day 10, these being fed and starved. Error bars represent ±2SE for mean standard lengths.
Figure 6.9 – Mean oil droplet volume (A) and mean gut fullness (B) for *Macquaria ambiguа* larvae aged 9 to 12 days, sampled every three hours. Larvae were split into two treatments from 0800 on day 10, these being fed and starved.
Figure 6.10 - A comparison of diel variation in predicted means for fraction of larval snapper brain cells dividing (A) or in S phase (B) or G2M phase, for larvae which were either fed or starved, and sampled every three hours from day 8 to 11 after hatching. Error bars denote least significant difference (lsd).
Variation in the mean fraction of cells in S phase (Figure 6.10) may be weakly explained by sampling point (p=0.05383). The effect of treatment upon the fraction of cells in S phase changed depending on the sampling point (p=0.0369). Fraction S was higher in larvae with swim bladder inflation (p=0.0018). It was also related to time point within a compressed 15 hour cycle (p=0.0307) in fed larvae only.

Variation in the fraction of cells in G2M phase (Figure 6.10) was related to sampling point (p=0.0000) and to treatment, although the latter relationship varied with sampling point also (p=0.01687). This variation was not diel in nature, as it did not differ significantly depending on the time of day. Figure 6.10 does however suggest a shorter rhythm or cycle of around 15 hours, and indeed there are significant differences between sampling time in fed larvae when the cycle is compressed to 15 hours (p=0.0000) as well as an interactive effect of CTP with gut fullness (p=0.00023) (Table 6.3). In fed larvae, the G2M fraction decreased for the first 9-12 hours and then increased again by the 15th hour of the cycle. These trends were not apparent in the compressed cycle for starved larave (p=0.1115) for which the pattern was irregular and non-cyclical between successive 15 hour periods (Figure 6.10). Variation in G2M was weakly explained by larval length (p=0.0585) and oil volume (p=0.0717), and by the interaction between gut fullness and sampling point (CID=48.08, df=23, p=0.0016).

6.3.2.3 Morphological and developmental effects: Day 23

For larvae aged 23 days (Figure 6.11) and sampled over 24 hours, there was a significant increase in mean length of larvae over the 24 hr period (p=0.0000) (Table 6.4). Swim bladder inflation was not determined in 23-day-old larvae. Larvae of this age appeared to feed during daylight hours and excrete gut contents entirely at night (Figure 6.12). Hence gut fullness was maximal from 1200 hr to 2000 hr and guts were completely empty from 0000 hr to 0800 hr.

6.3.2.4 Brain cell proliferation: Day 23

In post-flexion larvae aged 23 days sampled every 4 hours from 1200 to 0800 on day 24, there was no relation between FOCD and time of day but a strong relationship with both gut fullness (p=0.0114) and light (p=0.0108) whereby mean FOCD was lower
Figure 6.11 – A comparison of diel variation in: A) gut fullness and length of 23 day-old larval samples; with B) the fraction of cells in S, G2M or both (FOCD), for golden perch larvae sampled every 4 hours starting from 1200 h on day 23 after hatching. Error bars denote ±2SE.
when gut fullness was low and at night (Table 6.3, Figure 6.12). S phase fraction did not vary with time of day but was related to gut fullness (p=0.0269) and light (p=0.0254) in a manner similar to FOCD. The fraction of cells in G2M varied with time (p=0.0358), gut fullness (p=0.0081) and light (p=0.0077), being lower during the dark period.

6.4 DISCUSSION

The primary objectives of this analysis were to determine if brain cell proliferation in larval fish brain exhibited significant diel variation, whether this variation was circadian in nature, what triggered variation, and whether variation was stage or species specific. There was no evidence for a circadian cycle in cell proliferation parameters in pre-flexion larvae of either species. In 23 day old, post-flexion golden perch larvae, light and feeding period were positively related to the level of cell proliferation (and to each other). Given that darkness appeared to prevent feeding in larvae of this age, it is reasonable to suggest that these larvae might show a similar and cyclical pattern in feeding and, if feeding triggers DNA synthesis, cell proliferation. However, this can only be confirmed or refuted by a direct study sampling on consecutive days. Overall, the level of change over 24hr in these 23-day-old larvae was only in the order of 2%, and when assessed by time, was not significant. This clearly has less import for the interpretation of proliferation data than do the 10% “within day” changes that were evident in younger larvae of both snapper and golden perch. A sub-daily cycle in cell proliferation was noted in 9-12 day old golden perch, but this will be discussed later.

There was strong evidence in both species for significant variation in mean fractions of proliferating brain cells within 24-hour periods, and the extent and pattern of this variation was dependent on developmental stage. While 1-day-old snapper larvae showed significant change in mean fraction of cells in both S and G2M, levels of FOCD did not vary and overall, the level of S and G2M was very low when compared to older feeding snapper larvae. Variation in S phase was also linked to an oil-time interaction, suggesting that oil reserve usage may be linked to the initiation of growth, or in this case the initiation of DNA synthesis in the brain cells. For 4-day-old snapper larvae, FOCD and S phase fractions did not vary significantly over 24hours, nor does oil droplet diameter. Fraction G2M differed only slightly with time of day. Given that these
larvae were sampled from a single replicate tank only, a tank specific effect may have been present. Overall levels of cell division in larvae at this age were low and similar to those for one day old larvae (around 0.07).

The mean level of FOCD in older feeding dependant snapper larvae (and in 9- to 12-day-old *M. ambigua*) was almost double that of oil-dependent snapper larvae, and fluctuated significantly within 24 hr periods around a mean of 0.14-0.15 (Figure 6.6). However these larvae exhibited increased G2M and reduced S phase levels, which may be indicative of cell cycle arrest in G2M. In previous chapters such a pattern has been associated with low growth and higher mortality. These were both evident in the current experiment, with high daily mortalities resulting in few larvae remaining by day 11. The increase in mean length (over days 8-11) for larvae reared in the control treatment was only half that of larvae aged 8 to 11, reared at 21°C, in the temperature trial (see Chapter 5). Given the concerns over the health and physiological condition of these larvae, comparisons between the snapper data derived here with that from healthy snapper larvae should be treated with caution. G2M was much higher than mean S, and consequently FOCD tended to reflect patterns exhibited by G2M. In fully fed golden perch larvae aged 9 to 12 days, there was a consistent and repeating pattern of variation in mean fraction G2M that operated on a 12-15 hour cycle. There was some (non-significant) graphical evidence for a similar periodicity in snapper from 1100 hr (day 9). This sub daily cycle in brain cell division appears to be relatively novel, with little research on diel variability in neural tissue of any organisms present in the literature. Studies of the teleost retina, a known circadian pacemaker tissue, not surprisingly demonstrated a clear endogenous circadian rhythm in cell proliferation of retinal cells (Chiu *et al.*, 1995; Kwan *et al.*, 1996). However, cyclical environmental factors (light, feeding) did not appear to be driving this variation in younger larvae, leading to the suggestion that the variation is genetically based. Such a cycle seems unlikely to be hormone driven, given that cycles in hormone production, even in larvae, are generally circadian in nature (Ali, 1991; Falcon, 1999; Okimoto and Stetson, 1999). The possibility that this cycle is an endogenous, genetically driven hangover from the period of synchronous cell divisions that characterise the early embryological development of all vertebrates, seems unlikely, given that cell division synchronicity is lost very early in
embryogenesis of fish larvae (Kimmel et al., 1995). Clearly, the mechanisms behind such variation require further investigation.

Food availability and photoperiod are known entraining agents in circadian rhythms of cell proliferation (Rensing and Goedeke, 1976; Thompson et al., 1999; Van Dolah and Leighfield, 1999) and these two factors were manipulated in the current experiments in order to determine their effect upon diel variation in brain cell proliferation of fish larvae. Starvation clearly disrupted the 12-15h cyclical nature of variations in mean G2M and S phase fractions in golden perch larvae. With the exception of a dip on day 11 (1100 h) mean S and FOCD of starved larvae was relatively steady (unfluctuating). Therefore, feeding stimulus appears necessary for the propagation of this cycle. The absence of a significant decrease in cell proliferation levels in starved larvae cannot be explained by larval use of oil reserves, as oil droplet volumes were significantly higher in starved larvae, suggesting that oil reserves are used in conjunction with feeding (or at least are not utilised in response to short term starvation). Most likely, the starvation period was too short for the effects of nutrient deprivation to have an effect upon growth processes. Gut content only reached zero at the end of the starvation period. Retention of gut contents in the starved or intermittently fed larvae has been observed previously (Canino and Bailey, 1995). Golden perch larvae aged 9 to 12 days did not, however, show any correlation between diel changes in food intake and the mean fraction of dividing cells in fully fed larvae. This contrasts with the results from the snapper larva analysis, which shows a positive relationship between the number of rotifers ingested and the fraction of cells synthesising DNA (also seen in day 23 golden perch larvae). Increased S phase levels were also linked to decreasing oil reserves in 1-day-old snapper. Higher nutritional intake/absorption means a greater nutrient supply to fuel cell division. However, whether the entry of cells into DNA synthesis is a direct response to increased nutrient in the blood (one might expect a lag between ingestion and initiation of DNA synthesis) or a physiologically linked response to the feeding and intake of food itself, is unknown. The ingestion of rotifers is only one factor influencing variation, for while feeding is clearly circadian, variation in the fraction of cells in S phase is not. Finally, it should be noted that, the delayed feeding treatment for snapper larvae was most likely flawed in design. An observed phototactic response of rotifers to the light source and away from outflow pipe, thus maintaining their availability to feeding larvae throughout the day, may explain the lack of temporal difference in mean
rotifer ingestion, and the lack of difference in the mean FOCD, S or G2M levels between the delayed fed and control treatments.

Light has been shown to be a key stimulating and entraining factor in many circadian rhythms including those of cell proliferation (Kwan et al., 1996; Van Dolah and Leighfield, 1999). Cell proliferation in 23-day-old golden perch larvae was significantly higher during daylight than at night, but it is impossible to separate the effects of light and feeding. Extending photoperiod of snapper larvae might be expected to increase the time for feeding and growth per day. However, both length and S phase fractions are generally lower in larvae reared under an extended photoperiod. Extending light period reduces the time available for larvae to surface and inflate swim bladders (Martin-Robichaud and Peterson, 1998), and consequently, fewer larvae sampled from the extended photoperiod treatment had inflated swim bladders. Regardless of treatment, both S and FOCD are lower in larvae lacking swim bladders. Swim bladder inflation increases swimming efficiency, meaning that larvae from the extended photoperiod treatment may have had less energy to devote towards growth processes (i.e. cell division).

