Metabolism and Physiology During Ontogeny of Cultured Yellowtail Kingfish (*Seriola lalandi* Carangidae)

by

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Abstract

Various aspects of metabolism and physiology were investigated during the ontogeny of yellowtail kingfish (*Seriola lalandi*), a fish of growing aquaculture importance in both New Zealand and other countries. Incubation experiments between 18-24°C showed that developing eggs and larvae were heavily influenced by temperature. It appeared that at warmer temperatures larvae hatched smaller but grew on the yolk sac, whereas at cooler temperatures larvae grew inside the chorion. Oxygen consumption data supported this, with a negative correlation found between total embryonic oxygen consumption and temperature. A mechanism was proposed to explain the differential effect of temperature on ontogeny and growth. Like other marine fish with pelagic eggs, yellowtail kingfish were found to be heavily reliant on free amino acids as a source of energy. At 23°C the pattern of substrate utilisation in eggs was considerably different from that at 17-21°C, indicating that 23°C exceeded the tolerance for normal development. Inter-individual aggression by large individuals was associated with the development of size heterogeneity in juveniles. Although this aggression also affected the survival of smaller juveniles, it was not the primary agent of much of the mortality that occurs during this phase, as many of these individuals were on a degenerate developmental trajectory. Yellowtail kingfish fingerlings used for ongrowing were robust to the stressors imposed by live transport. The ontogenetic development of metabolic rate from 0.6 mg-2.2 kg did not follow the same scaling exponent as that observed for mammalian models of allometry, and has implications for interspecific studies of mass-dependent metabolism.

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List of Abbreviations

AIC	Akaike information criterion
ANOVA	analysis of variance
BP	break point
DPH	days post hatch
HPF	hours post fertilisation
LS	least squares
NIWA	National Institute of Water and Atmospheric Research
$\dot{M}O_2$	mass oxygen consumption rate (e.g. mg O_2/unit time)
$\dot{N}NH_3$	molar ammonia excretion rate (e.g. mmol NH_3/unit time)
$\dot{N}O_2$	molar oxygen consumption rate (e.g. nmol O_2/unit time
NQ	nitrogen quotient
RSS	residual sum of squares
SIC	Schwarz information criterion
$\dot{V}O_2$	volumetric oxygen consumption rate (e.g. ml O_2/unit time)

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Chapter 1: General Introduction

General overview of this thesis

This thesis describes how particular aspects of metabolism are related to ontogeny in cultured yellowtail kingfish, *Seriola lalandi*, Valenciennes 1883, Carangidae. A literal translation of ontogeny is 'the mode of production of a form' (Oxford English Dictionary, 2nd Ed), and relates to the physiological, morphological and behavioural development of a species within a lifetime. Fish are well suited for use in the study of ontogeny as they are a speciose and diverse lineage, and most develop from an egg spawned into the environment with little parental care thereafter. Such traits allow researchers to investigate a myriad of variables that can affect how an individual grows, adapts and reproduces, right from the point fertilisation. The same breadth of ontogeny cannot easily be investigated in most other vertebrates where early development occurs in an amniotic egg or *in utero*. An additional advantage in using fish to study ontogeny is that rapid growth rates may allow manipulative experiments to be carried out over a relatively short period of time.

The rapid growth rate of yellowtail kingfish is one attribute that has prompted the recent development of the culture of this species in New Zealand. Combined with a high market value, yellowtail kingfish are one of the few finfish species that represent a viable diversification away from salmonid culture in this country. Aquaculture of yellowtail kingfish in New Zealand is reliant on hatchery reared juveniles produced at the National Institute of Water and Atmospheric (NIWA) Research Ltd Bream Bay Aquaculture Park. An understanding of the developmental biology of this species is vital not only to secure a constant supply of high quality juveniles for grow-out, but also to ensure that ongrowing operations are both profitable and sustainable. For these reasons it is important that there is a thorough knowledge of how the metabolism of yellowtail kingfish changes during ontogeny, hence the impetus for this thesis.

There are two main themes addressed within this thesis. The first is an ecophysiological interpretation of the ontogeny of metabolism in yellowtail kingfish. Thus far, salmonids have been the model species used to develop much of our understanding of fish physiology, though there is a growing body of literature on the physiology of non-salmonid species. The findings of this thesis are compared and contrasted to that which is known for some other species, and are discussed with respect to the less well understood ecophysiology of yellowtail kingfish. The second theme of this thesis is the relevance of these investigations to the culture of yellowtail

kingfish and finfish in general. While all the findings have some practical value in improving commercial production, two chapters (4 and 5) deal specifically with overcoming problems in animal husbandry associated with metabolism at particular ontogenetic stages. In order to put many of the findings of this thesis into an ecophysiological and aquaculture context, it is first necessary to give an overview of what is known about yellowtail kingfish and the culture of this species.

Yellowtail kingfish biology and culture

Yellowtail kingfish (Figure 1) are members of the Family Carangidae (jacks and pompanos), which are fast swimming predators of the reef or open sea, and are generally characterised by compressed, streamlined bodies with high aspect ratio tails (Nelson, 1984). Yellowtail kingfish have a circumglobal distribution, are fast growing and can reach over a metre in length (Gillanders et al., 1999). There is relatively little known about the general biology of this species compared to sister species such as Japanese yellowtail (*S. quinqueradiata*), due mainly to the fact that the latter are more important to aquaculture and have therefore received more research effort.

Figure 1. Adult yellowtail kingfish (approximately 90 cm in length).

Virtually all of the research carried out to date on yellowtail kingfish has been related to the culture or fishery of this species. Poortenaar et al. (2001) studied the seasonal variation in gonadal condition and reproductive endocrinology of wild fish in order to better understand the reproductive physiology of broodstock. Individuals of this species

were found to spawn multiple times during the austral summer months, and generally reach sexual maturity around 80-90 cm (Poortenaar et al., 2001). Growth is rapid, with fish reaching 30-50 cm (2-3 kg) in the first year in the wild (Gillanders et al., 1999), a useful attribute for culture. Yellowtail kingfish eggs are positively buoyant (Smith, 2004), approximately 1.2-1.4 mm in diameter (Tachihara et al., 1997) and possess a single oil droplet (Figure 2, Hilton, 2002).

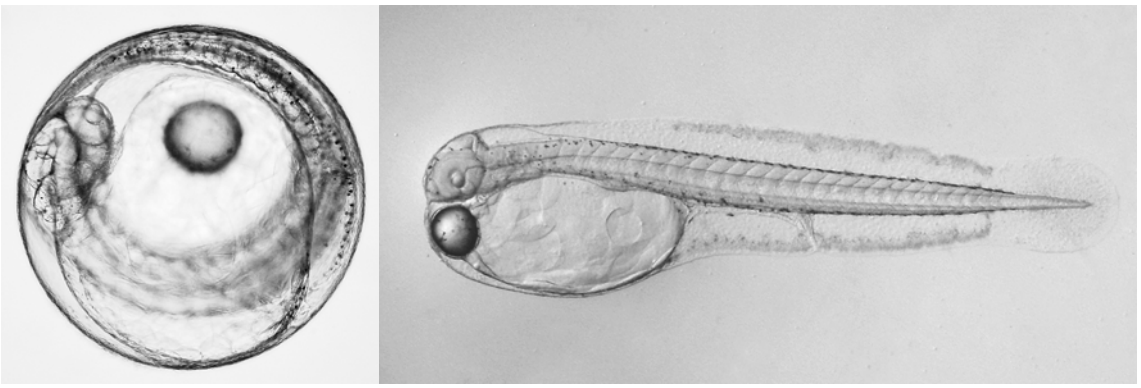

Figure 2. Yellowtail kingfish egg (~1 mm diameter) and hatched, pre-feeding larva (~5 mm total length).

The development of eggs is similar to that of most teleosts, with larvae hatching at 3-5 days post-fertilisation depending on incubation temperature (Figure 2, Smith, 2004). A study by Hilton (2002) found lipids to be an important energy substrate during the first feeding period, while protein was not. Water turbidity is an important determinant of larval feeding success in yellowtail kingfish (Carton, 2005), and larval and juvenile growth is rapid. The development of the gastrointestinal tract is complete by around 25 days post-hatch (DPH), one of the fastest rates found in fish to date (Chen et al., 2006a; Chen et al., 2006b). The rapid growth of juvenile yellowtail kingfish is correlated with the development of intense intra-cohort aggression and cannibalism, and represents a significant problem to the culture of this species (Ebisu & Tachihara, 1993). The exceptional growth rate of yellowtail kingfish is reflected in a high oxygen consumption rate (Partridge et al., 2003), which approaches that of some tuna species (Bushnell & Jones, 1994).

Figure 3. Example of intra-cohort aggression and size heterogeneity in yellowtail kingfish. All juveniles are 39 days post-hatch and the aggressor is approximately 25 mm in length.

The culture of yellowtail kingfish and most other *Seriola* spp. (Japanese yellowtail *S. quinqueradiata* Temminck & Schlegel 1845, goldstriped amberjack *S. auerovittata* Valenciennes 1833 and amberjack *S. dumerili* Risso 1810) occurs primarily in Japan, where fish are harvested at 2-5 kg and are sold mostly as sashimi for US$10-30 kg^{-1} (Nakada, 2002). All *Seriola* spp. are similarly accepted by the Japanese market, where product quality is generally more important than species (Nakada, 2002). According to the United Nations Food and Agriculture Organisation, Japanese yellowtail aquaculture generated nearly US$13 billion dollars globally in 2004, while other *Seriola* spp. generated around US$16 million dollars (FAO, 2006). In comparison, total New Zealand aquaculture production, which is composed mainly of green lipped mussel (*Perna canaliculus* Gmelin 1791), was worth US$120 million dollars in the same period (FAO, 2006). More recently, kona kampachi (*S. rivoliana* Valenciennes 1833) aquaculture has developed in Hawaii, and yellowtail kingfish are now produced in south Australia (Stehr Group, Port Lincoln) and New Zealand (NIWA Bream Bay Aquaculture Park). Yellowtail kingfish production in Australia and New Zealand is reliant on hatchery-reared juveniles for ongrowing as it is almost impossible to find individuals in the wild, whereas in Japan juveniles can be captured under floating kelp in some of the large sheltered bays (Nakada, 2002).

The viability of yellowtail kingfish aquaculture in New Zealand is contingent on a number of factors, the most important of which are market acceptance of the product and profitability. There is obviously already a strong market developed in Japan for

Seriola spp., but most production from Australia and New Zealand to date has been sold to American or European restaurant distributors. Anecdotal evidence suggests that this is a relatively large market with high returns, and there is positive feedback about product quality. Given that there appears to be a secured market and economic price for yellowtail kingfish, the next most important factor in production is securing the supply chain. This involves a constant level of production and quality that can be scaled up to meet market demands, which is in turn reliant on efficient juvenile supply and ongrowing. The lack of detailed information on the biology of yellowtail kingfish makes improving production quantity and quality difficult, hence the recent surge in research on this species (e.g. Carton, 2005; Chen et al., 2006a; Chen et al., 2006b; Ebisu & Tachihara, 1993; Hilton, 2002; Partridge et al., 2003; Smith, 2004). The information contained within this thesis is an important addition to this growing body of knowledge, providing details of how key physiological and metabolic characteristics change throughout ontogeny, from eggs through to adults.

Background to experiments in this thesis

There are five main experimental components to this thesis, each of which has been arranged into a chapter, and broadly follows the ontogeny of yellowtail kingfish. Chapter 2 investigates the effect of temperature on the morphological development of eggs and larvae. Temperature is probably the most important abiotic variable that affects embryonic development, both in the hatchery and in the wild. Some important findings are presented concerning the extent to which incubation temperature affects the rate of development and larval size at hatch and first feeding. A novel hypothesis is presented to explain the results, which involves temperature differentially affecting the rate of ontogeny and growth. Chapter 3 expands on this work by quantifying how embryonic metabolism and biochemical substrate use differs at a variety of incubation temperatures. The standard oxygen consumption rate was used as a measure of total metabolic turnover, the results of which were interesting, though somewhat unexpected. This required a reconsideration of traditional approaches used to interpret temperature-dependent physiological development. Marine fish with pelagic eggs are known to be heavily reliant on free amino acids (Fyhn, 1989), and lipid content is known be invariant in yellowtail kingfish during embryogenesis (Hilton, 2002). Given this, the focus of Chapter 2 was to quantify the pattern of free amino acids, protein and carbohydrate flux during development, as this would allow definitive statements to be

made about the pattern of substrate utilisation during embryogenesis, and how temperature might affect it.

Chapter 4 characterises how juvenile growth rates are affected by individual growth variation and aggression. The initial few weeks of development sees vast differences in growth rate between juvenile yellowtail kingfish of the same age (Figure 3), along with the development of intra-cohort aggression. Experiments were designed to separate the effect of these variables in order to elucidate the relative contribution of inherent growth differences and aggressive tendencies to the development of size heterogeneity within a cohort. Chapter 5 investigates the tolerance of juveniles to live transport, which represents one of the most intense stressors cultured fish encounter. One of the key limiting factors identified in improving live transports was the accumulation of carbon dioxide (CO_2) in a closed system. Two experiments were constructed to establish how robust yellowtail kingfish juveniles are to elevated CO_2 levels.

Chapter 6 studies the changes in aerobic metabolism during ontogeny, from 0.6 mg to 2.2 kg, a mass magnitude range equivalent to that between a mouse and an elephant. Several respirometry techniques were required to quantify oxygen consumption and ammonia excretion rates in yellowtail kingfish varying from larvae to sub-adults. The respirometric data set that was obtained is unique in that it is a single study that controls for activity level and temperature to a greater extent than other studies of intraspecific allometry that use combined data sets to obtain a wide mass range. The relationship between mass and metabolism is analysed using a number of models proposed by different authors, and compares models more rigorously than has previously been done. The all-important metabolic scaling exponent was considerably different to that of interspecfic allometries, and has important implications for the study of mass-metabolism. Chapter 7 discusses the findings of the previous chapters in terms of the interaction between ontogeny and physiology in yellowtail kingfish. Both the scientific and applied significance of the results are summarised, and suggestions offered about research directions that are worthy of further investigation.

Chapter 2: The Effect of Temperature on Morphological Development During the Endogenous Feeding Period

Introduction

Culture of yellowtail kingfish (*Seriola lalandi* Valenciennes 1883) is well established in Japan, where juveniles are predominantly sourced from wild populations (Nakada, 2002). The culture of this species has also been commercially established in Australia (Fowler et al., 2003) and in New Zealand, although seed production is totally reliant on hatchery reared juveniles. Despite the importance of larviculture in the production of yellowtail kingfish, aspects of the early developmental biology of this species are poorly known. Knowledge of early ontogenesis is crucial for understanding larval survival and recruitment in the wild, and important in aquaculture for optimising egg incubation and larval rearing protocols. One of the few studies of early development in yellowtail kingfish is that of Smith (2004), who described the timing of developmental stages during embryogenesis and first feeding, and the effect of incubation temperature on developmental rate. Smith (2004) observed the timing of ontogenetic check points to be highly dependent on incubation temperature. Temperature is one of the most important variables that affects fish egg development, and may have far reaching consequences on traits such as hatching time, body size, growth, differentiation of muscle (Blaxter, 1992) and vertebral formation (Wargelius et al., 2005).

The degree to which temperature increases developmental or metabolic rate is commonly expressed in terms of Q_{10}, that is, the rate of increase over 10°C (Schmidt-Nielsen, 1997), and temperature is reported to increase developmental rate in fish eggs by a Q_{10} value of 2.5-7.2 (Bermudes & Ritar, 1999; Galloway et al., 1998; Hamel et al., 1997; Hart & Purser, 1995; Kamler et al., 1998; Morehead & Hart, 2003). Smith (2004) estimated that temperature affected the time to hatch in yellowtail kingfish by a Q_{10} value of 2.78-6.07, though could not be more accurate as the Q_{10} analysis was based on multiple comparisons of hatching time between incubation temperatures.

The aim of this chapter is to describe the effect of temperature on the morphometric development of yellowtail kingfish during the endogenous feeding phase. Egg, oil droplet and yolk sac volume were measured together with larval length during the early growth phase at 18, 20, 22 and 24°C. Variables such as egg, oil droplet and yolk sac volume provide an indication of the endogenous resources available for development, and crucially, the energy reserves that will allow larvae to adjust to exogenous feeding. Oil droplets appear to be an important energy resource during the onset of

endogenous feeding of a number of fish species (Rønnestad et al., 1992a; Rønnestad et al., 1994; Rønnestad et al., 1998), and larger larvae are thought have a higher fitness as more energy reserves are available to extend the first feeding window (Miller et al., 1988; Pepin & Meyer, 1991). In addition to quantifying the effect of temperature on morphometric development, a re-analysis of Smith's (2004) temperature-hatch data is also provided in order to more accurately calculate the Q_{10} value for developmental rate. The work in this chapter is important for providing baseline information for further analyses of metabolism and substrate use during embryogenesis (Chapter 3), and also to gain a better understanding of the early developmental biology of this species.

Materials and Methods

Brood stock and egg collection

Brood stock were caught off the East Coast of Northern New Zealand between 1999-2001, and held at the National Institute of Water and Atmospheric Research Limited (NIWA) Bream Bay Aquaculture Park. The brood stock consisted of 14 fish with an average weight of approximately 17 kg (average fork length 1.07 m). The fish were held in a 70 m^3 circular tank with a depth of 2.5 m, and ambient temperature 10 µm filtered seawater was supplied at 12 m^3 h^{-1}. The photoperiod replicated the ambient photoperiod for latitude 35º 50'S using halogen lights to reproduce twilight periods, and fluorescent lights to reproduce daylight hours. The brood stock diet consisted of frozen pilchards and squid enriched with vitamins and tuna oil capsules. Spawned eggs were channelled from a skimmer on the surface of the brood stock tank into a separate100 l egg collector. An airlift was used to draw water through the egg collector and concentrate the eggs.

The effect of temperature on morphometric development

The trial was carried out in February 2004 at the NIWA Bream Bay Aquaculture Park. A sample of approximately 2000 eggs was collected from the egg collector within 5 h of spawning, and disinfected in 5 ppm Chloramine-T (Sigma-Aldrich, Australia) for 20 min. The eggs were transferred to four incubation tanks at a density of 280 eggs l^{-1}, initially adjusted to the spawning temperature of 18°C. The incubators were 100 l semi-conical tanks supplied with 1 µm filtered heated (26°C) and cooled (16°C) seawater (35-36‰). Temperature was maintained by mixing the water sources, and fine control was achieved using 2 kW bar heaters with digital thermostats (±0.1°C). Water flow was

maintained at around at 1 l min^{-1} and oxygen saturation above 85%. Light was supplied by fluorescent lights (250-500 lux at water surface) programmed to a 14 h light:10 h dark photoperiod. After the eggs were added to incubators, tank temperatures were elevated to 20, 22 and 24°C at a rate of 2°C h^{-1}.

Fifteen eggs or larvae were removed every 10-20 h for microscopic measurement of egg and oil droplet diameter, yolk sac dimensions and larval total length. Measurements were carried out over the period when larvae would start begin feeding. Digital photographs were taken of eggs and oil droplets using a Zeiss Axiolab compound microscope at 50 x magnification, and larvae were photographed using a Zeiss Stemi 2000C dissection microscope at 10-20 x magnification. Dimensions were determined from the digital photographs to the nearest 1 µm using the software SigmaScan Pro (Systat Software Inc., California, USA). Egg and oil droplet volumes were calculated using the equation for a sphere, and yolk volume at hatch was calculated using the equation for a prolate spheroid (Avila & Juario, 1987). Treatment differences in yolk volume at hatch and maximum larval length were determined using one-way ANOVA's (α=0.05), followed by Tukey's post-hoc tests to assign significant groupings. The relationship between incubation temperature and larval length and oil droplet volume at hatch (taken as the sample measured closest to 50% hatch) was investigated using linear regression. The time to 50% hatch was assigned using the data by Smith (2004), as this represents a more detailed analysis of the timing of hatch than the current study. As temperature dependent time (day degrees or effective day degrees, Kamler, 2002) was no better at standardising development between the different incubation temperatures than absolute time, age was expressed as HPF.

Reanalysis of Q_{10} from Smith (2004)

The developmental rate (time to 50% hatch) of yellowtail kingfish embryos as reported by Smith (2004) was regressed against temperature and the slope used to calculate the temperature coefficient Q_{10} (Kamler, 2002).

Results

Egg volume varied between 1.35-1.46 µl with no distinct trend over time, and there was high overlap between incubation temperatures at each sampling time.

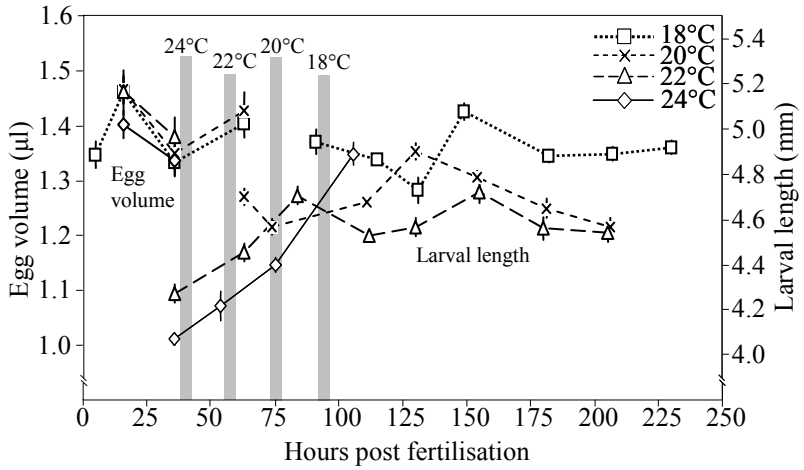

Figure 4. Variation in egg volume and larval length during incubation at four temperatures. Vertical bars indicate time of 50% hatch at each temperature. Data points represent mean ± SE determination of 15 individuals.

Larval length at hatch was negatively correlated with temperature (Figure 5), thus at 18°C hatched larvae were 18% longer than at 24°C.

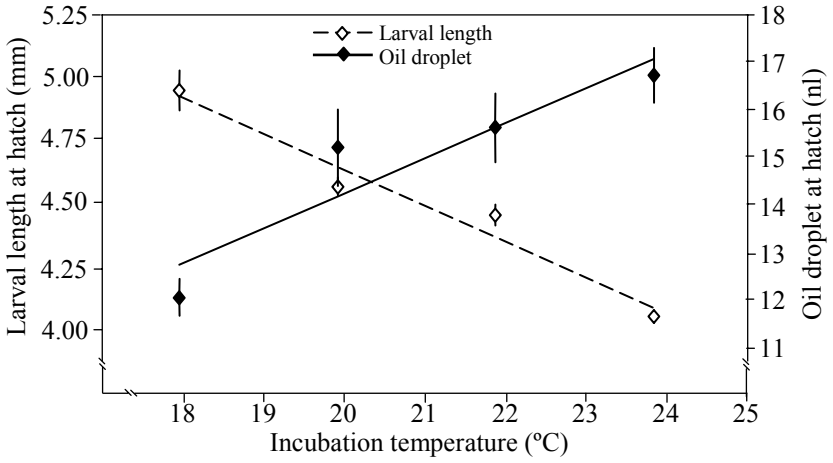

Figure 5. Variation in larval length and oil droplet volume at hatch. Data points represent mean ± SE determination of 15 individuals. Linear regressions for larval length represented by $y = -0.14 \cdot x - 7.56$, $r = -0.98$, and for oil droplet volume by $y = 0.72 \cdot x - 0.22$, $r = 0.93$.

At 22°C and 24°C larvae continued growing in length through the first feeding period, whereas at lower temperatures larval growth was minimal (Figure 4). Although there was a significant difference between the maximum larval length attained between incubation temperatures (ANOVA $p<0.05$), the magnitude of this difference was relatively minor (7%, Figure 6). Considerable variation was observed in yolk sac

volume at hatch at different incubation temperatures, however, there was a general trend for increasing yolk volumes with temperature (ANOVA $p<0.05$, Figure 6).

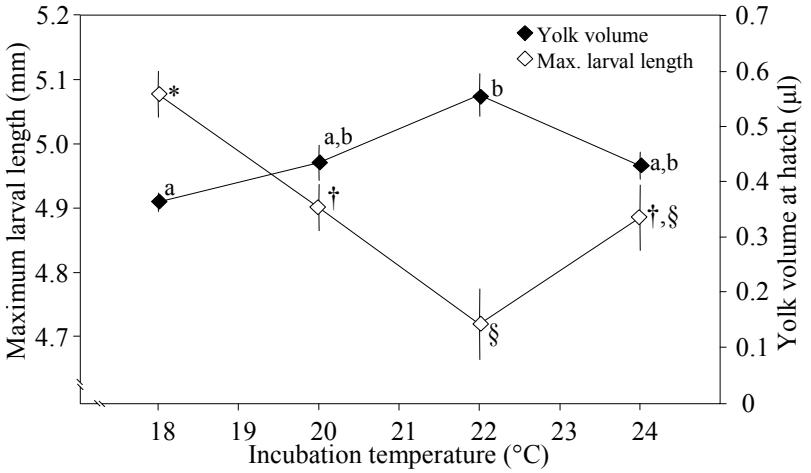

Figure 6. Variation in maximum larval length and yolk volume at hatch at four incubation temperatures. Data points represent mean ± SE determination of 15 individuals, and letters and symbols with the same script are not significant at $p<0.05$ (ANOVA).

The oil droplet volume was depleted steadily during embryogenesis (Figure 7), and was positively correlated with temperature at hatch (Figure 5). The oil droplet was larger and persisted for longer during the first feeding period at 18°C, and unlike the other temperatures was still present a number of days after hatch (Figure 7).

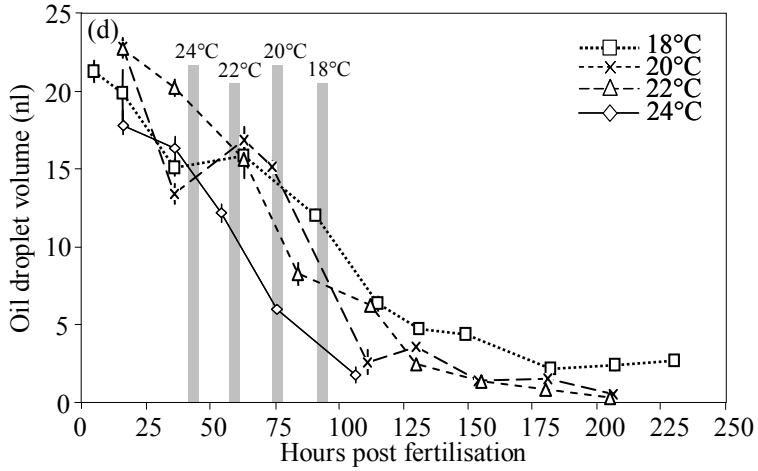

Figure 7. Variation in oil droplet volume during incubation at four temperatures. Data points represent mean ± SE determination of 15 individuals.

The time taken to reach hatch was negatively correlated with incubation temperature (Figure 8). The Q_{10} measure of thermal dependent developmental rate was calculated as 5.0.

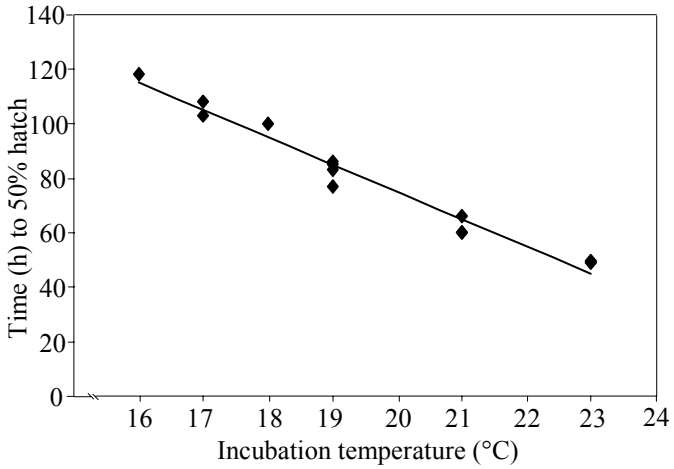

Figure 8. The effect of incubation temperature on time to 50% hatch. Data is taken from Smith (2004), and the linear regression is represented by the function y = -9.99 · x + 274.84, r = -0.98.

Discussion

The egg volumes recorded in the current study (1.25-1.66 µl) for yellowtail kingfish were similar to the range reported by both Smith (2004) and Tachihara et al. (1997). In general, yellowtail kingfish eggs are larger than those reported for other *Seriola spp* such as amberjack (*S. aureovittata* Valenciennes 1883: 1.31 µl, Fujita and Yogata 1984), Mediterranean yellowtail (*S dumerili* Risso 1810: 0.61-0.90 µl, Tachihara et al. 1993, Jerez et al. 2006) and Japanese yellowtail (*S. quinqueradiata* Temmick & Schlegel 1845: 1.02 µl, Fujita and Yogata 1984). The oil droplet volume in yellowtail kingfish (14-19 nl) was somewhat smaller than that reported for amberjack (21-24 nl, Fujita & Yogata, 1984), but larger than that of Mediterranean yellowtail (6-12 nl, Jerez et al., 2006; Tachihara et al., 1993), though these differences largely reflect interspecific differences in egg volume.