As mentioned in the methods, the current experiments had trouble in consistently isolating the preferred numbers of cells per sample. This immediately introduced an error into the data that were purely attributable to method rather than biological effects. For snapper, the slope and correlation of the relationship between cell number and cell fraction estimates was small, and did not overly affect the graphical representation of the data. However, the relationship for golden perch was closely correlated, and to allow more meaningful biological interpretation of graphed data, the cell fractions were standardised to 7000 cells using the regression of total cells with S phase estimation. This also meant that total cell number need not be entered as a variate in models. The latter method appears preferable, given that it accounts for variation due to cell number and allows more meaningful graphical representation. More advanced modelling programs have recently been developed which do not suffer from biases associated with variation in cell number.

The results presented here provide evidence for the occurrence of significant "non-circadian" variation in the level of cell proliferation in larval brain, with the pattern and
level of this variation dependant on developmental stage and species. This variation was related to food intake, but differences could also be explained by variation in larval size and other morphological variables between samples. A proportion of variation still remains unexplained. These results also indicate that firstly, the fraction of cells in S phase was related to nutrition (gut content or oil absorption), and as such was a more appropriate nutrition related growth index than G2M, whose levels varied in a less explainable and predictable manner. Secondly, the non-circadian nature of variation poses problems for the use of this technique as a growth index. If variation (at least in younger larvae) is not predictable on a diel basis, it becomes difficult to adjust for such variation and allow accurate interpretation of data as pertains to nutritional condition and growth status. Proliferation may operate on a compressed cycle in golden perch, but unless the triggering factors or a consistent starting point is determined, knowledge of this phenomenon does not help in interpreting data in relation to growth. Secondly, the lack of a circadian rhythm (or very low degree of change where one might be present, such as in 23-day-old M. ambigua) means that it is not easy to predict when cell proliferation might naturally peak in a 24 hr period and thereby allow better definition of starved and fed larvae, as hypothesised in the introduction.
CHAPTER 7

EFFECT OF STARVATION UPON THE PROPORTION OF BRAIN CELLS SYNTHESISING DNA IN PAGRUS AURATUS LARVAE.
7.1.1 INTRODUCTION

As outlined in Chapter 1 (Section 1.2), the primary aim behind the development of condition and growth indices for larval fish, is their application to investigations into the effect of environmental factors upon larval growth survival and eventual recruitment into the juvenile and adult populations (Ferron and Leggett, 1994). The idea that starvation will lead to reduced survival, as a result of higher predation pressures that are associated with reduced growth, increased stage duration and lowered escape capability, is currently subject to considerable debate (Elliott and Leggett, 2000; Suthers, 2000). However, starvation itself is a direct cause of mortality (Buckley, 1984) and it is important that growth indices look at the relationship between the parameter being measured (e.g. cell proliferation) and larval survival under conditions of nutritional stress. Histological indices have been able to determine the “point of no return” (PNR) at which larvae are too weak to feed and consequently can be presumed to be ecologically “dead” (Ehrlich, 1974; Ehrlich et al., 1976). Similarly, determination of a reduced level of cell proliferation as an indicator of starvation leading to mortality would aid in interpretation of field data pertaining to the survival probability of larvae. To date, investigations of the relationship between cell proliferation and starvation in larva have not attempted to link this index to mortality (Theilacker and Shen, 1993b; Westerman et al., 1999; Bromhead et al., 2000; Theilacker and Shen, 2001).

Previous studies of larval cell proliferation responses to starvation have both incorporated the fraction of cells in G2M within the index; Theilacker and Shen (2001) used the combined fraction of cells in S and G2M (termed the fraction of cells dividing or FOCD) as their index, and Bromhead et al (2000) used the ratio of the number of cells in G2M to the those in G01 (termed the cell division index or CDI) (see Section 1.3.3.4 for review). However, Bromhead et al (2000) cautioned that any evidence for G2 arrest in response to sub-optimal growth conditions might invalidate the use of this cell fraction in any cell proliferation measure that is intended as an overall growth index. The previous chapters have put forward substantial evidence that the mechanisms controlling the initiation and progression of larval brain cells through the cell cycle might differ for cells entering S to those entering G2. This included evidence that cells may arrest in G2 but not S, evidence that the fraction of cells in S phase correlates more
closely to overall growth, and evidence that fraction of cells in S and G2M are not closely interrelated. Hence the fraction of cells in S phase is the most appropriate growth index, but has yet to be tested in relation to its response to changes in duration of starvation in temperate marine teleosts.

The study of Theilacker and Shen (2001) targeted the critical period immediately post-hatch in a marine species, walleye pollock. They determined that the fraction of cells proliferating in larval muscle and brain tissue is highly sensitive to the nutritional condition and growth status (Theilacker and Shen, 1993; Theilacker and Shen, 2001). Bromhead et al. (2000) investigated the effect of starvation and refeeding in older larvae of freshwater species, G. olidus. However there has been no published investigation into how starvation affects cell proliferation in later larval stages of temperate marine species, which form the majority of species presently being targeted in recruitment studies. Latency and response of indices to changes in nutritional condition may change with age (Ferron and Leggett, 1994), and it is important to determine the response time of the fraction of brain cells synthesising DNA in order to aid in the prediction of past feeding history and current growth and condition status.

7.1.2 AIMS

Section 7.1.1 has highlighted the lack of investigation into the link between cell proliferation levels and larval growth and survival. It also discussed evidence that the fraction of cells synthesising DNA (S phase) represents a more appropriate overall growth index than do indices which incorporate the G2M fraction. Consequently, a short and simple growth and survival experiment was devised which aimed to:

1) Determine the latency and sensitivity of the response of the fraction of brain cells synthesising DNA, in response to changes in the duration of starvation and subsequent refeeding, in post-critical period (20-day-old) larvae of snapper, Pagrus auratus;

2) Determine whether the fraction of brain cells synthesising DNA is related to the level of daily mortality; and
3) Determine whether the fraction of cells in S phase relates to overall growth in starved and refed larvae.

7.2 METHODS

The following experiment took place as part of a larger trial which compared three different methods for the analysis of condition and growth, these being the spectrofluorometric analysis of RNA:DNA (using the methodology of Wagner et al. 1998), otolith increment analysis (Maillet and Checkley, 1989), and cell cycle analysis (Theilacker and Shen, 1993; Bromhead et al., 2000; Theilacker and Shen, 2001). The trial formed part of a collaboration with Dr Iain Suthers and colleagues from the University of New South Wales. Analysis of the otolith and RNA:DNA data are still in progress (Suthers and Bromhead; in prep). The following chapter deals only with the results generated by the cell cycle analysis, which was undertaken solely by myself.

7.2.1 Spawning, stocking and design

This experiment used facilities generously donated by Stewart Fielder at the N.S.W. Fisheries Port Stephens Research Centre. Larvae were supplied via adult snapper spawning as described in Chapter 2 (Section 2.2.1.1). Larvae were initially reared in a single external 10 ML rearing tank in coarse filtered water drawn directly from the adjacent estuary (Lemontree Passage, Port Stephens). Larvae fed on a mixed diet of predominantly rotifers and copepods until day 17, and were subject to the natural photoperiod for that time of year, approximately 0700 hr to 1900 hr.

On day 17, larvae were collected by 200µm dip-net, and 80 larvae transferred into each of 8 experimental tanks. Tank design and operation is as described in Chapter 2 (Section 2.2.1.2). Larvae were initially acclimatised for 20 minutes by flotation of capture buckets in tank water, before adding 30% tank water for 5 minutes, and then slowly releasing larvae into the tanks. These larvae were then allowed to acclimatise to the tanks over a period of three days prior to the implementation of experimental treatments. Environmental conditions were strictly controlled, with temperature range maintained at 20.2 to 20.5°C, salinity at 34.9 to 35.9 ppt, dissolved oxygen at 6.4 to 7.4
ppm, pH at 7.8 to 8.0, and a photo-period regime of 12L:12D. Dead rotifers and debris settled to the bottom of the tanks and were removed twice daily by siphoning.

Starting on day 20 after hatch, the 8 experimental tanks were subjected to experimental treatments. Larvae in two tanks were fully fed (group FF) twice a day for the duration of the experiment. Larvae in two more tanks were starved one day, then fed 5 days (group ST1), two tanks starved two days then fed four (group ST2), and two tanks starved three days and fed three days (group ST3).

7.2.2 Feeding and sampling

Larvae in FF treatments were fed twice a day, at 8am and 2pm, to maintain a density of rotifers at 5 per millilitre. This accommodated the higher feeding rates and requirements of the larvae at this developmental stage. An initial sample of one fed larvae per tank was taken at 8am on day 1. Starvation treatments were then imposed on relevant tanks. A random sample of 5 larvae were then sampled from each tank at 8 am each day for 6 days, equalling 10 larvae per treatment per day. At the end of this period (day 7), remaining larvae were counted and the experiment terminated. In total, 248 larvae were sampled for analysis. Upon sampling, larvae were individually transferred to a well plate with engraved 10mm line and their image captured by video and numbered according to larvae sample number. These images were later used to determine larval length.

Brain tissue was dissected as described in Chapter 2 (Section 2.3.3). Once the tissue was drawn into the needle, the cells were then transferred and gently triturated 4 times in 200µL of cryoprotectant (16.67 mL of foetal calf serum (FCS), 16.67 mL dimethyl sulfoxide (DMSO) and 66.67 mL of Eagles minimum essential medium (E!v!EM) to make a 100 mL stock solution) following the methodology of Theilacker and Shen (1993b). The dissociated cells were then snap frozen in cryotubes (Nunc) in liquid nitrogen dewars, which were then transferred back to the John Curtin School of Medical Research, and stored at -75°C.
7.2.3 Data collection, analysis and statistics

The processing of brain cell samples was essentially the same as that described previously (Section 2.3) but with cells dissociated in cryoprotectant prior to freezing. These samples were thawed quickly in a waterbath at 37°C, for two minutes and the cell suspension transferred to a cryotube containing 4 mL PBS. Cells were then centrifuged and washed twice in PBS, before being resuspended in 200µL PBS and fixed in 4 mL of 75% ethanol, for 4 hours. The washing and staining of cells in preparation for flow cytometric analysis is described in Chapter 2 (Section 2.3.3).

The flow cytometric settings for collection of brain cell data, as well as methods describing subsequent analysis of this data, are described in Chapter 2 (Section 2.3.4). In this experiment, the fraction of cells in S phase represents cell proliferation, and is the response variate in a mixed model analysis which uses treatment, age or developmental status as explanatory variables. Morphological growth, as measured by standard length (SL) and growth rate (GR), is also compared with fraction of cells in S phase to determine if this fraction is appropriate, either as a recent or instantaneous growth indicator for larvae subjected to various levels of nutritional stress. Given that the current data set is unbalanced, restricted maximum likelihood (REML) analyses of logit transformed cell fraction data have been used in preference to ANOVA, to determine the most appropriate model to explain variation in fraction S. A more detailed explanation for this statistical approach is provided in chapter 2 (Section 2.3.5). Both real and predicted data will be presented graphically to allow comparison of fitting data.

7.3 RESULTS

7.3.1 Growth and survival

There was no overall effect of treatment in explaining variation in larval length (CID=5.444, df=3, p=0.1420), however variation was explained by “day” (of experiment), with length increasing over the course of the experiment (CID=67.25, df=6, p=0.0000) (Figure 7.1A). The interaction between day and treatment did not explain any further variation (CID=17.83, df=15, p=0.2717). However, despite a lack of
significant difference among treatments, the mean lengths of larvae starved for three
days did appear to be consistently lower from day 3 onwards (Figure 7.1A). A
preliminary analysis of growth rate presented numerous instances whereby daily growth
was negative, in both starved and constant fed larvae. This was likely due to small
sample numbers and large size range variation and is discussed further in Section 7.4. It
was decided that growth rates would be determined at day 4 and at day 7 to calculate
mean daily growth rates for 3 day intervals (i.e. day 1 to 4 and 4 to 7) as well as overall
growth rates (days 1-7). Mean daily growth rate calculated for days 1 to 4 was highest
in ST1 larvae (0.33mm/d), followed by FF larvae (0.23mm/d), ST2 (0.16mm/d) and the
lowest growth rate over this period was for ST3 larvae (0.04mm/d). Over the next 3
days (days 4 to 7), when all larvae were feeding, fully fed larvae had highest growth
rate (0.597mm/d) followed by ST1 larvae (0.443), ST2 larvae (0.387) and ST3 larvae
(0.269).

Mortality levels were low (3 or less dead per treatment) in all treatments on days 1 and
2, but on day 3 higher mortalities were recorded for ST1 (9 dead) and ST2 (7 dead)
treatment groups (Figure 7.1B). The number of mortalities decreased thereafter in the
ST2 treatment, while another increase was evident on day 6 for ST1 larvae (10 dead).
ST3 larvae showed by far the highest mortality level, with 11 dead larvae recovered on
day 4 and 31 recovered on day 5. Mortality was consistently low in FF treatment, with
no more than 3 dead larvae recorded on any single day.

7.3.2 Fraction of brain cells synthesising DNA

Variation in fraction of S phase cells was explained by a “day” effect (CID=31.25,
df=6, p=0.0000) with overall levels higher on day 7 than on day 2 (Figure 7.2A,B), and
a standard length (SL) effect, with longer larvae having higher fraction of brain cells
synthesising DNA (CID=6.109, df=1, p=0.0134). There was a strong interactive effect
between day and treatment (CID=51.55, df=15, p=0.0000) even when variation due to
length was removed first (CID=30.12, df=13, p=0.0045). There was little difference in
mean predicted S phase level between treatments on days 2 or 3 (Figure 7.2B). On day
4, the predicted fraction of cells in S phase decreased in ST3 larvae to levels
significantly less than that seen in other treatments. Furthermore, FF and ST1 larvae had
Figure 7.1 – A) Mean standard length; and B) number of mortalities per day, for 20 day old *Pagrus auratus* larvae subjected to either constant feeding (FF), 1 day starved:5 day refed (ST1), 2 day starved:4 day refed (ST2), 3 day starved:3 day refed (ST3) nutrition regimes, and sampled at 8am every day. Arrows denote refeeding points for each regime (i.e. ST1 refed at arrow “1”, ST2 at arrow “2”...). Error bars denote ±2SE.
Figure 7.2 – Response and recovery of fraction of brain cells synthesising DNA, as represented by: A) actual means; and B) predicted means (from REML model); for 20 day old (start) Pagrus auratus larvae subjected to either constant feeding (FF), 1 day starved:5 day refed (ST1), 2 day starved:4 day refed (ST2), 3 day starved:3 day refed (ST3) nutrition regimes, and sampled at 8am every day. Arrows denote refeeding points for each regime (i.e. ST1 refed from arrow “1”). Error bars in A) denote ±2SE and in B) denote least significant difference (lsd). Both data sets are presented so as to allow visual comparison of model generated predicted values and actual raw values.
significantly more brain cells synthesising DNA on this day than do ST2 larvae. The latter trend was no longer apparent on day 5, although refeeding ST3 larvae still exhibited significantly lower S phase fractions that did larvae in other treatments. There were no differences among treatments on days 6 and 7, but a general overall increase in the fraction of cells in S phase from day 6 to 7 (Figure 7.2).

### 7.3.3 Survival and brain cell proliferation

The fraction of larval brain cells synthesising DNA was significantly related to the daily mortality of larvae subjected to different durations of starvation and recovery feeding (ANOVA, $F=7.19$, $n=24$, $p=0.0136$, $r^2=0.2464$) (Figure 7.3). The data mean for day 5 ST3 larvae appeared to have considerable leverage in this relationship. However, when this data mean (mortalities=31, mean $S=0.0248$) was excluded, the relationship was still significant (ANOVA, $F=5.41$, $n=23$, $p=0.0300$, $r^2=0.2050$). Larvae with higher mortality rates could have a large variation in the fraction of cells synthesising DNA. However, larvae with high $S$ fraction appeared to be associated with low daily mortality (Figure 7.3). Mortality rates of greater than 3 larvae per day were only apparent in groups with a mean fraction of S phase cells less than 0.040 ($\ln$ value $= -1.40$).

### 7.3.4 Growth rate and cell proliferation

The relationship between fraction of cells synthesising DNA and larval growth rate was assessed by comparing means for days 1-4 (treatments applied) and 4-7 (all groups feeding) (Figure 7.4). Over the first 4 days, mean growth rate and mean fraction cells in S phase were positively correlated (ANOVA, $F=18.66$, $n=4$, $p=0.0496$, $r^2=0.9032$). ST1 larvae had both highest mean growth rate and fraction of brain cells synthesising DNA, followed by FF larvae, ST2 and then ST3 larvae had the lowest growth rate and fraction S. However, between days 4 and 7, during which all larvae were feeding, there was no correlation between the fraction of cells in S and mean growth rate (ANOVA, $F=0.0003$, $n=4$, $p=0.9874$, $r^2=0.0002$). There was little difference in the mean fraction of cells in S phase, but mean growth rate appeared to be higher in larvae which had experienced less or no starvation period (Figure 7.4). Overall comparison of mean growth rate and cell fraction showed a positive relationship between growth rate and fraction brain cells synthesising DNA (Figure 7.4B). Larvae starved 3 days were most
Figure 7.3 – The relationship between the mortalities (per treatment per day) and mean fraction of brain cells synthesising DNA (lnS, for each treatment each day), for 20 day old (start) Pagrus auratus larvae subjected to either constant feeding, 1 day starved:5 day refed, 2 day starved:4 day refed, or 3 day starved:3 day refed nutrition regimes, and sampled at 8am every day. Regression line has correlation of 0.2464 (ANOVA, F=7.1932, n=24, p=0.0103).
CHAPTER 7

Starvation

Figure 7.4 – Comparison of mean growth rate with mean fraction brain cells synthesising DNA, as calculated for: A) Days 1-4 and 4-7; and B) overall (days 1-7) for 20 day old (start) *Pagrus auratus* larvae subjected to either constant feeding (FF), 1 day starved:5 day refed (ST1), 2 day starved:4 day refed (ST2), 3 day starved:3 day refed (ST3) nutrition regimes, and sampled at 8am every day. Number beside S and F denote number of days “Starved” and “Fed” respectively. Correlation values ($r^2$) are also stated.
effected, not increasing in length and having only 45% of the number of cells synthesising DNA when compared to day 1. After 3 days refeeding, these larvae were seen to increase significantly in length (to 7.95 mm) and the mean fraction of cells synthesising DNA had increased by 2.25 times to 0.0454, a level higher, but not significantly, than that of constant fed larvae of the same age.

DISCUSSION

The fraction of cells in S phase was significantly lower in larvae starved for three days (ST3), but did not differ from fed larvae (FF) when assessed at shorter starvation periods of one (ST1) or two (ST2) days. ST3 larvae were also associated with higher mortality and shorter mean lengths when compared to other treatments. There appeared to be a lag of 24-48 hours after refeeding before S phase fractions in both ST2 and ST3 larvae recovered to levels exhibited by constant fed larvae. After 3 days, mean growth rate and fraction of brain cells synthesising DNA showed a tight positive correlation. Between days 4 and 7, when all treatment groups were feeding, cell fractions were fairly uniform but overall growth rate was higher in constant fed larvae (although see below), and decreased as duration of starvation in each treatment increased. Overall the results suggest that brain cell proliferation, as indicated by a measure of the fraction of cells synthesising DNA, is a sensitive indicator of nutritional condition in snapper larvae.

Biochemical indices of growth and condition vary in the speed of their response to reduced nutritional intake. Determining the latency of this response both to feeding after starvation, and to starvation after feeding, is required to allow accurate interpretation of growth data as pertains to larval feeding history, condition and growth status (Ferron and Leggett, 1994). Changes in RNA:DNA, for example, have been detected within 24 hours (Wright and Martin, 1985). The physiological response of tissues to starvation has been proven to vary among tissues (O'Connell, 1976; Theilacker, 1978; Houlihan et al., 1988) and indeed Theilacker and Shen (2001) present some evidence to suggest that larval brain tissue, although significantly affected by starvation, may be spared to some extent when compared to muscle. The current analysis demonstrated a significant difference between fed and starved larvae in the fraction of brain cells synthesising
DNA, and this difference was apparent after 48 to 72 hours of starvation. The recovery time after refeeding was between 24 and 48 hours. Bromhead et al. (2000) found in their study of freshwater *G. olidus* larvae, that the response time to starvation was between 24 and 48 hours, and recovery time to pre-starved levels around 4 days. These larvae were starved for 7 days, which may explain the increased delay in recovery. Both fed-starved and starved-fed response times were found to be longer at lower temperatures (Bromhead et al., 2000), although this was partly due to lower proliferation rates at lower temperatures. However, that study used the ratio of G2M to G01 cells as the growth index, which in previous chapters has been shown not to be a particularly good indicator of overall growth (see Chapter 4). Therefore the brain tissue based indices used by Bromhead et al. (2000), and Theilacker and Shen (2001), may be less reliable with respect to predicting overall growth, as they incorporate the G2M fraction. Theilacker and Shen’s (2001) muscle based index will be discussed later.