The hatching times of yellowtail kingfish larvae were generally similar to those reported in other *Seriola spp* at equivalent temperatures (Fujita & Yogata, 1984; Lazzari, 1991; Tachihara et al., 1993). The reanalysis of Smith's (2004) data showed that the length of time to hatch was strongly correlated with temperature, a finding in concordance with many other studies (Blaxter, 1969; Falk-Petersen & Hansen, 2003; Kamler, 1992;

Lasker, 1964; Martell et al., 2005; Mihelakakis & Kitajima, 1994; Mihelakakis & Yoshimatsu, 1998). The temperature coefficient of developmental rate in yellowtail kingfish (Q_{10} = 5.0) is within the range reported in other teleost species, which varies between 2.5-7.2 (Bermudes & Ritar, 1999; Galloway et al., 1998; Hamel et al., 1997; Hart & Purser, 1995; Kamler et al., 1998; Morehead & Hart, 2003). The relatively high Q_{10} value and strong correlation coefficient of yellowtail kingfish indicates that development in this species is quite sensitive to variation in temperature.

In addition to affecting hatching time, temperature also exhibited a strong negative correlation with the larval size at hatch. This effect has been noted in some fish species (Bermudes & Ritar, 1999; Martell et al., 2005; Ojanguren & Braña, 2003; Otterlei et al., 1999; Van Eenennaam et al., 2005), but not in others (Pepin et al., 1997; Rombough, 1994). Ojanguren and Braña (2003) proposed that such a pattern may be attributable to lower yolk conversion efficiency at elevated temperatures. This does not appear to explain why yellowtail kingfish larvae hatched smaller at warmer temperatures, as the post-hatch growth rates were higher and resulted in similar maximum larval lengths (4.7-5.1 mm) across all incubation temperatures for the time period observed, a similar finding to that made by Martell et al. (2005) for haddock (*Melanogrammus aeglefinus* Linnaeus 1758). The practical implications of these findings for the larviculture of yellowtail kingfish is that if larval size at first feeding can be used as a measure of fitness at first feeding, then variation in incubation temperatures between 18-24°C should have minimal impact on first feeding success. Although no mass measurements were made in the current study, further evidence that smaller larval size was not solely due to increased catabolism of metabolites comes from the fact that larvae from warmer incubation temperatures generally hatched with larger yolk sacs and oil droplets, and also that the total embryonic oxygen demand of yellowtail kingfish decreased at warmer temperatures (see Chapter 3). This finding supports observations by Fuiman et al. (1998) that elevated temperature generally accelerates the rate of ontogeny independent of the rate of growth in larval fish.

Thus, it appears that the main effect of elevated temperature on hatching time and larval size is to cause earlier hatching of smaller larvae, which subsequently increase tissue mass as yolk sac larvae rather than pre-hatched embryos. The reason hatching time is advanced of tissue mass development is probably attributable to temperature modification of the two phases of hatching. In the first phase of hatching, choriolytic enzymes are released from hatching glands into the perivitelline space, which proceed

to partially digest the inner layer of chorion proteins (Yamagami, 1988). Visual observations have shown that the sequence of developmental events, including the formation of hatching glands, occur faster at warmer temperatures (Rechulicz, 2001). In addition, hatching enzymes have a higher activity at warmer temperatures, resulting in more rapid weakening of the chorion (Yamagami, 1988). The second phase of hatching requires the physical movement of the embryo in order to break the weakened chorion (Blaxter, 1969). Increased incubation temperature results in greater physical movement of the embryo in order to break down the oxygen gradient that forms in the perivitelline fluid (Kamler, 1992; Rombough, 1988b), which in turn leads to earlier rupturing of the chorion. For these reasons, larval length at hatch in yellowtail kingfish appears largely a function of the amount of time an embryo spends developing inside the chorion, which is in turn strongly temperature-dependent.

The negative correlation between oil droplet volume at hatch and incubation temperature is also likely to be a reflection of temperature advancing ontogeny ahead of growth. A previous study by Hilton (2002) has shown that lipid is not used as an energy substrate during embryogenesis of yellowtail kingfish, meaning that the depletion of the oil droplet represents the biosynthetic rearrangement of the lipids into body tissues or energy reserves. The relationship between a larger oil droplet at hatch and higher temperature appears to simply represent a lower biosynthetic requirement for lipids by smaller larvae. Interestingly, larvae at 18°C apparently conserved the oil droplet to a higher degree through the first feeding period. Given that the larvae from the four temperatures were not significantly different in size during this time, the larger oil droplet at 18°C probably relates to the lower energy requirements of larvae at cooler temperatures, as lipids are thought to be energetically important during this period (Hilton, 2002). A practical consideration of this finding is that the conservation of the oil globule at 18°C may mean that larvae raised at this temperature have a longer period available to start first feeding, as more endogenous energy reserves are available.

The findings of this study are important for a number of reasons. Firstly, given that varying temperature may result in embryos and larvae that are not necessarily the same size at comparable developmental stages, physiological studies of embryogenesis in yellowtail kingfish (and other species) will need to take this fact into account. Secondly, the finding that smaller larvae at hatch do not necessarily result in smaller larvae at first feeding is an important point in the assessment of larval fitness attributes for fisheries or ecological recruitment models (Chambers, 1997; Litvak & Leggett, 1992). Thirdly, evidence that temperature differentially affects growth and

ontogeny is important in interpreting latitudinal differences in temperature, growth and life history characteristics (Atkinson & Sibly, 1997; van der Have & de Jong, 1996; Yamahira & Conoverb, 2002).

Chapter 3: Energetics and Metabolism of Yellowtail Kingfish During Embryogenesis

Introduction

In the past two decades there has been considerable research into the sequence of substrate utilisation in developing marine fish eggs and larvae, mainly in an effort to improve the larviculture of species for commercial production (Finn et al., 1995b; Finn et al., 1995c; Finn et al., 2002; Rønnestad et al., 1992a; Rønnestad et al., 1992b; Rønnestad et al., 1994; Rønnestad et al., 1998; Sivaloganathan et al., 1998). This work has shown that the metabolic fuel use of fish eggs varies considerably between freshwater and marine species, and between marine species with demersal eggs versus pelagic eggs. This knowledge has in turn led to a better understanding of species-specific broodstock nutrition, egg quality (Lahnsteiner & Patarnello, 2004), and larval nutrition during first feeding (Rønnestad et al., 1999).

This study investigates the relative importance of various metabolic substrates in the early development of yellowtail kingfish (*Seriola lalandi* Valenciennes 1833), a species of considerable aquaculture value in Japan (Nakada, 2002) and developing value in Australia (Fowler et al., 2003) and New Zealand. The rationale for such a study stems from the fact that yellowtail kingfish production in the latter two countries is entirely dependent on larviculture, and therefore it is desirable to understand egg and larval physiology in order improve the production and quality of juveniles for on growing. Despite the importance of several species of the genus *Seriola* for aquaculture (Fowler et al., 2003; Nakada, 2002; Poortenaar et al., 2001), there has been little research into the early developmental energetics or metabolism of this group of circum-global pelagic piscivores. To date there are only a small number of published biochemical analyses of the eggs and larvae of *Seriola spp*, and most have been concerned with the effect of broodstock diets on the lipid, vitamin and carotenoid content of eggs from yellowtail (*S. quinqueradiata* Temminck & Schlegel 1845*,* Verakunpiriya et al. 1996; Watanabe et al. 1996; Verakunpiriya et al. 1997a; Verakunpiriya et al. 1997b). The only study with a developmental aspect is the unpublished work of Hilton (2002), who concluded that proteins and lipids were not important energy substrates during embryogenesis in yellowtail kingfish.

Carbohydrates are important metabolic substrates during the first few hours of development for several fish species (Finn et al., 1995c; Finn et al., 1995d; Vetter et al., 1983), so it would be expected that glucose and glycogen have a role in fuelling early development in yellowtail kingfish. Non-protein-bound free amino acids (FAA) are

also known to be significant energy substrates for developing embryos and first hatch larvae of marine fish with pelagic eggs (Rønnestad et al., 1999). Energy budgets derived for Atlantic cod (*Gadus morhua* Linnaeus 1758, Finn et al. 1995a), Atlantic halibut (*Hippoglossus hippoglossus* [Linnaeus 1758], Finn et al. 1995c), European sea bass (*Dicentrarchus labrax* [Linnaeus 1758], Rønnestad et al. 1998), lemon sole (*Microstomus kitt* [Walbaum, 1792], Rønnestad et al. 2002a) and turbot (*Scophthalmus maximus* [Linnaeus 1758], Finn et al. 1996) show that 60-90% of energy requirements of embryos and early larvae are met through FAA catabolism. There are, however, no published data on the egg or larval FAA content in *Seriola spp*.

This study aims to assess the relative importance of carbohydrates (glucose and glycogen), FAA, and protein in the development of yellowtail kingfish incubated at different temperatures. Lipid or fatty acid content were not surveyed as these do not appear to be energetically important during embryogenesis (Hilton, 2002). Yellowtail kingfish produce small buoyant eggs (1.4 mm diameter) with a single oil droplet at fertilisation (Smith, 2004), so it would be expected that the sequence of substrate utilisation should follow the general pattern for fish species with pelagic eggs. As fish eggs are generally fully aerobic during early development (Finn et al., 1995b; Finn et al., 1995d), the oxygen consumption rate ($\dot{N}_{O_2}$) was used as a measure of metabolic turnover. In addition to characterising the $\dot{N}_{O_2}$ and flux of metabolites during embryogenesis, the effect of temperature on these factors was also investigated. Temperature is one of the most important abiotic variables that affects fish eggs in both the wild and in captivity (Blaxter, 1992; Kamler, 2002), having far reaching effects on diffusion properties and the rates of biochemical reactions, though little has been published on how temperature affects metabolic fuel use in eggs. The effect of temperature on biological processes is typically expressed in terms of Q_{10}, that is, the rate of metabolic change over a 10°C increase, and most biological processes are reported to have a Q_{10} of between 2-3 (McNab, 2002). The $\dot{N}_{O_2}$ of fish embryos and larvae are reported to have Q_{10} values near 3, indicating marked temperature sensitivity (Rombough, 1988b). This analysis is, however, complicated by the simultaneous effects of temperature on both developmental rate and metabolism (Barrionuevo & Burggren, 1999; Rombough, 1988a). In addition to reporting the pattern of substrate utilisation in yellowtail kingfish during embryogenesis, this chapter uses a novel method to account for the differential effect of temperature on growth and ontogeny found in Chapter 2.

Materials and Methods

Egg incubation

All experiments were carried out in February 2003 at the NIWA Bream Bay Aquaculture Park. Brood stock, egg collection methods and egg incubation tanks were the same as that outlined in Chapter 2. Eggs were collected from the broodstock tank 2 h post–fertilisation (HPF), disinfected in 5 ppm Chloramine-T (Sigma-Aldrich, Australia) for 20 min and transferred to 4 egg incubation tanks at an approximate density of 100 eggs l^{-1}. The incubation tanks were set at 17°C, the same temperature as the broodstock tank the eggs were spawned into. A sample of eggs were taken for respirometric and biochemical analysis, while three incubation tanks were elevated to 19, 21 and 23°C at a rate of 2°C h^{-1}. Eggs and larvae were incubated under a 14 h light:10 h dark photoperiod, and samples were taken in daylight every 10-14 h during embryogenesis for respirometric and biochemical analyses until hatching was complete. Larvae at 17°C were maintained until ready for first feeding in order to obtain samples for amino acid analysis from the entire yolk dependency period.

Respirometry and biochemical sampling

The $\dot{N}_{O_2}$ was determined by closed respirometry on groups of 20-30 eggs or 4-10 larvae (*n*=6 test replicates). Four blank replicates were used to subtract background oxygen consumption. At the beginning of each experiment, a sample of eggs or larvae were removed from the incubator, washed in UV sterilised seawater that had been equilibrated to the rearing temperature, and transferred to 2.8 ml glass respirometry vials filled with air-saturated water. The vials were sealed with rubber stoppers and placed on their sides in a container within the rearing tanks for the course of the closed respirometry period. In respirometers containing eggs, a small glass ball (4 mm diameter) was placed inside the vial and gently tipped every 15 min to ensure the water inside the respirometers was evenly mixed. The glass ball did not come into contact with the positively buoyant eggs, hence there was no risk of physical damage. Respirometers containing larvae did not require active mixing as the larvae routinely swam around the vial. Oxygen saturation was measured after 1.5-3 h using a polarographic oxygen sensor (Strathkelvin Model 1302, Strathkelvin Instruments, Glasgow, Scotland) housed inside a temperature regulated microcell (Strathkelvin Model MC100). Oxygen saturation in the respirometers was measured in the microcell by injecting two 250 µl samples of respirometer water with a gas-tight temperature-

equilibrated syringe (Hamilton Inc, Nevada, U.S.A.) to ensure that the previous sample was purged. Approximately 50 eggs or 10-30 larvae were taken midway through each respirometric measurement period and stored at -70°C for biochemical analysis.

Biochemical analyses

The glucose and glycogen content of eggs and larvae were quantified in 3 replicate samples of 5 individuals according to the method of Finn et al (1995d). Protein, ninhydrin positive substances (NPS: FAA, ammonia and small peptides) and ammonia (NH_3) was quantified from 3 replicate samples of 5-10 individuals. The samples were suspended in 1.2 ml of distilled water, the chorion punctured with forceps and then sonicated. Triplicate 100 µl aliquots were removed for ammonia quantitation using the method of Bower and Holm-Hansen (1980). Triplicate 200 µl aliqouts were removed and 5% trichloroacetic acid (TCA) added for protein and NPS analysis. The samples were left overnight at 4°C and centrifuged (20 min, 12 000 x *g*), and the supernatant removed for NPS analysis using the method of Moore (1968) with norleucine as a standard. The pellet was dissolved by heating at 56°C for 30 min in 500 µl 1 mol l^{-1} NaOH, followed by neutralisation with 300 µl 1.67 mol l^{-1} HCl. Protein concentration was determined using Coomassie Brilliant Blue Dye (Biorad Laboratories, California, U.S.A.) with bovine serum albumin as a standard. Individual FAA were quantified in eggs and larvae from the 17°C incubation in order to give a general indication of the quantity and sequence of utilisation. The supernatant (containing the FAA) was collected from TCA precipitated samples from 3 replicates of 5 individuals according to the methods above, and analysed using reverse phase high performance liquid chromatography (RP-HPLC) based on the protocol developed by Hubbard (1995). 10 µl of supernatant were derivatised with phenyl isothiocynate and 1-4 µl injected. Individual amino acids were identified using calibration standard AAAS18 (Sigma-Aldrich, Australia), with taurine added. The detection limit of the injected sample was approximately 6 pmol. Taurine and arginine could not be reliably resolved, however, these amino acids are not especially important in the FAA pool of the eggs of marine fish with pelagic eggs (Rønnestad et al., 1999) so were reported as a combined concentration using arginine as a standard.