There was a close positive correlation between the mean fraction of brain cells synthesising DNA and the mean daily growth rate as calculated for days 1-4 of the experiment, with constant fed larvae and 2- and 3-day starved larvae having lower fraction S and lower mean growth rate than other treatment groups. Furthermore both 2- and 3-day starved larvae exhibited an increase in the fraction of cells in S phase and in growth rate for the period days 4-7, in response to refeeding. The 2 and 3-day starved data means for the period days 4-7 fell very close to the regression line for days 1-4 data means (Figure 7.4A). Combined, these data suggest that fraction S is a very sensitive index of overall larval growth rate and will reflect nutritional condition. However, larvae which had been fed for 6 days, or starved 1 day:refed 5 days, had an increased mean growth rate in the absence of concurrent increases in fraction S. The sudden increase in mean lengths between day 6 and 7, which represented a dramatic increase in growth trajectory when compared to that of the previous 5 days (Figure 7.1A) suggested that perhaps there was a sampling bias towards larger larvae in these treatments on the last day. No change in growth trajectory for snapper larvae of this age had been reported in previous growth trials (Fielder et al., in preparation) so it seems unlikely this was a real effect representing a natural sudden increase in mean growth rate. However, if this was a real increase in length, then the data suggest that growth rate and fraction S might become dissociated above a given fraction. In other words, overall larval growth rate might continue to increase after brain cell proliferation rates (or fractions) have reached
their upper limit. Given the close linear relationship over a greater range of S phase fractions (up to 0.17) with mean growth rate (see Chapter 4) this appears unlikely. However, Chapter 4 dealt with younger larvae.

The comparison of survival data with mean fraction S data suggests that if larvae have a high fraction of cells synthesising DNA, then mortality levels can be assumed to be low. However, the opposite does not hold true for larvae with a low fraction of cells dividing where the associated range of daily mortality is quite broad. It should also be noted in the current analysis however, that S phase levels were quite low in comparison to those seen for *P. auratus* in Chapters 4 and 5, despite overall growth rates being comparable. This reduction in S phase may be a tissue-specific response to the transfer of larvae from greenwater estuary fed tanks to aquaria tanks, where both conditions and feed were different. The acclimatisation period prior to start of the experiment may not have been sufficient. It is more likely that this period in snapper development may represent a phase of lower cell proliferation in the brain. In Chapter 4, 21-day-old larvae exhibited lower cell fractions, however this was assumed to be a response to disease. Snapper larvae in the current experiment showed no evidence of disease at all. It might be useful to reassess the relationship between cell proliferation and survival over a more normal growth range in starved and fed larvae.

In summary, while the fraction of cells in S phase in both starved and fed larvae was relatively low, this parameter was still sensitive to effect of starvation, significantly so after 3 days, and produced a lag in recovery (after larvae started feeding again) of two days. Overall, the results back those from other chapters suggesting that the fraction of brain cells in S phase relates closely to overall growth, and to a lesser extent, survival, and is an appropriate index of nutritional condition and growth status in snapper larvae.
CHAPTER 8

GENERAL DISCUSSION

8.1 INTRODUCTION

The ultimate aim of the research presented in this thesis was to assess the feasibility of flow dynamic analysis of cell cycle transitions in liver tissue. This, in a potential tool for the analysis of whole liver growth. Such a tool would be invaluable in defining the influence of environmental agents upon liver growth rate and eventual recruitment success (Thilagan and Sheer, 2004). To achieve this, investigations were first conducted to further define optimal methodological criteria and then apply these in assessing the influence of numerous environmental, developmental and physiological factors upon brain cell proliferation of larval insects, and the relationship with overall larval growth. The data arising from these investigations has shed considerable light upon the effects of salinity, temperature, and the effect of cell proliferation and whole larval growth, as well as the development, age and sex related responses of the index. Perhaps most importantly, it has revealed important differences in the nature and response of the two "proliferating" components of the cell cycle, S and G2M, to the various conditions. This aspect is detailed below, which serves as a base by which to discuss firstly, the overall influence and significance of environmental and developmental factors upon cell proliferation in larval brains; and secondly, the overall suitability of this approach for investigations into larval growth and recruitment dynamics. This discussion will also highlight those areas requiring further research to be highlighted, and place the findings in the context of current literature.

8.2 SUMMARY OF RESULTS

8.2.1 Optimisation of methods

Three areas were identified as crucial to precise and accurate estimation of cell cycle fractions in larval brain cells. (1) Preparation (2) proliferation parameters, and (3) data modelling. Appropriate cryopreservation methods, gentle dissociating techniques, and scrupulous fixation (to ensure all cells had uniform immunochromic quality) were the primary preparative issues identified. Ideally, a minimum of 10000 cells per sample should be
8.1 INTRODUCTION

The ultimate aim of the research presented in this thesis was to assess the suitability of flow cytometric analysis of cell cycle fractions in larval teleost brain, as a potential tool for the analysis of whole larval growth. Such a tool would be invaluable in defining the influence of environment upon larval growth rate and eventual recruitment success (Theilacker and Shen, 2001). To achieve this, investigations were first conducted to further define optimal methodological criteria, and then apply these in assessing the influence of numerous environmental, developmental and physiological factors upon brain cell proliferation of larval teleosts, and the relationship with whole larval growth. The data arising from these investigations has shed considerable light upon the effects of salinity, temperature, time of day and starvation upon brain cell proliferation and whole larval growth, as well as detail the developmental, age and size related responses of the index. Perhaps most importantly, it has revealed important differences in the nature and response of the two “proliferating” components of the cell cycle, S and G2M, to these aforementioned factors. A summary of the most important results is detailed below, which serves as a base by which to discuss firstly, the overall influence and significance of environmental and developmental factors upon cell proliferation in larval brain, and secondly, the overall suitability of this approach for investigations into larval growth and recruitment dynamics. This discussion will allow those areas requiring further research to be highlighted, and place the findings in the context of current literature.

8.2 SUMMARY OF RESULTS

8.2.1 Optimisation of methods

Three areas were identified as crucial to precise and accurate estimation of cell cycle fractions in larval brain cells: 1) preparation; 2) collection parameters; and 3) data modelling. Appropriate cryopreservation methods, gentle dissociative techniques, and use of fixatives (to ensure all cells had uniform fluorochrome uptake) were the primary preparative issues identified. Ideally, a minimum of 10000 cells per sample should be
analysed, at less than 200 cell/sec on a low flow (12µL/sec) setting within a period of 1 to 18 hours after staining. Accurate modelling estimates are also aided by gating out debris, aggregates and potential circulatory cells. An apoptotic population was identified and an index created to quantify the level of apoptosis occurring.

8.2.2 The effect of salinity upon cell proliferation in brain

This investigation looked at the relationship between salinity and cell proliferation in brain tissue of snapper larvae from age 6 days through to 21 days. Mean growth rate was most closely correlated with mean fraction of cells in S phase ($r^2=0.63$) while G2M showed no significant correlation with growth rate or with fraction S. There was some evidence for G2M arrest in response to disease which affected larvae late in the experiment. The effect of salinity upon fraction S phase was most apparent in larvae 12 days of age or older, while the overall level of cells in S (and FOCD) varied with age of the larvae, being generally higher when aged 9 and 15 days. The developmental and length data supported the conclusions from the cell cycle data, that growth and condition was optimal at 30 ppt. Cell proliferation was lowest, and apoptosis highest, at 10 ppt.

8.2.3 Effect of temperature upon cell proliferation in larval brain tissue

This investigation looked at the relationship between temperature and cell proliferation in brain tissue of snapper larvae from age 6 days through to 14 days. The optimal temperature for brain cell proliferation in larval snapper decreased as the larvae got older, despite the fact that overall growth and development was most rapid at the higher temperatures, regardless of larval age. Consequently, the fraction of cells in S phase and FOCD showed a low correlation with mean growth rates. Survival, however, was lower at the highest temperatures. There was a suggestion of possible brain cell cycle arrest in G2M at the highest and lowest temperature extremes. Apoptosis was higher at lower temperatures initially but was lowest in larvae reared at 15°C by day 14.
8.2.4 Diel variation in brain cell proliferation in snapper and golden perch larvae.

This chapter detailed two experiments. The first investigated whether there was any diel variation in cell proliferation of pre-feeding, mixed feeding and exogenous feeding snapper larvae, with the latter also subjected to changed feeding time and extended photoperiod treatments. The second experiment investigated how starvation might affect patterns in diel variation of brain cell proliferation in golden perch larvae, and also looked at diel variation at two developmental stages, mid- and post-flexion. There was little evidence for a circadian cycle in cell proliferation in either species except for post-flexion golden perch. The change in mean cell proliferation between day and night was only 2%. The first experiment (snapper) may have been affected by poor larval quality, but demonstrated strong diel variation in G2M, and a much lower level of variation in S phase fractions. Differences in number of cells per sample explained some of the variation in both experiments. However, variation in fraction of cells in S phase was also related to feeding (gut fullness), swim bladder inflation, and photoperiod length. The latter result was supported by trends in length data. Golden perch may exhibit a sub-daily rhythm (12-15 hr) in cell proliferation, which is disrupted in starved larvae. Overall the pattern and extent of diel variation appears to depend on species and developmental stage. The fraction of brain cells in S phase shows the least variability and highest correlation with feeding and length measures.

8.2.5 Effect of starvation upon brain cell proliferation in larval snapper

This investigation looked at the relationships between starvation, survival and overall growth and the fraction of brain cells synthesising DNA in 20- to 27-day-old snapper larvae. From day 1, larvae were either fully fed (FF), starved 1 day (ST1), starved 2 days (ST2) or starved 3 days (ST3), then re-fed until day 7. Despite seemingly lower overall levels of brain cell proliferation (when compared to snapper in salinity and temperature experiments), the fraction of brain cells synthesising DNA was significantly lower in ST3 larvae on day 4 than in other treatments and exhibited a lag in recovery to FF levels of nearly 48 hr. The fraction of cells synthesising DNA appears a sensitive indicator of overall growth rate and also can be related to mortality.
8.3 ASSESSING LARVAL CELL PROLIFERATION AS A POTENTIAL TOOL IN RECRUITMENT STUDIES

The introduction chapter (section 1.2.1) highlighted three assumptions that are critical to the theory linking growth measures with recruitment prediction. These were that:

1) The technique being used to measure a particular growth parameter is accurate, precise and reliable;
2) The growth parameter being measured is a suitable indicator of growth status of the whole larvae; and
3) Growth rate is positively related to survival probability and recruitment success.

By addressing these assumptions in the light of the current results, as well as the existing literature, the following discussion will highlight the strengths and weaknesses of the current flow cytometric cell cycle approach, determine which areas require further research, and assess the current status and overall suitability of the index for its intended application.

8.3.1 The technique being used to measure a particular growth parameter is accurate, precise and reliable.

Theilacker and Shen (1993a,b, 2001) investigated and refined specific methodological aspects pertaining to the dissection, dissociation and cryopreservation of larval brain cells. However, neither they nor Bromhead et al. (2000), reported any investigation into specific flow cytometric requirements of the system being analysed, relying instead upon general guidelines for flow cytometric analysis of cell cycle fractions. The current investigations have determined the ideal cell numbers, concentrations, flow rates and other cytometric factors which serve to maximise the precision (reduce error about mean on repeated measures) and accuracy (reduce the coefficient of variation, CV, of the G01 peak) of signal collection and subsequent modelling estimates of cell fractions. Gating out debris, aggregates, and circulatory cells increases the estimation precision of the modelling program and ensures nontarget “events” are excluded from the analysis.
The finalised protocol for brain tissue ensured consistent CVs of around 4.5%, well below the recommended maxima of 8% (Shankey et al., 1993) or even 5% (Shapiro, 1988), and ideal reduced chi-square (RCS) values generally between 1 and 3. In contrast to brain analyses, the analysis of muscle nuclei was hampered by difficulties in isolating clean muscle nuclei preparations (see appendix II). However, Theilacker and Shen (2001) have recently presented a simple and sensitive methodology for the isolation and analysis of muscle nuclei from larval fish, the implications of which are discussed in “Future Directions” section.

The validity of using flow cytometric cell cycle analysis as a means to assess cell cycle fractions has been established conclusively in other fields of research such as medicine (Shapiro, 1988), and the current investigations serve merely to tailor the generalised protocols that have arisen from these fields to the new system to which they are being applied (Shapiro, 1988). It has also defined the maximal speed of processing that is possible without decreasing accuracy and precision of the index. In conclusion, the current results, in combination with previous larval and non-larval cell cycle analysis investigations, have demonstrated that flow cytometric analysis of brain cell DNA content will provide an accurate and precise index of cell proliferation in larval brain tissue. However, strict guidelines pertaining to cell preparation, preservation, cytometric and collection parameters, as well as to the gating and modelling analysis of the cytometric data, must be followed to ensure precision and accuracy.

8.3.2 The growth parameter being measured is a suitable indicator of growth status of the whole larvae.

Theilacker and Shen (1993b, 2001) proposed that the fraction of cells in S and G2M phases (termed the fraction of cells dividing – FOCD), would provide a suitable index of cell proliferation in a larval teleost tissue, and that this index would accurately reflect the growth status of the larvae as a whole. This was in part based on cell culture evidence that suggested that cells having entered S phase will progress through the cell cycle independently of external conditions (Hartwell and Weinert, 1989; Murray and Kirschner, 1989) and either arrest in GO (Fernandes et al., 1997), or undergo apoptosis after nutrient depletion (Hale et al., 2000; Koyama et al., 2000). This is supported by evidence that nutrient deprivation can be used to synchronise cell cultures at G01
(Kumada et al., 1995). However, the in vivo results presented here, suggest that numerous suboptimal growth conditions, such as those associated with disease or suboptimal temperature, may cause an increased level in G2M with decreased S phase in brain tissue of larval fish. This pattern of response is highly indicative of cells arresting in G2M (Stewart et al., 1995; Martinez-Botas et al., 1999), and has been reported in fission yeast, in response to nutrient deprivation (references in Murray and Hunt, 1993). Furthermore, the fraction of cells in G2M generally showed little correlation with fraction of cells in S phase, and a lower correlation with overall mean growth rates than apparent for fraction of cells in S phase. Together these results suggest that the mechanisms controlling the progression of cells into and through each of these two phases differ in how they respond to environmental conditions and how they relate to overall growth. Consequently, combining S and G2M fractions does not represent the most precise measure of cell proliferation in brain tissue. Given its much closer correlation with overall growth rate (excepting temperature data, discussed later) the fraction of cells in S phase should be used as the index of cell proliferation in larval brain tissue. The following discussion will focus on fraction S as a growth index.

If the fraction of brain cells in S phase is to accurately reflect the growth of whole larvae, those factors influencing whole larval growth must similarly influence the fraction of cells entering S phase. The effects of varying salinity, photoperiod and of starvation are similarly reflected in both overall growth and the fraction of brain cells synthesising DNA, but this relationship was not apparent for larvae reared at different temperatures. In contrast to DNA synthesis in brain cells, overall somatic growth of older snapper larvae appeared to be accelerated by higher temperatures, despite higher mortality levels at these temperatures suggesting detrimental physiological effects were occurring. However, the upper temperatures at which the relationship between brain cell proliferation and overall growth became dissociated were much higher than larvae from local temperate stocks would naturally encounter or be adapted. Furthermore the appropriateness of length as a morphological growth measure might be questioned given that it may not necessarily indicate changes in larval mass. Numerous studies have shown that optimal temperatures for growth and for survival of teleost larvae generally differ (Bestgen, 1996; Hart et al., 1996; Rombough, 1996). The current results suggest that low survival was associated with low brain cell proliferation, but overall growth did not always hold the same relationship (i.e. high growth at high temperatures
was associated with reduced survival). This relationship will be discussed later. Overall, the fraction of brain cells in S phase appears to be a good indicator of larval growth, but the relationship between these two variables at different temperatures needs to be better defined.

The assumption that measuring cell proliferation will relate to the overall growth of a tissue or whole larvae, does not account for the possibility that the level of cell death (apoptosis) may vary also (Baserga, 1985; Bromhead, 1996). The fraction of proliferating cells will represent the level at which new cells are being added to the population. However, overall tissue growth is a balance between the addition of new cells and the loss of dying cells (Baserga, 1985). The current research identified a substantial population of apoptotic cells in the larval teleost brain, and then refined a protocol by which the level of apoptosis could be determined. However, subtracting the fraction of apoptotic cells (FAp) from the fraction of cells dividing (FOCD) showed less correlation to mean growth rates than did mean S phase fractions (see section 4.3.2.2).

There is some evidence that apoptosis may be induced as a normal part of development and modelling of the central nervous system in early stage vertebrates (Krueger et al., 1995; Blaschke et al., 1998; Clarke et al., 1998), but also induced by environmental stress such as lack of nutrition (Boza et al., 1999; Tessitore et al., 1999; Koyama et al., 2000). Hence apoptosis may be low when conditions are appropriate for maintenance only, but high when stressed, or when conditions are ideal for growth and development. This theory could explain why the net tissue growth index (NTGI=FOCD-FAp) does not correlate more closely with overall growth than does the fraction of cells in S phase. Cell proliferation (Fraction S) appears to have a simpler positive linear relationship with overall growth and therefore is, by itself, an appropriate index of tissue and overall larval growth.

The current results indicate that the fraction of cells synthesising DNA in larval brain is a suitable indicator of whole larva growth. In the nutrition experiment, the mean fraction S for starved and re-fed larvae correlates with mean growth rate, when calculated over three day periods. This supports the idea that cell proliferation should reflect past feeding history and current growth status (Theilacker and Shen, 1993b; Bromhead et al., 2000). However, the findings of Theilacker and Shen (2001) have also suggested that cell proliferation in larval brain may be defended partially from the
effects of sub-optimal growth conditions. A study of effects of starvation in walleye
pollock larvae demonstrated that the FOCD was lower in brain than in muscle for
feeding larvae, but higher in brain (compared to muscle) than in starved larvae. It is
tempting to suggest that this result may have been due to brain-specific cell cycle arrest
in G2M in response to starvation. However, evidence for arrest has been noted in
muscle (see Appendix II), and evidence for brain being similarly defended in fish has
come from other histological and biochemical studies (e.g. Theilacker, 1978; Houlihan
et al., 1988).

The following conclusion can be made regarding the suitability of measuring cell
proliferation levels in larval brain as indicators of whole larval growth. Firstly, the index
should be derived only from the fraction of cells in S phase, given that G2M cells
appear capable of arrest, and apoptotic responses appear complex and require further
research before their meaning in the context of net tissue growth can be accurately
interpreted. Secondly, the fraction of cells in S phase show high correlation with overall
growth. Overall, these findings suggest that brain tissue is a good indicator of whole
larval growth.

8.3.3 Growth rate is positively related to survival and recruitment success.

Only field-based studies will determine the suitability of cell proliferation indices in
assessing recruitment success. However, the results presented in Chapters 4, 5 and 7
suggest a relationship between the fraction of brain cells in S phase, and the survival
capacity of larvae subjected to varying salinities, temperatures or duration’s of
starvation. These results indicate that low mean fraction S and high G2M (signalling
arrest of cells in G2M) are associated with higher mortality levels. While S and overall
growth were not correlated in larvae subjected to a wide range of temperature
treatments, decreases in S phase fractions were apparent on those days and in those
treatments which showed higher mortality levels. Hence it would seem that
physiological condition and growth of brain might be more closely linked to survival
than overall growth, as measured by length. Given the critical role of the brain in
virtually every aspect of larval biology, from behaviour to physiology (e.g. Okimoto
and Stetson, 1999) it is not surprising that this might occur. Certainly the evidence
presented here suggests that this relationship might deserve further investigation.
8.4 SNAPPER (*P.auratus*): RELEVANCE OF FINDINGS FOR GROWTH AND SURVIVAL IN THE FIELD

This thesis has investigated the effects of numerous environmental factors upon brain cell proliferation, overall growth and (occasionally) survival, in larval snapper, and the following discussion draws this information together and discusses its relevance for possible elucidation of recruitment influences in this species, and in doing so highlight the potential of the technique for use in actual field recruitment studies. This species distribution ranges from the warm temperate or subtropical waters off Australia and New Zealand, up through Asia to China and Japan (Paulin, 1990). They are an important species for fisheries and aquaculture and have already been the focus of large scale restocking programs in Japan as well as recruitment studies off New Zealand (see Fukuhara, 1985; Francis, 1997).

Environmental factors have already been shown to play a large role in the growth rate of fish larvae (see Ferron and Leggett, 1994), a fact supported by the current findings for snapper larvae subjected to various temperature, salinity, photoperiod and nutrition regimes. Factors that reduce growth rate will lead to increased stage duration (see Leggett and Deblois, 1994). Thus the reduced growth rate for snapper larvae at lower salinities or lower temperatures was reflected in a lower percentage of larvae achieving flexion by the end of experiment. Increased stage duration in teleost larvae has been linked to higher predation and starvation susceptibility due to associated effects such as reduced swimming speed and foraging ability (Bailey and Yen, 1983; Yin and Blaxter, 1987; Bailey and Houde, 1989). Hence growth rate may be linked to survival probability and eventual recruitment success (Bailey and Houde, 1989) (See Chapter 1, Section 1.2.2).