Data handling and statistical analysis

Oxygen consumption was calculated as nmol O_2 consumed ind^{-1} h^{-1} according to the methods of Rønnestad et al (1992a), and an exponential function used to describe the relationship between $\dot{N}_{O_2}$ and developmental time. Developmental time was

expressed as both an absolute measure (HPF), and relative measure (proportion of time to hatch, fertilisation=0, hatch=1). The time to 50% hatch was assigned using the data by Smith (2004), as this represents a more detailed analysis of the timing of hatch than the current study. The van't Hoff equation for Q_{10} was used to quantify the effect of temperature on oxygen consumption (McNab, 2002). The Q_{10} was calculated using a number of measures of developmental time in order to derive meaningful measures of the effect of temperature on metabolic rate. Although some authors have used the metabolic scaling exponents of $\dot{N}O_2$ -mass relationships to calculate the Q_{10} between eggs and larvae raised at different temperatures (Finn et al., 2002; Walsh et al., 1991), this was not employed in the present study because mass was not deemed a useful measure of egg development given that such a large proportion of the yolk is metabolically inert (Konstantinov, 1980). An attempt was made to calculate Q_{10} from scaling exponents derived from log-linearised $\dot{N}O_2$ and HPF, however, as the scaling exponents were not equal there could be no meaningful analysis of the intercepts and therefore no calculation of Q_{10}. The only method of calculating Q_{10} from absolute developmental time was to compare the metabolic rate of eggs from time periods for which there were paired $\dot{N}O_2$ measurements (9, 26, 35.5, and 49 HPF). The second measure of metabolic rate used to calculate Q_{10} was the $\dot{N}O_2$ at hatch (estimated from the exponential function), which represents a developmental point independent of absolute time. The third measure of metabolic rate used to calculate Q_{10} was total oxygen consumed prior to hatch. The amount of oxygen consumed prior to hatch was calculated by estimating the area under the $\dot{N}O_2$ -HPF curve using the trapezoid rule (Burden & Faires, 2000). Significant differences in $\dot{N}O_2$ at hatch and O_2 consumed to hatch were analysed by linear regression of these variables against incubation temperature using Statistica v. 6.0 (StatSoft Inc., Oklahoma, U.S.A.). The concentrations of glucose, glycogen, NPS and ammonia were quantified as nmol of glucose, glycosyl units, NPS and NH_3 ind^{-1} ± SE, respectively, and protein was quantified as µg protein ind^{-1} ± SE. As with $\dot{N}O_2$, the flux of biochemical constituents were compared across incubation temperatures using the proportion of time to hatch as a measure of development.

Results

The time to hatch was negatively correlated to incubation temperature, with 50% hatch occurring at 50, 65, 80 and 102 HPF at 23, 21, 19 and 17°C, respectively. It was observed that eggs became negatively buoyant in the hours prior to hatch, however hatched larvae were positively buoyant. Respiration increased semi-linearly during the first 40 h of development for all incubation temperatures, and rose sharply around the time of hatch (Figure 9a). The $\dot{N}O_2$ was higher at warmer incubation temperatures when plotted against HPF (Figure 9a). A comparison of the $\dot{N}O_2$ at 9, 26, 35.5 and 49 HPF resulted in Q_{10} values ranging from 0.3-48.5. Higher temperatures were seen to initially raise metabolic rate when $\dot{N}O_2$ was scaled against relative developmental time (proportion of time to hatch), however, at around 70% of the time to hatch the relationship switched such that higher incubation temperatures resulted in lower metabolic rates (Figure 9b). The estimated $\dot{N}O_2$ of individuals at hatch showed strong and significant negative correlation with temperature (r=-0.97, p<0.05), as did the oxygen consumed to hatch (r=-0.98, p<0.05, Figure 9c). The estimated $\dot{N}O_2$ of eggs at hatch at 23°C was only 63% of that at 17°C, and corresponded to a Q_{10} of 0.5. Similarly, the total oxygen consumed to hatch at 23°C was only 52% of that of at 17°C, and corresponded to a Q_{10} of 0.3.

The glucose concentrations were generally low (1-5 nmol ind^{-1}) throughout ontogenesis for all temperatures except for individuals at 23°C (Figure 10a). Eggs incubated at this temperature demonstrated a considerable increase in glucose content to 8.6 nmol ind^{-1} mid-development, which was soon followed by a rapid decline to less than 1 nmol ind^{-1} pre hatch and thereafter. Glycogen content fluctuated between 5-9 nmol glycosyl units ind^{-1} during most of embryogenesis at 17-21°C, and increased somewhat prior to hatch (Figure 10b).

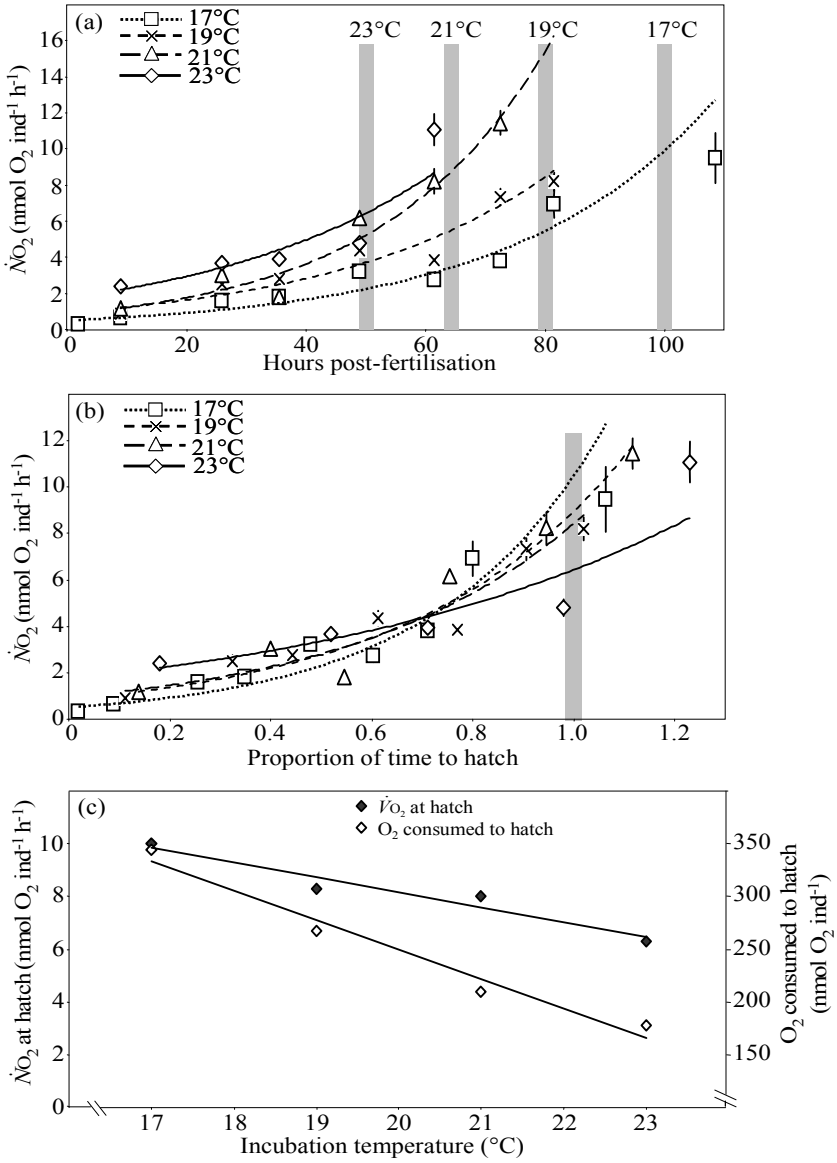

Figure 9. Oxygen consumption of eggs and larvae of yellowtail kingfish incubated at different temperatures over (a) absolute developmental time, and (b) relative developmental time. Each data point represents mean $\dot{N}O_2$ ± SE of 6 replicates of 20-30 eggs or 4-10 larvae, and hatch time is indicated by the vertical grey bar. The exponential functions are as follows: (a) 17°C, $y=0.507 \cdot \exp^{(0.030x)}$, r=0.96; 19°C, $y=0.938 \cdot \exp^{(0.028x)}$, r=0.96; 21°C, $y=0.857 \cdot \exp^{(0.036x)}$, r=0.94; 23°C, $y=1.753 \cdot \exp^{(0.026x)}$, r=0.94, and (b) 17°C, $y=0.507 \cdot \exp^{(3.029x)}$, r=0.96; 19°C, $y = 0.938 \cdot \exp^{(2.196x)}$, r=0.96; 21°C, $y=0.857 \cdot \exp^{(2.346x)}$, r=0.94; 23°C, $y=1.753 \cdot \exp^{(1.390x)}$, r=0.94. The $\dot{N}O_2$ at hatch and O_2 consumed to hatch at different incubation temperatures are shown in (c), and are represented by the linear regressions y=-0.57x+19.55, r=-0.97 and y=-27.91x+807.94, r=-0.98, respectively.

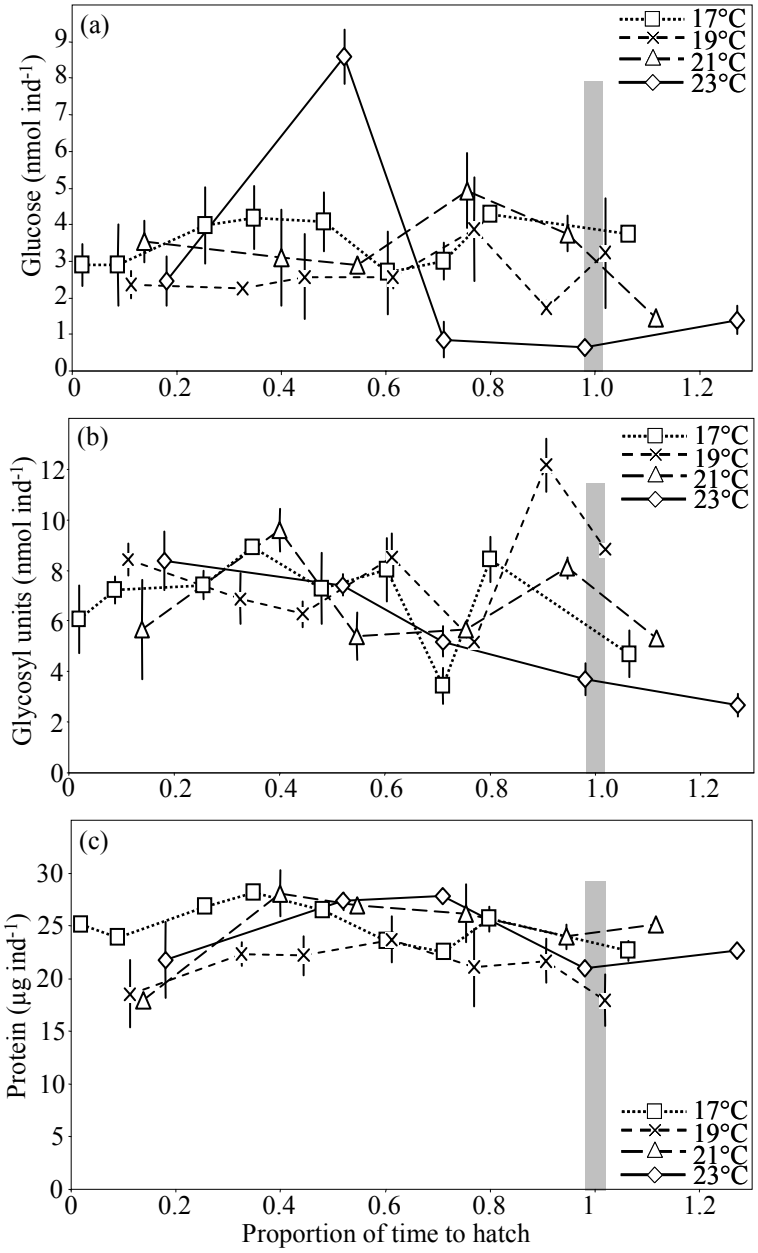

Figure 10. Changes in (a) glucose, (b) glycogen and (c) protein content of eggs and larvae of yellowtail kingfish incubated at different temperatures. Data points represent the mean concentration ± SE of 3 replicates of 5-10 individuals, and hatch time is indicated by the vertical grey bar.

In contrast, glycogen content decreased over time in eggs incubated 23°C (Figure 10b). The protein content of eggs incubated at elevated temperatures decreased relative to the 17°C samples initially, but after approximately mid-embryogenesis

protein content was relatively similar and fluctuated between 17-27 ug ind^{-1} (Figure 10c).

The initial FAA pool as determined by RP-HPLC was 181 ± 15 nmol ind^{-1}. The main FAA present in eggs 2 HPF were glycine (20%,), alanine (10.3%), leucine (9.9%), serine (7.7%), valine (7.5%) and isoleucine (6.7%), which together comprised over 62% of the pool. Following incubation at 17°C nearly all FAA decreased 5-10% within the first 9 h of development, though glycine and valine decreased 82% and 95%, respectively (Figure 11). Following a slight increase at 50 HPF, all FAA decreased rapidly during embryogenesis and were near exhaustion at hatch (Figure 11). The predominant FAA in yolk sac larvae were glycine, serine, valine and unresolved arginine/taurine. The total concentration of FAA during the first feeding period varied between 20-40 nmol ind^{-1}.