Significant differences in snapper growth and fraction of cells dividing were generally only apparent for larvae reared in salinities that differed by greater than 10ppt. Older larvae appeared more sensitive to these effects. Significant differences in length and the fraction of brain cell synthesising DNA were apparent in larvae reared in temperature treatments that differed by only 3°C. Such changes in temperature can be encountered in the open ocean, however changes in salinity of 10 ppt or more are generally only
encountered in near coastal or estuarine environments. Given that mature snapper migrate to shallow coastal waters to spawn, and maturing larvae and juveniles are known to inhabit shallow bays and estuaries (see Hecht et al., 1996) such variation in environmental temperatures and salinities might be commonly influencing larval snapper growth, and possibly survival and recruitment, in their native environments. Fraction of brain cells synthesising DNA in larvae reared at different salinities was generally higher at around 25-35 ppt, which matches the optimal salinity range for survival in this species (Fielder et al., in preparation). Likewise, survival has been found to be higher at lower temperatures around 18°C for snapper larvae (Fielder et al., in preparation) again in good accordance with current brain cell synthesis data for this species.

Snapper larvae hatch relatively early and undeveloped (Pankhurst et al., 1991), feeding on zooplankton. In culture, larvae absorb their yolk sac by day 2 and oil globule by day 6 by which time external feeding has commenced (Battaglene and Talbot, 1992). This suggests that the window in which snapper larvae have to find a suitable food source is fairly small (Pankhurst et al., 1991). The current analysis of starvation did not examine starvation responses of snapper larvae during this critical period. However, the 20-day-old larvae examined still exhibited significant decrease in brain cell DNA synthesis (apparent before significant difference in lengths) along with increased mortality for larvae not fed for 3 days.

The relationship between the fraction of brain cells synthesising DNA and overall growth (as indicated by length) under variable temperatures, appeared to become uncoupled at higher temperatures. However, the adult snapper used to supply these larvae are from the local temperate stocks and it is unlikely that their larvae would naturally encounter or be adapted to temperatures in the range of 24-27°C or above. Removing these higher temperature data from the analyses markedly improved the correlation between growth and fraction S. Hence, under the normal temperature range this species would encounter in the field, the index is still sensitive to differences in growth rate. It is possible that a measure of cell proliferation in muscle, which might be expected to grow in line with increasing length, may relate even more closely to overall growth, however investigation is required to confirm this theory. Furthermore, growth
in brain appears more indicative of survival probability than overall length, adding to its potential value in recruitment studies. However, given variation associated with age and developmental status, it is clear that aside from monitoring environmental variables (i.e. temperature, salinity), analyses of field data should take into account larval age (otolith), size and developmental status. The fraction of snapper brain cells synthesising DNA (control) appeared to vary significantly for part of the diel analysis time period, but was otherwise relatively steady. Some of the variation was explained by gut content (feeding). However, it is difficult to interpret the significance of these results to larval growth in the field, as high mortality rates indicated that larval quality (and physiological condition) might have been poor. Furthermore, whether diel variation is specific to laboratory associated conditions, or is also apparent in the field, requires investigation. Theilacker and Shen (2001) found no evidence in field-sampled larvae for diel variation in muscle cell proliferation for walleye pollock sampled at sea.

8.5 FUTURE DIRECTIONS

The results presented in this thesis have highlighted the potential for flow cytometric analysis of cell proliferation in larval brain cells as a tool for assessing overall larval growth. However, it has also raised a number of issues pertaining to both the methodology and theory behind this approach which require further research. Advancing cytometric technologies will likely aid in future studies. The following points outline the key areas requiring further research to enable the future development and acceptance of cell cycle approach to estimating larval growth status:

1) Methodology: A study which compares modelling based estimation of cell cycle fractions against straight gating based estimates (i.e. setting a box gate around the S and G2M populations and determining the number of cells falling within this gate) is required. Such a study would determine if modelling based estimates are necessary, given that they appear to be biased depending on the number of cells in the sample (see Chapter 6).

2) Cell growth and proliferation: The current analyses provided strong evidence for cell cycle arrest in larval brain cells. This could be more conclusively
proven by an investigation in which starved and fed larvae were pulse labelled with bromodeoxyuridine (BrdU) (see Moore et al., 1994), a fluorescing uridine analogue which can be measured using flow cytometry. Such a study could also help to determine the length of the cell cycle in brain or other tissues, by assessing the time it takes for BrdU to progress through the cycle to mitosis. This would aid in determining the response time of the index to changes in growth conditions. Recent advances in cytometric technology may also allow the analysis of the proportion of growth that is attributable to hyperplasia or to hypertrophy, and how this might change with development. This could be achieved by preparing serial sections of larva, stained with propidium iodide, and analysing them using laser scanning cytometry (LSC). This technique combines the capabilities of histological sectioning with flow cytometry and would allow assessment of changes in cell size and cycle status.

3) Morphological growth comparisons: Length is not necessarily the most sensitive or appropriate index of overall larval growth. However, using mass based measures such as dry weight are not practical in association with flow cytometry, which requires wet tissue for analysis. However, a dry weight comparison might be possible if samples were dried and weighed, then fixed and stained, and cell proliferation levels analysed using serial sections and laser scanning cytometry. However, this approach is likely to add considerable processing time to what is meant to be a fast and simple fisheries tool.

4) Comparison of methodologies: Assessing cell proliferation is a very sensitive and appropriate approach to determining larval growth status. However, there are numerous methods by which cell proliferation can be assessed and a comparison of these would prove very useful in pinpointing the most sensitive and efficient method. Cell proliferation levels can now be assessed using the MPF spin filter assay of Westerman et al. (1999), or by analysis of cyclins using flow cytometry (Darzynkiewicz et al., 1994). Cyclin analysis appears likely to avoid complications that DNA based cell cycle analysis encounters with G2 arrest. Another methodological
comparison required is a parallel analysis of current fluorometric RNA/DNA techniques with cell proliferation methods, so as to more easily highlight the relative advantages and shortfalls of the two methods (Suthers and Bromhead; in preparation).

5) Target tissue: Theilacker and Shen (2001) presented some evidence to suggest that larval muscle tissue might be a more sensitive indicator of larval growth than is brain. However, muscle cell proliferation still needs to be assessed to determine if it is subject to cell cycle arrest, and how it is effected by temperature. There is some evidence that the relationship between hyperplasia and hypertrophy in larval muscle tissue is dependant on temperature (e.g. Hanel et al., 1996) and the implications for using this tissue to assess larval growth needs to be investigated.

6) Field investigations: Morphological indices of condition are considered problematic due to differences in morphology of field and laboratory reared larvae (see Ferron and Leggett, 1994). However, given that tissue form and mass arise from cell growth and division processes, the predominance of hyperplasia and hypertrophy in larval tissues might possibly differ between laboratory and wild individuals. This requires investigation. Whether diel variation in cell proliferation might simply be a product of conditions imposed in laboratory also requires investigation, as Theilacker and Shen (2001) found no evidence for diel variation in field caught walleye pollock larvae (muscle cell proliferation).

7) Environmental effects: Determining the effects of temperature and diel variation upon the relationship between cell proliferation and overall larval growth is critical to the final acceptance or rejection of this method as an appropriate tool for assessing the effects of environmental variables of larval growth, survival and recruitment success. As such, the effects of temperature and diel variability on cell proliferation and its interpretation in relation to overall larval growth require further investigation to determine if the results from this thesis were tissue specific (i.e. not relevant to other tissues such as muscle).
8.6 FINAL CONCLUSIONS

As Westerman et al. (1999) noted, the use of biochemical and metabolism based indices of growth, which currently are predominant in larval growth analyses, may be limited by the fact that they measure processes which can be, but are not always direct measures of actual somatic growth processes. In young developing organisms, cell proliferation is the predominant method by which somatic growth is achieved. The current results have demonstrated that flow cytometric cell cycle analysis provides an accurate and sensitive measure of the proportion of cells in G01, S and G2M phases of the cell cycle, providing that strict methodological criteria are met. In doing so, it has been shown that, firstly, larval brain cells may arrest in G2M in response to certain environmental conditions and subsequently that the fraction of cells in S phase offers a more appropriate index of larval growth than does previous measures which have incorporated the G2M fraction (Theilacker and Shen, 1993; Theilacker, 1993; Bromhead, 1996; Bromhead et al., 2000; Theilacker and Shen, 2001). This has been further confirmed by a close correlation between the fraction of larval brain cells synthesising DNA and larval growth rate (as measured by increases in mean length). Overall, the method presented in this thesis represents a highly sensitive and appropriate tool for use in larval growth studies, such as might be incorporated in recruitment analyses of wild fish stocks.
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APPENDIX I – THE EFFECT OF DIFFERENT FREEZING AND DISSOCIATION METHODS ON SUBSEQUENT WHOLE BRAIN CELL RECOVERY

INTRODUCTION AND AIM

Experiments that involve intensive sampling regimes may not always allow time for the investigator(s) to dissect larval tissues out prior to preserving. Bromhead (1996) used snap freezing of larvae in liquid nitrogen while sampling in the field. However, the method by which cells are preserved will effect the subsequent cell recovery level (the number of whole intact cells available to be analysed by flow cytometry from each larva). It would be useful to determine whether the level of cell recovery possible from snap frozen whole larvae differs greatly from that attained using dissociation of cells in cryoprotectant (Theilacker and Shen, 1993).

The method by which cells are dissociated can also affect cell recovery levels. Bromhead (1996) forced brain tissue through a 60 µm filter cup, while Theilacker and Shen (1993) used a pipette to dissociate fresh brain cells. A comparison of cell recovery levels between these two methods would aid in finalising a protocol for the preparation of larval brain cells for analysis by flow cytometry.

METHODS

The first experiment preserved 5 snapper larvae by snap freezing in 1 mL of 35 ppt tank water in cryotubes in liquid nitrogen. Five more larvae from the same tank had their brains dissected out and dissociated in 200 µL of cryoprotectant (16.667 mL FCS, 16.667 mL dimethyl sulfoxide (DMSO) and 66.667 mL of Eagles Minimum Essential Medium (EMEM) to make a 100 mL stock solution). These were then frozen at -20°C (Theilacker and Shen, 1993b). One week later, samples were thawed, and dissociated brain preparations stained with propidium iodide and analysed by flow according to
protocol of Bromhead et al. (2000). The data was then analysed for the relative proportion of debris to whole cells, as a measure of cell recovery. A second experiment simply involved analysing recovery of brain cells from frozen whole larvae 12 months after initial freezing, and comparing these with 1 month frozen samples.