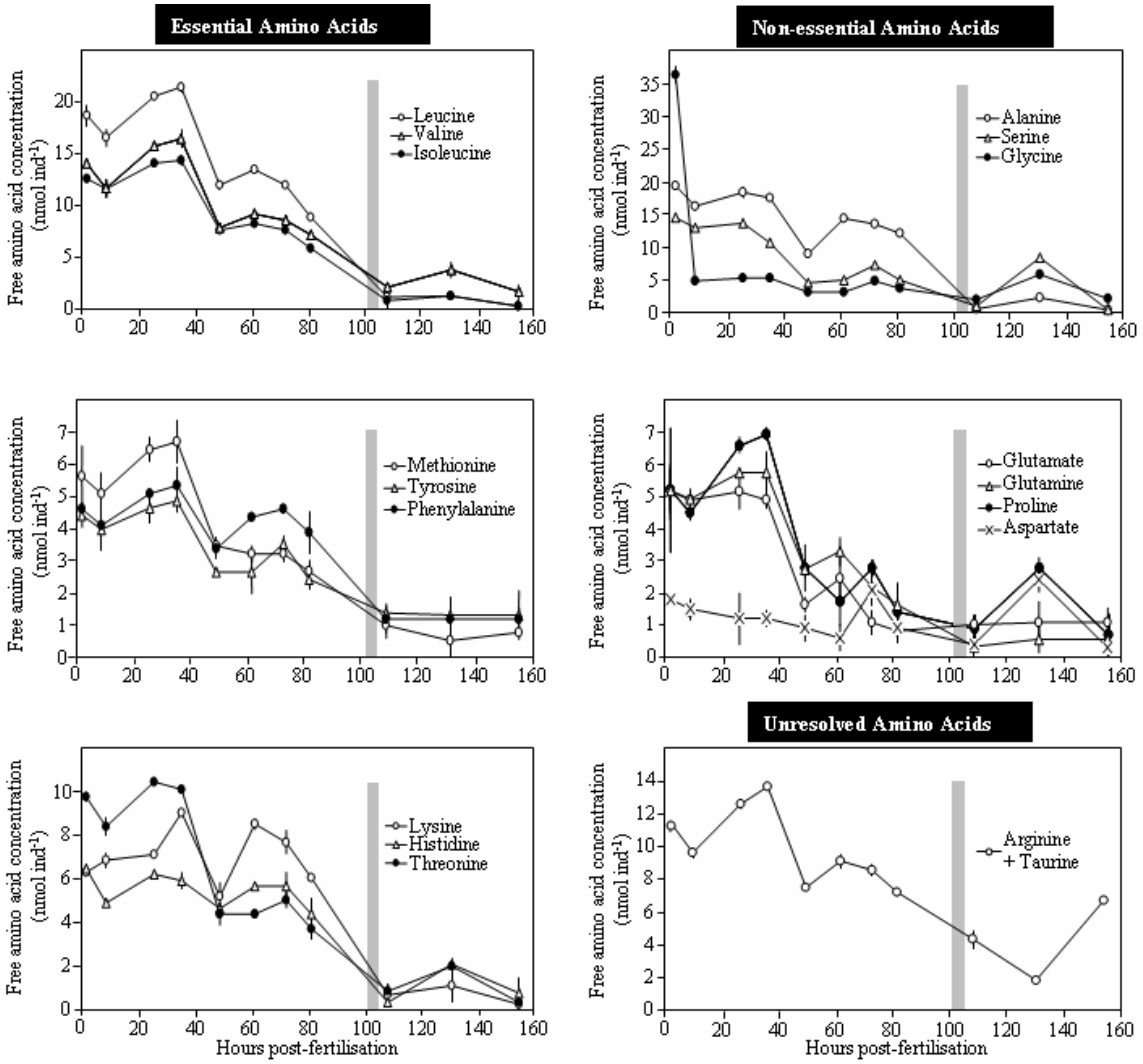

Figure 11. Changes in the free amino concentration of egg and larval yellowtail kingfish incubated at 17°C, as determined by RP-HPLC. Data points represent the mean determination ± SE of 3 replicates of 5 individuals. Arginine and taurine could not be reliably resolved so are reported as combined concentrations using arginine as a standard. Hatch time is indicated by the vertical grey bar.

The concentration of NPS at 2 HPF was 212 ± 23 nmol ind^{-1}, and rose rapidly to approximately 340 nmol ind^{-1} during the first quarter of embryogenesis in eggs incubated at 17, 19 and 21°C (Figure 12a). The NPS concentration dropped rapidly during mid embryogenesis at these temperatures, stabilised somewhat at 60-160 nmol ind^{-1} during late embryogenesis and decreased to 25-45 nmol ind^{-1} around hatch. In contrast to the similarity in pattern displayed at the lower incubation temperatures, eggs at 23°C demonstrated a continual decrease in NPS from the first measurement, though finished with a comparable concentration at hatch (Figure 12a). Ammonia

accumulated within eggs at a near constant rate for all incubation temperatures, starting at 20-70 nmol ind^{-1} and increasing to 250-300 nmol ind^{-1} at hatch (Figure 12b).

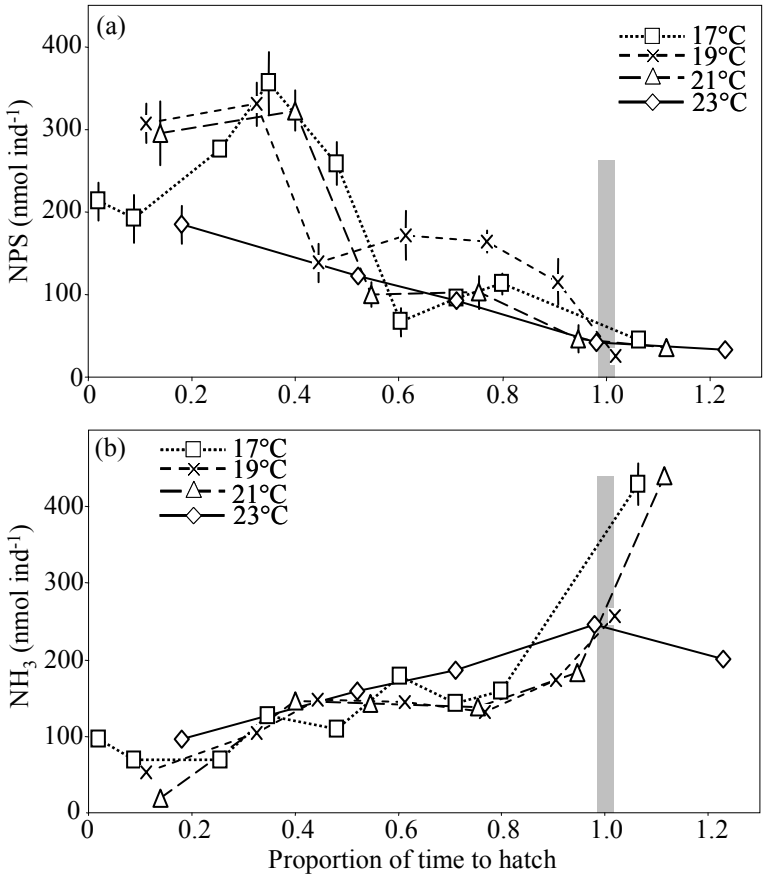

Figure 12. Changes in (a) ninhydrin positive substances and (b) ammonia in eggs and larvae of yellowtail kingfish incubated at different temperatures. Data points represent the mean concentration ± SE of 3 replicates of 5-10 individuals, and hatch time is indicated by the vertical grey bar.

Discussion

This chapter investigated the energetics and sequence of substrate utilization of yellowtail kingfish during embryonic development at different temperatures. The range of respiration rates exhibited by yellowtail kingfish eggs and first hatch larvae were broadly similar to those reported in other marine fish eggs (Finn et al., 1995d; Rønnestad et al., 1992b; Rønnestad et al., 1994; Sivaloganathan et al., 1998). The trend of a steady increase in $\dot{M}O_2$ during embryogenesis followed by an increase around hatch has also been observed in the development of Atlantic halibut (Finn et

al., 1991), gilthead seabream (*Sparus aurata* Linnaeus 1758, Rønnestad et al. 1994) and turbot (Rønnestad et al., 1992a).

The relationship between temperature and embryonic respiration rate appeared unusual when using absolute developmental time to compare temperature effects. The range of Q_{10} values calculated from $\dot{N}O_2$ at equivalent absolute developmental time points were highly variable and well outside the range typically reported in developing fish embryos and larvae (Rombough, 1988b). The wide and generally elevated range of Q_{10} values reported above for yellowtail kingfish illustrates the fact that direct comparison of metabolism using absolute developmental time ignores the simultaneous effects temperature exerts on both developmental rate and metabolism (Barrionuevo & Burggren, 1999). Development stage has been standardised in larvae using weight (Finn et al., 2002), however, the fact that much of the yolk is metabolically inert means mass is not a particularly informative measure of development in eggs (Konstantinov, 1980). Other attempts to standardise developmental time have included using temperature independent units such as day degrees or effective day degrees (Weltzien et al., 1999), or adding a constant to metabolic rate to standardise to a single temperature (Walsh et al., 1991), though neither of these solutions are particularly precise (Barrionuevo & Burggren, 1999; Rombough, 1988a). In addition, there is evidence that temperature accelerates embryonic ontogenetic rate more than that in yolk-sac larvae (Pepin, 1991). The use of relative developmental time (proportion of time to hatch) in the current study is novel and circumvents these problems.

When $\dot{N}O_2$ was measured against relative developmental time, higher incubation temperatures initially had a predictable effect on metabolic rate, namely that oxygen uptake increased (Figure 9b). This relationship switched at around 70% of the way though egg development such that $\dot{N}O_2$ was lower at higher incubation temperatures. At hatch the $\dot{N}O_2$ of larvae at 23°C was only 63% of that of larvae at 17°C, and over the entire course of embryonic development eggs raised at 23°C only consumed 52% of the oxygen of eggs raised at 17°C. Converting these comparisons to Q_{10} values yield rates of only 0.5 and 0.3, respectively. The juxtaposition posed by having a negative relationship between metabolic rate and temperature can be resolved in light of the fact that yellowtail kingfish hatch shorter at higher water incubation temperatures (Chapter 2), an effect that has also been noted in other fish species (Bermudes & Ritar, 1999; Van Eenennaam et al., 2005). It was proposed in Chapter 2 that the

smaller hatch size at warmer temperatures was a function of increased hatching activity rather than decreased yolk conversion efficiency, as the yolk sac was larger on these newly hatched larvae and there was little difference in maximum larval size between incubation temperatures. The observation that larvae incubated at warmer temperatures consumed less oxygen during embryogenesis supports this hypothesis, as it indicates that yolk sac larvae were not just smaller due to increased substrate catabolism. Additional support for this hypothesis comes from a survey of larval fish developmental studies by Fuiman et al. (1998), who concluded that for several fish species elevated temperatures accelerates ontogeny somewhat more than the rate of growth. The decoupling between mass and ontogeny has important implications not only in interpreting metabolic Q_{10} values, but also for the develop of universal models of biological time that are contingent on temperature, mass and ontogeny (Gillooly et al., 2002).

The effect of temperature on the metabolic constituents of eggs was varied. Glucose content was 1-5 nmol ind^{-1} at 17-21°C, but increased mid development at 23°C before dropping to low levels prior to hatch (Figure 10). The glucose pool size was within the range of that reported in other marine fish species, and although the pattern of glucose flux during development was not particularly similar to that of other studies, there has been considerable variation reported (Finn et al., 1995c; Finn et al., 1995d; Lahnsteiner & Patarnello, 2004; Seoka et al., 1997). The concentration of glycogen was relatively constant during development at 17-21°C, fluctuating between 3.4-12.2 nmol glycosyl units ind^{-1}, whereas at 23°C eggs showed a continual decline in glycogen content. As with glucose, the glycogen concentration was similar to the range reported in other marine teleost species with similarly sized eggs (0.2-8 nmol glycosyl units ind^{-1}), and although the pattern of flux was different to other fish species there is considerable variation reported (Finn et al., 1995c; Finn et al., 1995d; Seoka et al., 1997; Vetter et al., 1983). The different pattern of glucose and glycogen content of eggs at 23°C indicates a change in metabolic flux from the low temperatures. Given that typical summer sea surface temperatures for northern New Zealand are between 18-21°C (unpublished data, Leigh Marine Laboratory, University of Auckland, New Zealand), and temperatures around 23°C would be exceptional, it appears that yellowtail kingfish eggs are compromised at high temperatures and do not develop normally. In general, the relationship between glucose and glycogen pool content and rate of catabolism is poorly understood in fish eggs, though the broad consensus is that these low concentration substrates are energetically only important immediately

post fertilisation in marine fish (Finn et al., 1995c; Finn et al., 1995d; Rønnestad et al., 1992a).

The protein content of eggs and first hatch larvae fluctuated during development but remained between 18-28 ug ind^{-1}, corresponding with previous estimates (Hilton, 2002). Protein levels have been shown to be stable or slightly increased during embryogenesis (Finn et al., 1991; Finn et al., 1995d; Finn et al., 1996; Lahnsteiner & Patarnello, 2004; Rønnestad et al., 1992b; Rønnestad et al., 1994; Rønnestad et al., 1998; Sivaloganathan et al., 1998; Vetter et al., 1983), and are important as an energy substrate during the onset of first feeding in Atlantic cod (Finn et al., 1995a), lemon sole (Rønnestad et al., 1992b), turbot (Finn et al., 1996), seabass (*Lates calcarifer* [Bloch 1790], Sivaloganathan et al., 1998) and gilthead sea bream (Rønnestad et al., 1994).

The profile of FAA found in yellowtail kingfish eggs was similar to that reported in other marine teleosts which produce pelagic eggs (Finn et al., 1995b; Finn et al., 1995d; Finn et al., 1996; Rønnestad et al., 1992b; Rønnestad et al., 1998; Sivaloganathan et al., 1998). The neutral amino acids leucine, valine, serine, isoleucine and alanine dominated the FAA pool, accounting for 50-85% of the pool prior to hatch. While the relative composition of these five FAA is highly conserved in most marine fish species with pelagic eggs (Rønnestad et al., 1999), the high level of glycine present in yellowtail kingfish eggs 2 HPF is somewhat anomalous (Figure 11). Glycine levels had decreased rapidly by 9 HPF, after which time it more closely matched the relative pool contribution measured in other species (Rønnestad et al., 1994; Rønnestad et al., 1998; Sivaloganathan et al., 1998). The increase in FAA concentration at 50 HPF is correlated with a decrease in protein content around the same time. This period marks the 20 myomere stage, so it is likely some egg protein content was hydrolysed to provide AA for both the growth and respiration of the growing embryo. Rapid depletion of all FAA followed until hatch along with a concurrent increase in protein, representing the formation of the advanced pre-hatch embryo. By hatch almost all FAA were exhausted to between 1-4 nmol ind^{-1}, and remained low during the first feeding period. Given the role of FAA in the buoyancy of marine pelagic fish eggs (Craik & Harvey, 1987), it is likely that the large decrease in FAA is responsible for the observation that yellowtail kingfish eggs became negatively buoyant near hatch. The ammonia content of the eggs increased alongside the loss of FAA and peaked after hatch, reflecting the catabolism of AA for energy. Wax esters and triacylglycerol have been shown to

decrease markedly during the first days after hatch (Hilton, 2002), and are probably the predominant metabolic fuel utilized during the first feeding period.