To determine the best method by which to dissociate larval brain cells, the brains from 10 golden perch larvae (from single tank) were dissected out. Five brains were then combined and then dissociated by filter cup method of Bromhead et al. (2000) and 5 by the trituration method of Theilacker and Shen (1993b). The two samples were then fixed and later stained with PI for flow cytometric analysis (as in Section 2.3.3). Collected data was then analysed by Modfit for effects of dissociative method upon relative cell cycle fraction and overall cell recovery.

**RESULTS AND DISCUSSION**

Snap freezing of whole larvae, when compared to slow frozen cryoprotected and dissociated cells, showed a significant decrease of around 7% in the fraction of whole cells recoverable for flow cytometric analysis (ANOVA, n=20, F=14.1, p=.0014). Mean recovery for cryoprotected and dissociated cells was 85.75% (± 0.85SE) compared to 78.65% (±1.68SE) for snap frozen whole larvae. Cell recovery fractions were down to less than 40% in all snap frozen whole larval samples after 12 months, stored at -75°C. Dissociating larval brain cells using the filter cup method of Bromhead et al. (2000) resulted in a lower whole cell recovery, as well as lower G2M, S and possibly apoptotic fractions (Figure 1), when compared to the trituration dissociative method of Theilacker and Shen (1993b).

The decrease in cell recovery by 7% for whole frozen larvae still allows a reasonably high cell recovery rate, and for larvae of sufficient size, this remains an efficient method for use in large-scale, rapid and field-based sampling. However, samples are not as stable for long periods as those dissociated and preserved in cryoprotectant, and therefore, if samples will not be analysed for many months after collection, they should be dissociated and preserved in cryoprotectant following the protocol of Theilacker and Shen (1993). The method by which cells are dissociated also clearly effects the whole cells recovery levels, and may also selectively fragment cells in particular stages of the
cell cycle. Using the trituration method appears to be the most gentle and efficient in small larvae.

Figure 1 – Density plots the effects of a) trituration and b) meshing (70 µm filter cup) upon whole cell recovery and upon cell cycle fractions in brain tissue isolated from golden perch larvae. FL2-W is the width if the fluorescence pulse and FL2-A the area under the pulse. It would appear that meshing reduces the number of intact cells dissociated (per 10000 events) and selectively fragments apoptotic, S and G2M cells.

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APPENDIX II – THE EFFECT OF SALINITY UPON MUSCLE NUCLEI PROLIFERATION IN SNAPPER (PAGRUS AURATUS) LARVAE.

INTRODUCTION

Muscle is considered the most appropriate single tissue for the determination of growth and condition in fish, due to its large mass and positive allometry to body size (Bone, 1978), and its function as an energy store and primary tissue source of protein during starvation (Loughna and Goldspink, 1984). In addition, muscle has high RNA levels and protein retention efficiency (Houlihan et al., 1988), and muscle protein synthesis rates have been shown to be sensitive to starvation (Smith, 1981). In larvae, muscle mass can increase by over 25%/d in some species (Alami-Durante et al., 1997). Furthermore, early stage muscle growth has been shown to influence size and growth rate in subsequent stages (Johnston et al., 1998; Galloway et al., 1999).

In mammals and birds, recruitment of new fibres to muscle (hyperplasia) is typically absent post-natal, except in the case of injury (Bischoff, 1986). However, in fish, both hypertrophy (increase in fibre size) and hyperplasia play a role from hatching (Galloway et al., 1999) right through to adulthood, and possibly also a third process, fibre splitting (Koumans et al., 1993). However the structure of muscle in larvae and adults differs considerably. The three fibre types (white, red and pink) only become evident as the larval stage progresses (Galloway et al., 1999). Both hypertrophy and hyperplasia are reliant on a myogenic population of precursor muscle cells, the myoblasts, which can be divided into embryonic/foetal myoblasts which fuse to form myotubes (hyperplasia), and adult myoblasts or satellite cells, which donate nuclei to fibres (Feldman and Stockdale, 1992; Koumans and Akster, 1995). This allows maintenance of the cytoplasm:nuclei ratio which limits muscle fibre growth (Koumans and Akster, 1995). Satellite cells are located between the sarcolemma and the basal lamina of muscle fibres (Bischoff, 1986). Which process contributes more to overall muscle growth depends on the species (Alami-Durante et al., 1997), developmental stage (Gibson and Johnston, 1995; Alami-Durante et al., 1997; Killeen et al., 1999)
environmental factors (Johnston, 1993; Gibson and Johnston, 1995; Johnston and McLay, 1997; Matschak et al., 1998; Johnston, 1999) and parental origin (Johnston and McLay, 1997). Some species larvae appear to achieve early growth predominantly via recruitment of new fibres (Weatherly et al., 1980; Matsuoka and Iwai, 1984; Alami-Durante et al., 1997) while others achieve growth predominantly through hypertrophy (Veggetti et al., 1993). The regulatory mechanisms that govern these two processes are still little understood (Koumans et al., 1991), although hyperplastic stimulation in muscle has been linked to growth hormone in fish (Fauconneau et al., 1997) and IGFs in mammals (Lawlor et al., 2000). In white muscle, new fibres appear between the larger pre-existing fibres (Koumans and Akster, 1995). It is generally agreed however that species with large adult size achieve a large proportion of their early muscle growth through hyperplasia. Fish that have high hyperplasia, grow faster and reach a greater length than fish growing mainly by hypertrophy (Weatherly, 1990). Hyperplasia decreases with increasing length in studies of juvenile carp muscle (Koumans et al., 1993).

The ability to determine the growth status of muscle tissue by analysis of cell proliferation has been greatly enhanced in the last decade by the development of techniques to both isolate muscle nuclei (Pinset and Montarras, 1994; Theilacker and Shen, 2001), culture muscle tissue (Bischoff, 1986; Hagiwara and Ozawa, 1994; Fauconneau and Paboeuf, 2000), and label cycling cells in vivo (Johnston et al., 1995; Fauconneau and Paboeuf, 2000). However, while numerous studies have demonstrated that protein and RNA levels in larval muscle are sensitive to starvation, studies of the effect of nutrient deprivation upon muscle cell proliferation in larvae are relatively scarce. Fauconneau and Paboeuf (2000) extracted and cultured muscle tissue from juvenile rainbow trout, and subjected these cultures to serum deprivation. They then used BrdU labelling and nuclei counts to create a proliferation index. However, the toxic nature of BrdU means that it can not be reliably applied in vivo (although see Johnston et al., 1995), and it does not allow assessment of proliferation in wild samples. As discussed earlier, Theilacker and Shen (2001) used flow cytometric analysis of cell cycle to determine the fraction of dividing muscle nuclei in starved and fed walleye pollock larvae, with good success. This approach appears likely to yield the best indices of muscle proliferation, which can be used non-invasively.
As discussed in the introduction, muscle tissue is also an appropriate candidate for assessment of nutritional condition and growth. In this regard a cell proliferation study of muscle growth requires the development of a protocol by which sufficient muscle nuclei can be isolated for flow cytometric analysis. Numerous methods have been used for isolation of muscle nuclei from vertebrate tissues, ranging from mechanical to enzymatic digestion (Kuehl, 1975; Pinset and Montarras, 1994). Only very recently have they been applied to isolating nuclei from larval fish (Theilacker and Shen, 2001). Such a method could prove exceedingly valuable for the development of a cell proliferation index as being investigated here. The current analysis presents a method by which to isolate muscle nuclei and analyse by flow cytometry. It aims to determine the effect of salinity upon larval growth and muscle nuclei proliferation.

**METHODS**

This experiment used the same larvae that were analysed in Chapter 4 for cell proliferation in brain tissue. All rearing, maintenance and experimental design details are described in Chapter 4 (Section 4.2).

**Isolation and staining of muscle nuclei**

Muscle nuclei were isolated from thawed snapper larvae using a modification of the method used by (Hahn and Covault, 1990) for mouse muscle nuclei. Briefly, connective tissue (skin, tail and fin spines etc) was removed from muscle using 30G needles, on a well slide in cold muscle nuclei isolating buffer (MNIB) (see Hahn and Covault, 1990). Muscle was then minced into <1 mm pieces with needle, scalpel or scissors in cold MNIB. Fragments were transferred into a 5 mL FACs tube containing 2.5 mL cold MNIB, using fine forceps and pasteur pipette. Muscle was homogenised in MNIB using motor driven blade (small blade 8G) at settings low, low-medium and medium, for 15 seconds each. The homogenate was then centrifuged at 3000rpm (Beckman JA-20 rotor) for 5 minutes. The supernatant was poured off and pellet resuspended in 2.5 mL MNIB for 15 secs at medium setting. 2.5 mL of MNIB (10 mL total) was then added. 50 µL of Triton X-100 [0.5% (v/v)] was mixed into the suspension using pasteur pipette trituration. The homogenate filtered through a 70 µm filter cups (nylon mesh)
into a 5 mL FACs tube and spun down at 2000 rpm for 5 minutes. The supernatant was then poured off, 4 mL PBS added, and the resulting suspension centrifuged at 2000 rpm for 5 minutes. This PBS wash was repeated twice, spun down and the nuclei resuspended in 400 µL PBS, 50 µL RNAse and 50 µL propidium iodide (PI). The use of ethanol fixation was not required given that nuclei are permeable to PI without this treatment step (Robinson, 1993). Flow cytometric settings determined as ideal for the initial examination of muscle nuclei data are shown in Table 1. Flow rates and cell concentrations for muscle nuclei analysis were based on those determined from brain cells from Chapter 3.