The concentration of NPS were used as a proxy of FAA concentration, and predictably followed the same pattern of depletion as that measured by RP-HPLC. While the initial concentration of FAA estimated by RP-HPLC and ninhydrin at 17°C was relatively similar (181 and 212 nmol ind^{-1}, respectively), the concentration of NPS was higher than concentrations measured from RP-HPLC during early to mid embryogenesis (2-62 HPF). This difference is a possible artefact arising from the reaction of ninhydrin with ammonia and small peptides (Moore & Stein, 1948). However, as the concentration of ammonia was too low to account for the NPS balance, small peptides associated with tissue protein synthesis may have been responsible for the apparent deficit. The FAA concentration estimated by RP-HPLC and NPS were in good agreement from the advanced embryo stage (80 HPF) until first feeding. This is presumably because the majority of tissue mass is developed and there is only a low concentration of small peptides associated with polypeptide formation.

While no attempt was made in the current study to derive a stoichiometric energy budget during embryogenesis, there are a number of factors that indicate that, as for other marine fish with pelagic eggs (Finn et al., 1991; Finn et al., 1995d; Finn et al., 1996; Rønnestad et al., 1994), the early development of yellowtail kingfish is reliant on FAA as the predominant energy source. Firstly, the contribution of carbohydrates to metabolism is likely to be minimal given the low concentrations. Secondly, the lipid content of eggs and yolk sac yellowtail kingfish larvae was relatively constant and therefore probably not energetically important (Hilton, 2002). Thirdly, there was a large rapidly decreasing pool of FAA during embryogenesis with no concomitant increase in protein content. In addition, there was also a considerable increase in internal ammonia concentration, which together indicate catabolism of FAA. The development of yellowtail kingfish eggs appeared to have a particularly high reliance on FAA catabolism for energy. Sivaloganathan et al (1998) proposed an inverse relationship between ecological temperature and the contribution of FAA catabolism to metabolism for most fish species with pelagic eggs studied to date. Yellowtail kingfish seemed not to conform to this trend as this temperate water species is likely to be closer to cold water species such as Atlantic cod or halibut, which derive a high percentage (65-75%) of energy from FAA catabolism (Sivaloganathan et al., 1998).

Eggs incubated at 17-21°C had a similar pattern of NPS flux during development, however, at 23°C NPS depleted constantly and rapidly without the characteristic increase mid-way through development. One possible explanation is the smaller number of NPS measurements made at 23°C resulted in an increase not being sampled. Alternatively, these eggs may not have contained a particularly large pool of small peptides, or the peptides may have been quickly turned over. The atypical spike in glucose content around the same time may represent the gluconeogenic conversion of some FAA to glucose. Another possibility is that given that the metabolic rate of fish eggs is highly dependent on temperature (Blaxter, 1992), the energy requirements of eggs at 23°C may have been so high that FAA were catabolised. This is quite plausible given the comparatively high $\dot{N}O_2$ and ammonia concentration of eggs from the 23°C incubator during the initial development period. As was evident from the trend in glycogen and glucose flux, yellowtail kingfish eggs incubated at 23°C appear to undergo a different pattern of substrate utilization compared to lower incubation temperatures. This temperature may have exceeded the upper limit for normal development.

The paucity of information on the effect of temperature on marine fish egg biochemical development makes comparisons difficult. The highest incubation temperature resulted in a considerably different pattern of NPS and carbohydrate flux compared to the other three incubation temperatures. The reason for this is unclear due to uncertainties about FAA pool size and protein synthesis and turnover rate, but for larviculture purposes egg incubation at or below 21°C has little effect on substrate utilization. It would certainly be useful to carry out more work on fish egg and larval energy metabolism using temperature as a covariate. Given the importance of FAA as both a metabolic fuel and a substrate for protein synthesis in pelagic marine fish eggs (Rønnestad et al., 2003), and the highly temperature dependent nature of metabolic rate, it would be interesting to see whether temperature has a greater effect on the biochemical development on pelagic eggs than those reliant on lipid as a fuel source, namely demersal eggs. Such studies would not only be useful for developing a better understanding of egg quality and early larval nutrition for larviculture, but would also be important in investigations concerning sea temperature variations and larval fish survival.

Chapter 4: Growth Variation and Aggression in Juvenile Yellowtail Kingfish

Introduction

For many fish species there is a marked divergence in body size soon after first feeding, a phenomenon that has been observed in both wild (Adams & Huntingford, 1996; Sakakura & Tsukamoto, 1996; Sakakura & Tsukamoto, 1998a; Sakakura & Tsukamoto, 1999; Smith & Reay, 1991; Swain & Riddell, 1990) and hatchery reared populations (Baras, 1999; Baras et al., 2003; Baras et al., 1999; Baras et al., 2000a; Baras et al., 2000b; Hoglund et al., 2005; Kadri et al., 1997; Qin & Fast, 1996; Ruzzante, 1994). The first feeding period represents a rapid phase in growth as the larvae switch from endogenous to exogenous feeding and undergo the considerable physiological, anatomical and behavioural changes that mark metamorphosis into juveniles. Aggressive interactions between juveniles often develop alongside the size heterogeneity that results from inter-individual differences in growth rate (Kestemont et al., 2003).

This chapter investigates the role of individual growth variation and aggression in the development of cultured larval and juvenile yellowtail kingfish (*Seriola lalandi* Valenciennes 1833). As with many other carnivorous fish species, one of the main difficulties in the larviculture of this species, and an important population modifier in the wild, is high mortality associated with intra-cohort aggression (Ebisu & Tachihara, 1993). A common precursor to aggression in juvenile populations of many fish species is rapid size differentiation after the introduction of *Artemia* as a food source, which is soon followed by aggressive behaviour in the form of larger dominant individuals tracking smaller subordinates. The development of this behaviour has been observed in greater amberjack (*S. dumerili* Risso 1810, Papandroulakis et al., 2005), Japanese yellowtail (*S. quinqueradiata* Temminck & Schlegel 1845, Sakakura and Tsukamoto, 1996), Japanese flounder (*Paralichthys olivaceus* Temminck & Schlegel 1846, Sakakura and Tsukamoto, 2002), and Pacific yellowtail (*S. mazatlana* Valenciennes 1833, Benetti, 1997). Eventually the dominant individuals start chasing and nipping subordinates, which in turn leads to yet greater size heterogeneity and high mortality as subordinates are stressed (Sakakura et al., 1998), have less time to feed or suffer physical trauma (Greaves & Tuene, 2001). Many species also exhibit intra-cohort cannibalism which in turn can lead to mortality of both the recipient and aggressor (via suffocation) (Smith & Reay, 1991), as has been observed in yellowtail kingfish (Ebisu & Tachihara, 1993). Size grading is a standard practice used in finfish culture to reduce size heterogeneity and aggression in juveniles (Baras, 1999; Baras et al.,

2000a; Qin & Fast, 1996), and has been used to significantly improve the survival and production of Japanese yellowtail during the cannibalistic period (Yamazaki et al., 2002). Additional factors that are known to influence aggression include light intensity, larval and prey density and temperature (Kestemont et al., 2003).

Although there are several studies describing the development of aggression in both wild and cultured Japanese yellowtail (Sakakura & Tsukamoto, 1996; Sakakura & Tsukamoto, 1998a; Sakakura & Tsukamoto, 1998b), and observations of cannibalism in yellowtail kingfish (Ebisu & Tachihara, 1993), there are no published accounts of the development of aggression in yellowtail kingfish. The present study was therefore undertaken to describe the development of size heterogeneity and aggression in yellowtail kingfish from commercial culture tanks, and to quantify the effectiveness of grading in reducing size heterogeneity and aggression. Graded and ungraded larvae were compared to partition the relative effects of individual growth rate and aggression on size differentiation within a cohort. The RNA:DNA ratio was used as an instantaneous measure of juvenile growth rate. Unlike a single measurement of weight or length, a single measurement of RNA:DNA ratio can give information on the growth rate, and may be applied to individual juveniles with high accuracy (Weber et al., 2003).

Materials and Methods

Egg collection and rearing

All work was carried out from November 2004 to March 2005 at the yellowtail kingfish production facility at the National Institute of Water and Atmospheric Research Limited Bream Bay Aquaculture Park in northern New Zealand. Juvenile yellowtail kingfish were produced from a naturally spawning brood stock of 15 adult fish, and observations had shown most spawning events arising from of a single female and male. Eggs were collected within 24 h of spawning and transferred to 100 l incubation tanks with natural photoperiod (14 h light: 10 h dark) and temperature (17-21°C). Newly hatched larvae (3-6 d post-spawn depending on incubation temperature) were transferred to 10 m^3 nursery tanks at an approximate density of 10-25 larvae l^{-1}, and the temperature elevated to the 21-23°C rearing temperature over 3 d. The tanks were lit with a combination of diffuse sunlight and fluorescent lights, with a light intensity of 5-10 x 10^3 lux at the water surface. The water flow into the tank was 20 l min^{-1} and

circulation within the tank was provided by air stones. Larvae were raised under standard green water algal culture techniques, with Rotiselco-Alg™ (Inve Ltd, Thailand) enriched rotifers *Brachionus plicatilis* introduced as a first feed 3 d post-hatch (DPH) and fed four times daily to maintain a concentration of approximately 10 ml^{-1}. Super HUFA™ (Salt Creek Inc., Utah, U.S.A.) enriched *Artemia* were introduced at 12 DPH with a 7 d weaning period between live feeds. *Artemia* were administered four times daily at a concentration of approximately 1 ml^{-1}, which were generally consumed within 30 min, and dry feed (Proton™ 2/3, Inve Ltd) was co-fed in increasing amounts from 16-18 DPH. *Artemia* input was reduced from 30 DPH, with weaning completed by 35 DPH. Metamorphosis occurred at 20-23 DPH at which point fish were termed juveniles. Larvae and juveniles were generally fed to satiation, but not overfed as this often caused bacterial contamination of the gut. First grading typically occurred at 30-35 DPH.

Size heterogeneity and aggression during commercial rearing

The aim of the first part of the study was to describe the development of size heterogeneity and aggression in three commercial production batches that were known to be from single spawning events. Size heterogeneity was quantified every 4-7 d by taking a sample of larvae or juveniles from a commercial larviculture tank with a 5 l jug and dispatching via an overdose of Aqui-S™ (Aqui-S, New Zealand). Twenty individuals were selected at random and the total length measured under a binocular microscope to nearest 0.1 mm. Aggression was monitored on the same day prior to the first feed at 0800 h, and at 1600 h after the last feed. Aggressive intensity was quantified by observing the number of aggressive interactions exhibited or encountered by a randomly selected individual in a 30 s period. Aggression was categorised as: an aim against a conspecific (typically a J-posture, similar in appearance to that described by Sakakura & Tsukamoto, 1996); a chase (which may include nipping, Ebisu & Tachihara, 1993); or an ingestion (Ebisu & Tachihara, 1993). Ten individuals were observed per sampling period. When size variation was large enough to distinguish size grades ten individuals of each grade were observed, and the size grade of the recipient or aggressor involved in the aggressive interaction also noted. No distinct size grades were visually apparent prior to the addition of *Artemia*. Three size grades were apparent after 2-3 d of feeding on *Artemia*. Very small fish were often more yellow in colour and less agile in swimming ability, and large fish were distinctly larger than the rest of the population (for example see Figure 3 of Chapter 1). The medium grade fish were those that were intermediate in size between the large and small grade. For each size grade 10 individuals were observed for 30 s each and

the aggressive interactions recorded. Observations continued until commercial grading occurred, at which time different batches were often mixed.

Grading trial

The aim of the second part of the study was to use grading to separate the effect of size heterogeneity and aggression on the growth and survival of the three different size grades. The grading trial was conducted in 12 black 45 l plastic tanks located in a temperature controlled room. Water flow into the tanks was approximately 200 ml min^{-1} at a temperature of 22°C. A small air stone assisted water circulation and a central drainage standpipe used to adjust water volume. Fluorescent lights were placed 0.5 m above each tank to provide illumination (light intensity 2.5 x 10^3 lux at the water surface), and were programmed to follow the natural photoperiod. The juveniles used in the grading trial (~3 000 individuals, 28 DPH) were from a single spawning (separate to those used in the first part of this study) and were raised in a 5 m^3 tank according to the protocols above. On the morning of the commencement of the grading trial, Aqui-S™ was added to the rearing tank at a concentration of 5 ppm. Three sub-samples of the population were randomly selected (~300 juveniles $sample^{-1}$) to act as ungraded controls. The control samples were set aside while the remaining population was hand graded using beakers into three visual size grades of large, medium and small juveniles (using the criteria outlined above). As the fish were graded they were placed into recovery tanks. Following re-anaesthetisation (to reduce stress during transfer), three samples of 205 juveniles from the medium and small grades were randomly assigned to trial tanks. Only around 60 juveniles were transferred to each of the three randomly assigned large grade trial tanks as there were insufficient large juveniles available in the stock tank. Tank volume was adjusted to 35 l in the small and medium grade tanks, and 15 l in the large grade tanks in order to maintain similar stocking densities (3-6 juveniles l^{-1}). For the three control tanks water volume was adjusted to 35 l and 220 randomly selected juveniles were added to each tank from the three samples taken prior to grading. Five individuals were taken from each of the graded tanks for measurement of blotted wet weight and then stored in liquid nitrogen for RNA:DNA analysis. From each control tank 20 individuals were randomly removed and weighed. The entire process of grading and sampling took around 5 h to complete. Later in the day the juveniles were administered two feeds of *Artemia* and a small amount of dry feed. The weight distributions of the graded groups were later analysed to check that they represented statistically distinct sub-populations (see Results).

The juveniles were raised in the grading trial tanks for 12 d. *Artemia* and dry feed was administered 4 times daily, with increasing amounts of dry feed as the weaning period advanced. Weaning of *Artemia* was complete by 35 DPH, representing day 7 of the grading trial. Tanks were cleaned twice daily, and the mortalities collected and counted. The mortalities could not reliably be sized as often they were degraded or bitten. The aggression was monitored on days 1, 6 and 11 of the trial, in both the early morning (pre-feed) and in the afternoon (15 min post-last feed) according to the behavioural criteria mentioned above. For the control tanks five random observations were taken of large, medium and small sized juveniles. For the graded tanks five observations were made per tank. The aggression observation process took around 45 min to complete. The order of observations for behaviour was not randomised as the distribution of grades between tanks was assigned randomly, and the time difference between the first and last observation was relatively short. The trial finished on the morning of day 12, and from each of the graded and control tanks 10 and 20 individuals, respectively, were sampled for weight. From the large and small grade tanks five individuals were taken and stored for RNA:DNA analysis, and from the medium and control tanks 15 and 20 individuals were taken, respectively. Finally, the number of individuals remaining in each tank was counted.

In addition to using mass gain as a measure of the growth and condition of juveniles, whole body RNA:DNA ratio was also used. Following lyophilisation and re-weighing of whole fish, nucleic acids were quantified using the fluorimetric protocol of Weber et al. (2003). Each sample was analysed in triplicate, and inter- and intra-assay variability, and spike and recovery tests were carried out.