Table 1 - The following flow set-up regimes were determined as the ideal settings for the acquisition of data for PI stained muscle nuclei.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Muscle cells (PI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytometer</td>
<td>FACScan</td>
</tr>
<tr>
<td>Laser Power (mW)</td>
<td>15.15</td>
</tr>
<tr>
<td>Laser Current (A)</td>
<td>5.50</td>
</tr>
<tr>
<td>Sample Pressure (V)</td>
<td>6.03</td>
</tr>
<tr>
<td>Threshold parameter &amp; level</td>
<td>FL2 - 16</td>
</tr>
<tr>
<td>FSC detector</td>
<td>E00</td>
</tr>
<tr>
<td>SSC PMT voltage</td>
<td>315</td>
</tr>
<tr>
<td>FL1 PMT voltage</td>
<td>580</td>
</tr>
<tr>
<td>FL2 PMT voltage</td>
<td>541</td>
</tr>
<tr>
<td>FL3 PMT voltage</td>
<td>150</td>
</tr>
<tr>
<td>FSC amplifier</td>
<td>LOG</td>
</tr>
<tr>
<td>SSC amplifier</td>
<td>LOG</td>
</tr>
<tr>
<td>FL1 amplifier</td>
<td>1.00</td>
</tr>
<tr>
<td>FL2 amplifier</td>
<td>1.00</td>
</tr>
<tr>
<td>FL3 amplifier</td>
<td>1.00</td>
</tr>
<tr>
<td>DDM parameter</td>
<td>FL2</td>
</tr>
<tr>
<td>FL-x area gain</td>
<td>1.00</td>
</tr>
<tr>
<td>FL-x width gain</td>
<td>9.99</td>
</tr>
<tr>
<td>All Compensations (e.g., FL1-%FL2)</td>
<td>0.0</td>
</tr>
</tbody>
</table>
Data analysis

Muscle cell nuclei analysis followed the same basic protocol as that for brain cells (Chapter 2), with the exception that no gating was required for circulatory population, which does not appear to be present in these samples. This is most likely due to the initial mincing of muscle tissue which would act to “bleed” any circulatory cells into the large initial dissection volume, before removal of these pieces to fresh buffer.

Initial investigations indicated that some nuclei were not completely detaching from muscle fibre fragments during processing. These fibre fragments appeared to have had a nominal amount of PI uptake, such that a shoulder formed on the left side of the G01 peak (Figure 1). This population of G01 nuclei modelled as S phase, and so it was determined that nuclei/fibre complexes would be gated from the analysis (Figure 1). A typical DNA frequency histogram as generated by PI stained muscle nuclei, using the current protocol and collection parameters, is presented in Figure 1.

Models

- **FOCD**
  - Fixed model: Salinity + age + (salinity\times age).
  - Random model: Tanks

- **S**
  - Fixed model: Salinity + length + age + (salinity\times age).
  - Random model: Tanks

- **G2M**
  - Fixed model: Salinity + age.
  - Random model: Tanks
Figure 1 – Muscle nuclei preparations exhibits a population of events staining at a G01 intensity but with larger size, and showing a slight “lean” in their staining as they got larger (A). This population, identified by fluorescent microscopy as nuclei still attached to fibre fragments, created a distinct shoulder on the G01 peak (B). This shoulder models as S phase, even though they are clearly G01. Gating out all larger “non-nuclei” fragments yields a much cleaner histogram with improved CV (C).
RESULTS

Cell proliferation

REML analysis determined there is a significant interactive effect of both larval age and rearing salinity upon the proportion of dividing muscle nuclei in larvae of *Pagrus auratus* (CID=26.86, df=12, p=0.0046). Mean proportion of dividing muscle nuclei was lower in older larvae but there is little consistent pattern and few significant differences in mean FOCD between treatments (salinity levels) (Figure 2A). Age by itself had a significant effect upon mean muscle FOCD (CID=213, df=2, p=0.001). With the exception of 10 and 15 ppt larvae, mean FOCD was significantly higher on day 15 when compared to day 12 larvae. Mean FOCD then decreased very significantly for all treatment levels by day 18 and remained at this level to day 21.

Salinity by itself had no significant effect on mean FOCD (CID=14.3, df=12, p>0.1), but the salinity by age interactive term shows a significant effect. There is no significant difference between the mean FOCD between treatments on day 12 while only 20 and 45ppt treatments differ significantly from each other on day 15. On day 18, only 10ppt larvae have significantly higher FOCD than larvae in any other treatments (20, 35 and 45 ppt). However, 10, 15 and 20 ppt all show significantly higher mean FOCD than 30 and 35 ppt larvae after 21 days. Overall it can be seen that the salinity at which mean FOCD is highest varies depending on the age of the larvae. There is little significant difference in the proportion of dividing nuclei in younger larvae but older larvae appear to achieve the highest division at lower salinity levels.

The mean fraction of cells in S phase tends to mimic the patterns displayed by FOCD (Figure 3). There is also, after variation due to age and salinity age interaction is taken into account, a weakly significant length effect (CID 8.796; df=2; p=0.0123). This would appear to indicate that the fraction of cells in S decreases as larval length increases (over the size range studied) (Figure 4). There is no significant difference in the mean FOCD for 12 and 15 day old larvae, but a very significant decrease between day 15 and 18. With the exception of 30 ppt treatment, there is no significant change in FOCD between day 18 and 21 (Figure 2B). Up to day 15, mean S is higher (>0.10)
Figure 2 – REML based predicted means for the; A) fraction of cells dividing (FOCD); and B) fraction of cells in S phase, for nuclei isolated from muscle of larval Pagrus auratus reared at 7 salinities and sampled 12, 15, 18 and 21 days after hatching.
Figure 3 – Comparison of cell cycle fractions of muscle nuclei isolated from larval Pagrus auratus which had been reared at salinities ranging from 10 ppt to 45 ppt and sampled at ages 12, 15, 18, and 21. Error bars are +/-2SE.
Figure 4—Comparison of *Pagrus auratus* larval size (standard length) with the fraction of larval muscle nuclei in S, G2M or both (FOCD) for larvae reared at different salinities (using pooled samples ages 12, 15, 18 and 21).
than mean G2M (<0.10). However, on day 18 there is little difference between mean levels and on day 21, the fraction of cells in S phase is significantly lower than fraction in G2M for larvae reared at 25 ppt or above (Figure 3).

A comparison of the fraction of nuclei either dividing (FOCD), in S or in G2M, with larval length (for larvae reared at each salinity level) (Figure 4), demonstrates 3 main trends. Firstly, with the exception of larvae reared at 10 ppt, FOCD and S fractions decrease as larval length increases. There is very little change in G2M with respect to larval length.

**Comparison between tissues**

Correlation between respective levels of S, G2M and FOCD in brain cells and muscle nuclei is very low in each instance (Figure 5). Of these, the highest correlation, and only significant relationship, is evident for the fraction of muscle and brain cells in S phase (ANOVA, F=16.22, p=0.0001, r²=0.063).

Mean growth rate (mm/d) is compared with FOCD, S and G2M in muscle, and two main trends are evident (Figure 6). Mean FOCD (F=20.30, p=0.0001, r²=0.4385) and S (F=22.08, p=0.0001, r²=0.4593) increase as larval growth rate increases. Mean G2M shows no significant relationship with mean growth rate.

The fraction of muscle nuclei in G01 shows a close negative correlation with fraction S (ANOVA, F=2804, p=0.0000, r²=0.918), while the fraction of G2M cells is also negatively correlated with G01 (ANOVA, F=62.39, p=0.0000, r²=0.207) (Figure 7). There is no significant relationship between fraction of cells in S and G2M (ANOVA, F=8.39, p=0.0041, r²=0.033). Apoptosis was not investigated in muscle tissue.
Figure 5 – Comparison of brain and muscle cell cycle fractions of *Pagrus auratus* larvae. There is little significant correlation between the fraction of cells in S phase (A: $r^2 = 0.063$), G2M phase (B: $r^2 = 0.004$) or in both combined (C: $r^2 = 0.014$).
Figure 6 – Comparison of mean growth rates with mean cell cycle fractions for muscle nuclei isolated from *Pagrus auratus* larvae. Each point represents the mean for a single sampling of larvae from a particular age and salinity treatment. $R^2$ values are placed at end of each regression line.
Figure 7 - Comparison of the fraction of *Pagrus auratus* muscle nuclei in different stages of the cell cycle. A) S and G01 ($r^2 = 0.918$). B) G2M and G01 ($r^2 = 0.207$). C) S and G2M ($r^2 = 0.033$).
DISCUSSION

Analyses of muscle nuclei data revealed some unexpected results. Firstly, the correlation between mean FOCD, or S, and growth rate was substantially lower in muscle when compared to brain (see Chapter 4). Secondly, there was less significant effect of salinity upon muscle nuclei proliferation, generally, with no effect of salinity until day 18, and only a few instances of significant differences in division between salinities on days 18 and 21. These differences on day 18 and 21 show that muscle nuclei proliferation was higher at the lower salinities, in particular at 10 ppt. This trend contrasts directly with that seen in brain tissue, in which cell proliferation was highest in larvae reared at 30 ppt. Finally, comparison of brain FOCD, S and G2 fractions with the respective fractions from muscle showed little correlation.

The evidence presented for brain FOCD suggests that brain tissue growth reacts to salinity in line with growth and development of the whole larvae. At first inspection the proportion of dividing muscle nuclei does not! There are a number of possible explanations for these trends and the lack of correlation between the two tissues. Firstly, the technique used to dissociate muscle nuclei did not yield particularly clean preparations. These were characterised by significant debris and clumping, increasing the likelihood of misclassification of cells and lowering modelling precision. Incomplete dissociation of nuclei from fibre fragments effected staining (see methods) and led to gating removal of this population. Such data manipulation may also reduce the sensitivity of the index. However, it may simply be that muscle tissue is less effected by differences in environmental salinity; i.e. it may have a much higher tolerance level that brain tissue. Where salinity related differences were evident, these occurred in older larvae, further supporting the conclusions from brain analysis that there is some ontogenetic variation in the salinity tolerance of snapper.

An explanation for the occurrence of higher mean muscle FOCD at lower salinities may rest in the nature of larval muscle growth and how it changes in response to environmental conditions. It is unexpected that muscle nuclei proliferation is less sensitive to the effects of salinity and that it shows a lower correlation with mean growth than brain FOCD. It shows a much higher proportion of cells dividing and
therefore might be expected to be more sensitive (i.e. show greater latitude in variation possible etc) to the effects of factors upon growth. In addition it is the largest, fastest growing tissue mass which has been previously shown to have a close allometry with body size in fish. In contrast to the results presented here, a recent paper by Theilacker and Shen (2001) demonstrated that cell proliferation in muscle tissue is closely aligned with overall larval growth. Therefore the current results may be more due to the comparatively unrefined nature of the technique used to process the muscle nuclei and tissue.

Summary

Muscle cell proliferation appeared less sensitive to the effects of salinity, and while showing reasonable correlation with overall growth rate (S=0.41), did not indicate 30 ppt to be optimal, contrasting with the conclusions gained from the brain cell proliferation analysis and developmental and length data (Chapter 4). There was little correlation between fraction S in muscle and in brain. The suitability and sensitivity of the methods applied to determine muscle cell cycle fractions have been seriously questioned.

REFERENCES


Appendix II

Muscle Analysis