Data handling and statistical analyses

The growth rates of fish from three commercially reared batches were modelled as exponential functions and an ANCOVA of log transformed larval length was used to test for significant differences in growth rate (H_0 rejected at $P<0.05$). The coefficient of variation of length (CV_{length}) was used to compare size variance over time. The development of aggression was monitored in the same commercial batches of fish, though for brevity data for Batch 1 was presented. Aggressive behaviour was calculated as the number of aggressive interactions (e.g. aim, chase etc.) exhibited by an individual per 30 s^{-1}, and the non-parametric Kruskal-Wallis test used to detect significant differences between groups (H_0 rejected at $P<0.05$), with a Dunn's test for post-hoc comparisons. The development of aggression was presented in tabular form using the mean number of aims or chases for each observation period (count 30 s^{-1}

ind^{-1}) as an index of aggressive intensity. Individual variation in aggressive intensity was presented as the minimum number of individuals (calculated as a proportion) that could account for at least 75% of the observed aims or chases. Both aiming and chases were categorized according to size grade where possible.

Growth in the grading trial was calculated using blotted wet weight rather than total length because the juveniles were sufficiently large for this to be accurately determined. As the weight distributions were highly skewed, the non-parametric Kruskal-Wallis test was used to analyse significant differences between replicates, treatments and time periods, with a Bonferroni correction for multiple comparisons (H_0 rejected at $P<0.002$), and Dunn's tests used for post-hoc comparisons. The variance between replicates at day 0 (t_0) or day 12 (t_{end}) was not significant, so the data from the three replicates of each treatment was collapsed to simplify further analyses. Changes in weight variance of the different treatments and time periods were interpreted using the mean CV_{weight} of the three replicates. Survival was quantified by calculating the mean survival (derived from the daily mortalities counts) from the three replicates within each treatment and plotted against trial day. Differences in aggression between sampling periods and treatments were analysed in the same manner as that used in the analysis of aggression in commercial batches. Aggression in the grading trial was presented using the mean number of chases exhibited by large juveniles for each treatment (count ind^{-1} 30 s^{-1}) as an index of aggressive intensity, and 25% and 75% quartiles used as a measure of variability between replicates.

As has previously been reported, larval and juvenile weight has a significant effect on RNA:DNA ratio, and therefore direct comparison of juveniles of very different sizes can lead to erroneous conclusions (Buckley et al., 1999; Clemmesen, 1996). Buckely et al (1999) suggest that, where possible, nucleic acid levels should be normalised against size or developmental stage to account for the effect of ontogeny on nucleic acid ratios in juvenile fish. The juvenile weights in the present study varied by a factor of 7 between treatments, so the size effect was accounted for by log transforming the data (an appropriate transformation for growth data, Underwood, 1997) and plotting the mean RNA:DNA ratio against mean wet weight ± 95% confidence intervals for each treatment and time period (i.e. weight was used as a covariate). A regression line was fitted to the individual data but excluded the small grade, as the growth rate was distinctly different in this group.

Results

Size heterogeneity and aggression during commercial rearing

The growth rate of Batch 2 was significantly lower than the other two batches (ANCOVA $P<0.05$, Figure 13a), although the CV_{length} was similar between batches (Figure 13b). Both the growth rate and size heterogeneity increased around 18 DPH (Figure 13a & b).

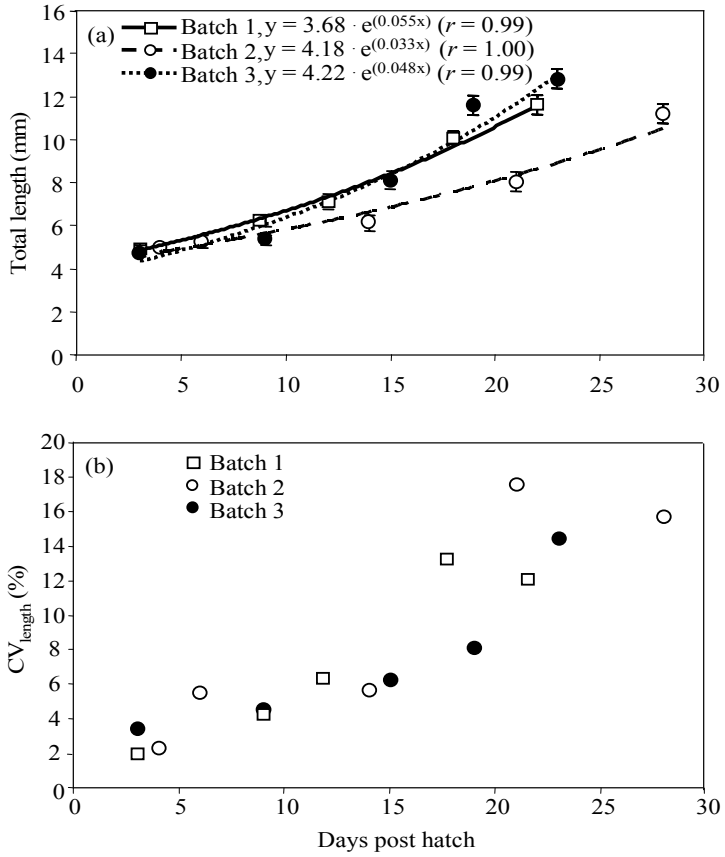

Figure 13. Growth rate of three commercial batches of yellowtail kingfish cultured between 21-23°C. (a) Total body length during development (mean ± SE, n=20 for each data point). (b) The coefficient of variation of body length during development.

The development of inter-individual aggression was evident at 12 DPH, with both the large and medium grade displaying aiming behaviour (Table 1). Aiming behaviour was generally higher pre-feeding, and the frequency increased significantly during development for the larger grade ($P<0.05$, Table 1).

Table 1. Aiming behaviour shown by different size grades of larvae and juveniles pre- and post-feeding. Data are presented using the mean number of aims exhibited (count ind^{-1} 30 s^{-1}) as an index of aggressive intensity (*n*=10 per observation period). Numbers in parentheses represent the proportion of the grade carrying out at least 75% of the chasing.

		Size grade of aggressor		
DPH		Small	Medium	Large
12	Pre-feed	0	0.4 (30%)	0.7 (30%)
	Post-feed	0	0.2 (20%)	0.4 (20%)
19	Pre-feed	0	0.3 (30%)	0.8 (20%)
	Post-feed	0	0.1 (10%)	0.3 (30%)
22	Pre-feed	0	0.3 (30%)	1.6 (40%)
	Post-feed	0	0.1 (10%)	0.5 (20%)

No small individuals were seen to aim. Only individuals of the large size grade were observed to chase, a behaviour which first became evident around 19 DPH (Table 2). Though variable between individuals, chasing behaviour was generally directed at smaller individuals, and later in development chases occurred irrespective of feeding state (Table 2). The variability in chasing behaviour between large individuals was illustrated by the fact that during any one sampling period at least 75% of the recorded chasing events could be attributed to only 10-20% of the large size grade. No incidences of cannibalism were observed when recording data, but were incidentally observed on an infrequent basis at the juvenile stage from around 30 DPH. In ungraded populations cannibalism has been observed to cause high mortality at 40 DPH or later.

Table 2. Chasing behaviour of large grade juveniles pre- and post-feeding, and the size grade of the chase recipients. Data are presented using the mean number of chases exhibited by the large juveniles (count ind^{-1} 30 s^{-1}) as an index of aggressive intensity (*n*=10 per observation period). Numbers in parentheses represent the proportion of individuals carrying out at least 75% of the chasing.

		Size grade of chase recipient			
DPH		Small	Medium	Large	Total
19	Pre-feed	0.4	0	0	0.4 (10%)
	Post-feed	0.1	0	0	0.1 (10%)
22	Pre-feed	0.5	0.2	0	0.7 (20%)
	Post-feed	0.2	0.1	0	0.3 (20%)

Grading trial

The within-treatment variance of blotted wet weight was not significant at t_0 or t_{end} (Kruskal-Wallis, $P< 0.001$) allowing the data for each of the three replicates to be pooled. The large grade at t_0 was equivalent to 8.3% of the original ungraded population, and the medium and small grades 50.0% and 41.7% of the original population, respectively. The size grades differed significantly in weight at both t_0 and t_{end}, and with the exception of the smallest grade, juveniles from all treatments had increased in weight by the end of the trial (Kruskal-Wallis, $P< 0.001$; Figure 14). At t_{end} no juveniles smaller than 36.5 mg were recorded from the control tanks (Figure 14). In contrast, 63% of the juveniles from the smallest grade were below this size.

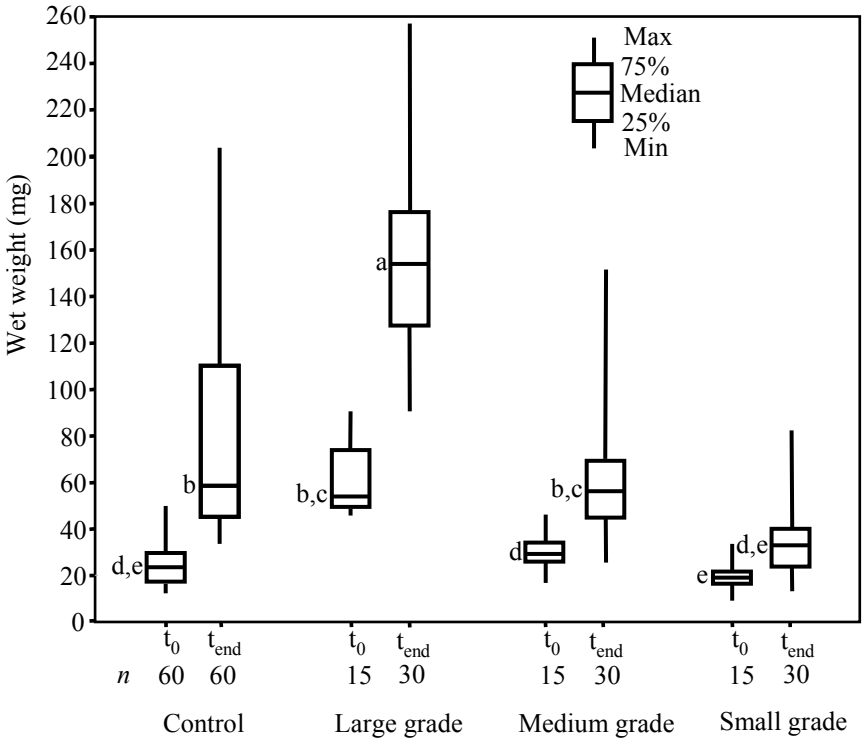

Figure 14. Changes in juvenile wet weight at t_0 and t_{end} of the grading trial. Data are displayed as median weight ± 75 and 25 percentiles, and minimum and maximum recorded values. Letters denote significant differences (Kruskal-Wallis, $P<$ 0.01).

The mean CV_{weight} was similar for all graded treatments at the start of the trial (large: 24.0%; medium: 24.5%; small: 23.2%), and considerably lower than that of the ungraded control group (34.9%). At the end of the trial the CV_{weight} had increased substantially for all treatments (medium: 39.7%; small: 46%; control: 54.6%) except the large graded treatment (26.4%). Juveniles from all treatments showed a poor level of survival in the first days of the trial, though the large grade was apparently less affected than the other treatments (Figure 15). Survival stabilised at day 4 for the large grade (80% survival), and to a lesser degree for the medium grade and control group (70% and 68% survival, respectively, Figure 15). The small grade had a relatively constant decrease in survival rate throughout the entire trial, whereas the medium grade and control group showed a decline in survival midway through the trial (Figure 15). Survival was poor for the control, small and medium grade treatments (15%), whereas 55% of the large grade survived.

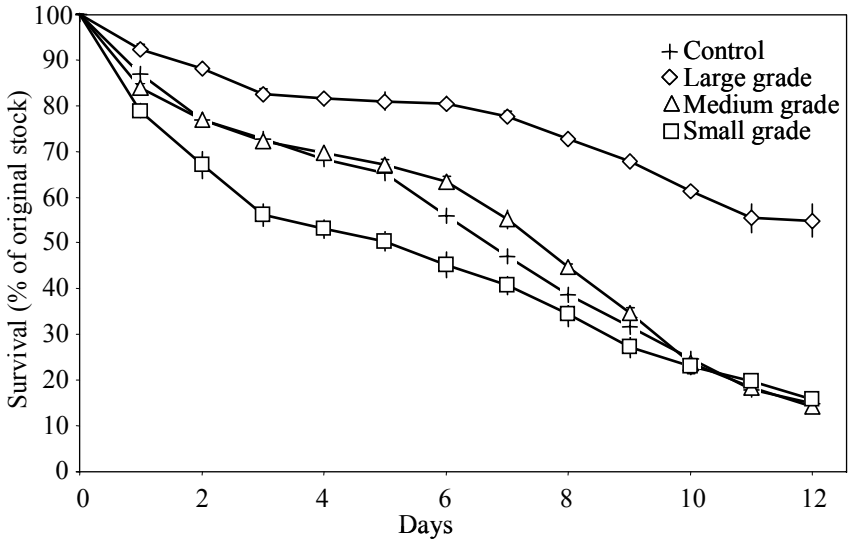

Figure 15. Juvenile survival during the grading trial. Survival is shown as the mean survival from three replicate tanks ± SE.

There was no significant difference in chasing behaviour between replicates Kruskal-Wallis, P<0.001). Chasing did not develop to a significant level until midway through the trial, after which it became very intense in the ungraded tanks, in particular pre-feed (Figure 16). As observed in the commercial culture tanks, chasing was carried out only by large individuals in the grading trial. Chasing behaviour was generally more intense in the control tanks of the grading trial (0.2-1.6 counts ind^{-1} 30 s^{-1}) than both the graded tanks (<0.1 counts ind^{-1} 30s^{-1}) and commercial culture tanks (0.1-0.7 counts ind^{-1} 30 s^{-1}).

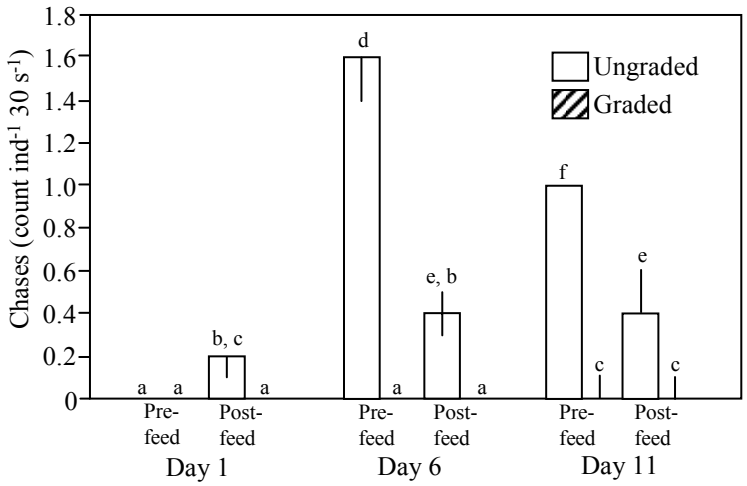

Figure 16. Chasing behaviour exhibited by large juveniles during grading trial. Chases are expressed as the median number of chases (count ind^{-1} 30 s^{-1} ± 25 and 75% quartiles) observed per tank (*n*=3). See text for description of how chasing observations were made. Letters denote significant differences (Kruskal-Wallis, *P*< 0.001).

Inter- and intra-assay variability in the RNA:DNA assay was 8.3% and 10.7%, respectively, but neither were significantly different (ANOVA, *P*<0.01). Spike and recovery tests yielded 92.6% and 101.3% of the DNA and RNA, respectively. The linearity of response of the assays were as follows: RNA + ethidium bromide *r*=1.00, DNA + ethidium bromide *r*=1.00 and DNA + Hoechst *r*=0.97. The RNA:DNA ratio increased in both the large and medium grade trade treatments over the course of the trial, however small grade juveniles decreased in RNA:DNA ratio (Figure 17). The ungraded control juveniles at t_{end} had a similar RNA:DNA ratio as the medium and large grades at equivalent mean weights. A linear regression of the data from all treatments and time periods (excluding the small grade) resulted in a near isometric (*r*=0.73, *n*=120, Pearson's r <0.01) relationship between log transformed wet weight and log RNA:DNA ratio (Figure 17).

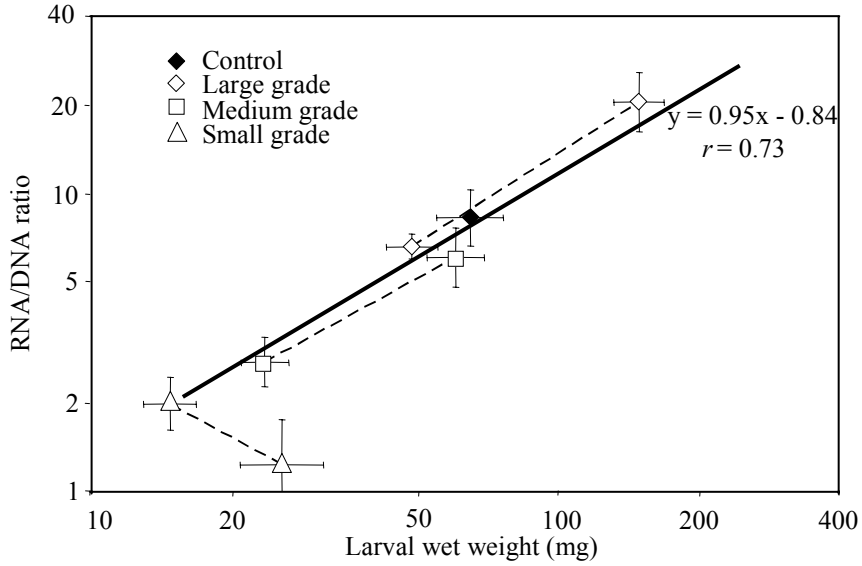

Figure 17. Relationship between larval wet weight and RNA:DNA ratio at t_0 and t_{end}. Data are displayed on logarithmic axes as the mean wet weight and RNA:DNA ratio with 95% confidence intervals. The linear regression represents all data except that from the small grade (see explanation in Methods). Sample sizes (t_0, t_{end}) are as follows: large, medium and small grade *n*=15, 30; control (t_{end}) *n*=60.

Discussion

The growth rate of larvae and juveniles presented in this chapter confirms the findings reported by Ebisu and Tachihara (1993) for yellowtail kingfish. Aiming behaviour was first observed 12 DPH, and chasing behaviour 19 DPH. Japanese yellowtail exhibit a similar pattern of growth and aggressive development, with J-posturing occurring from 15 DPH, and chasing and cannibalism from 23 DPH (Sakakura & Tsukamoto, 1996; Sakakura & Tsukamoto, 1999). The key differences between the two species are 1) the presence of aiming behaviour in yellowtail kingfish even after chasing sets in (this study), and 2) cannibalistic behaviour did not occur in yellowtail kingfish until around 30 DPH (this study and Ebisu & Tachihara, 1993). Large juveniles were found to be the dominant aggressors carrying out all of the chases, and small individuals were the main targets, although later in development medium sized individuals also became targets. Subordinate Japanese yellowtail are also typically smaller individuals (Sakakura & Tsukamoto, 1999), and have been shown to have elevated levels of the stress hormone cortisol (Sakakura et al., 1998), which has in turn been correlated to lowered growth (Jentoft et al., 2005) and immunocompetence in fish (Iguchia et al., 2003). This means that although chasing behaviour may not lead to the mortality of

recipients in the same direct manner that cannibalism does, the stress of being chased can increase the likelihood of mortality through growth inhibition, starvation and infection.

The increase in size heterogeneity and aggressive behaviour in yellowtail kingfish coincided with the switch to *Artemia* as a feed, and accelerated during the co-feeding of *Artemia* and dry feed (20-30 DPH). Changes in feed are known to be a precursor to increased size heterogeneity and aggression, as fish that are slightly larger or more capable of feeding are reported to gain a size advantage when moving onto advanced, and often more nutritious, diets (Kestemont et al., 2003). While the simultaneous presentation of foods with differing nutritional content undoubtedly augmented the development size heterogeneity during the co-feeding period, this was necessary to allow juveniles of varying feeding and digestive abilities to progress from live prey to dry feed. This strategy is also used in the culture of Japanese yellowtail (Sakakura & Tsukamoto, 1996; Yamazaki et al., 2002). Feeding state had little effect on the intensity of chasing behaviour later in development, and there was no limitation to food availability as the fish were fed to satiation. These factors indicate that the aggression is unlikely to have been related to juveniles developing cannibalistic behaviour to fulfil nutritional requirements, a similar conclusion reached by Sakakura and Tsukamoto (1998b) for Japanese yellowtail.

The large individuals, which carried out the majority of the aggression, made up 8% of the population, and were 2-3 times the weight of the subordinates prior to grading. The small grade were the main recipients of aggression and comprised 42% of the population, while the medium grade, which encountered a low to moderate level of aggression, composed 50% of the population. These figures are similar to the observations of Sakakura and Tsukamoto (1998a) on the hierarchical composition of cultured Japanese yellowtail. As has been noted with other fish species (Francis, 1983; Greaves & Tuene, 2001; Sakakura & Tsukamoto, 1998a; Sakakura & Tsukamoto, 1998b; Schjolden et al., 2005), there was considerable individual variation in aggression by yellowtail kingfish, such that at any one time only 1% of the entire population was carrying out 75% of the aggressive interactions.

Mortality in the grading trial was higher for all treatments (40-85% mortality) than would normally be expected in commercial culture (~10-40% mortality at NIWA Bream Bay Aquaculture Park) and may be due to a number of factors. Firstly, there was a considerable amount of handling involved in hand grading the juveniles, and this is

likely to have caused a high level of stress and possible mechanical damage, though the use of beakers to sort fish would have minimised the latter. The high mortality in the days after transfer despite low levels of aggression appears to support this, though it was interesting to note that the large fish were apparently more amenable to handling than smaller fish. Secondly, the aggression in the ungraded control tank was more intense than in commercial tanks, possibly because the small volume provided less spatial refuge for subordinates. Thirdly, experience shows yellowtail kingfish larviculture is generally less successful in small tanks than large tanks as small tanks are more prone to fluctuations in the physicochemical properties of the water. Despite these limitations the grading trial provided meaningful data on the relationship between growth, aggression and juvenile size. As was observed in commercial rearing tanks, agonistic interactions were only recorded by large grade juveniles, and only to any significant degree in control tanks. Size heterogeneity increased substantially in these tanks, such that by the end of the trial the largest juveniles were 4-6 times the weight of the smallest juveniles. In contrast, the weights within the other treatments only varied by a factor of 1.5-2.5. This indicates that size heterogeneity plays an important role in the severity of aggression, as has been reported for other species (Baras et al., 2000a; Kestemont et al., 2003; Sakakura & Tsukamoto, 1998b; Smith & Reay, 1991).

There was no chasing behaviour or obvious dominance hierarchy exhibited by the small or medium grade juveniles, despite the fact that at the end of the trial these juveniles were at the same size as the dominant large grade at t_0. Additionally, the size heterogeneity and weight ranges of juveniles from the medium grade tanks at t_{end} were likely to have been similar to that of the ungraded tanks midway though the trial, yet unlike the control tanks there were virtually no agonistic interactions by this grade. It is unclear whether the failure to establish a new dominance hierarchy by medium or small graded juveniles extends from a learned social position as an intermediate or subordinate (Sakakura & Tsukamoto, 1998b), or whether some or all large juveniles are intrinsically more aggressive.

While this study was unique in the use of RNA:DNA ratios to partition the effects of growth potential and aggression in juvenile growth, several studies have used this condition index for investigating nutritional aspects of larval and juvenile fish growth. An important factor in the interpretation of the RNA:DNA ratio data in the current study was the use of weight as a covariate to compare different treatments. While RNA:DNA ratio has been found to increase during development in larvae and juveniles of a number of fish species (Clemmesen, 1996; Gwak et al., 2003; Khemis et al., 2000;

Mercier et al., 2004; Rooker & Holt, 1997), the strength of the relationship in the current study was somewhat more pronounced. Had weight not been used as a covariate, the higher RNA:DNA ratios of large individuals would simply have been interpreted to mean that this grade benefited from higher protein synthesis rates, as the juveniles were all the same chronological age. In fact, large juveniles did not have a significantly different RNA:DNA ratio to medium grade or control juveniles at equivalent weights, meaning that the higher growth rate of this grade was attributable to higher food capture or assimilation traits. The RNA:DNA ratios of the large grade at t_{end} (>20) were considerably higher than that reported for fed juveniles of other species at similar stages of development, which are generally between 2-10 (Chícharo, 1998; Gwak & Tanaka, 2001; Gwak et al., 2003; Khemis et al., 2000; Mercier et al., 2004; Raae et al., 1988; Wright & Martin, 1985). Rooker and Holt (1997) caution against comparing RNA:DNA values between studies due to methodological differences (e.g. tissue preparation, standards), so it is possible that the high RNA:DNA values of large individuals at t_{end} may be attributable to methodological factors. It is equally plausible that the high RNA:DNA ratio of the large, rapidly growing grade of yellowtail kingfish juveniles was attributable to the comparatively warm rearing temperature and fast growth characteristics of this genus (Nakada, 2002). The RNA:DNA ratios of the other juvenile grades in this study were similar to that recorded by Weber et al. (2003), who reported RNA:DNA ratios of 3-8 for juvenile fathead minnow (*Pimephales promelas* Rafinesque 1820) and rainbow trout (*Oncorhynchus mykiss* [Walbaum, 1792]). Given that this study used an identical methodology to that of Weber et al. (2003), it would seem that the results can be treated with confidence.

The RNA:DNA ratio for the small grade at t_{end} (1.02) was similar to that reported for starving juveniles of other species (Caldarone, 2005; Clemmesen, 1994; Mathers et al., 1994). As the grading trial was carried out over the dry feed weaning period, the poor survival and growth of many of the smaller individuals may represent a failure to properly adapt to dry feed. The development of the gastrointestinal tract in yellowtail kingfish is complete at around 30 DPH with the formation of the pyloric caecae (Chen et al., 2006b), which coincides with the weaning period. Small individuals may lack a properly formed gastrointestinal tract at the time of weaning, and dry feed may not be as digestible or assimilable as *Artemia*. Alternatively small individuals may be less competent at identifying dry feed as a food source or ingesting it. It is unlikely that the nutritional quality of the food was an issue given the high growth rate of other individuals within the cohort. Irrespective of the reason for the failure to grow, it was evident from both the poor weight gain and low RNA:DNA ratio that many small

individuals were starving in the presence of an abundant food supply and no aggression. A comparison of the weight distribution at t_{end} showed that no juveniles smaller than 36.5 mg survived the ungraded treatment, a weight which was above 63% of the small grade. The fact that most of the small grade failed to survive the ungraded treatment implies that aggression can be estimated to attribute to 25-30% of the mortality of the original population. However, given that the weight and RNA:DNA ratio data indicated that most juveniles of this grade were on a poor developmental trajectory, it seems that aggression may simply quicken the demise of this proportion of the population. If this hypothesis is extrapolated to the original population, it can be estimated that 40% of a pre-weaned batch is unlikely to survive due to inherent developmental or feeding problems.

The findings from this study have some important considerations for larviculture of yellowtail kingfish. Firstly, given that the main aggressors were the large individuals, initial grading need only be carried out on the upper 10% of the population, which can be three or more times the weight of most of the remainder. This is an advantage considering most commercial graders are not particularly effective on body widths 2-3 mm (which equates to around 10-20 mm total length), the size range of most subordinates at the onset of chasing. Ebisu and Tachihara (1993) reported that cannibalistic mortality of yellowtail kingfish at 35 DPH could be reduced by grading large fish, however, the current study shows that earlier grading (e.g. 28-30 DPH) of particularly large individuals may also be useful for lowering mortality associated with non-cannibalistic aggression. Secondly, grading and the minimisation of aggression seem important only for the 40-60% of the population that make up the medium grade juveniles, as at least some of the individuals in this grade appear to have the potential to follow a viable growth trajectory (as determined by RNA:DNA ratios). Thirdly, 30-40% of the population (the smallest individuals) appear to have developmental problems that will ultimately lead to mortality, regardless of the aggressive environment. It is unclear why such a large proportion of the population is set on a degenerative developmental trajectory. Obvious answers would be differences in egg quality or genetic composition, although currently there is not enough known about these subjects to determine the relative contribution of either. In summary, while aggression is an important factor in modifying population size structure and is correlated with high mortality periods during yellowtail kingfish culture, much of the mortality may occur irrespective of the aggressive environment, albeit later in development.

Chapter 5: Stress Physiology During Transport

Introduction

The road transport of kingfish fingerlings (*Seriola lalandi* Valenciennes 1833) from the National Institute of Water and Atmospheric Research Limited (NIWA) Bream Bay Aquaculture Park to commercial on-growing clients is one of the most important components of the supply chain. The cost and risk involved in live transport is high compared to most other aspects of production, and is one of the most intense stressors cultured fish encounter. A high demand for oxygen by the transport biomass combined with an accumulation of waste products in a closed system presents a significant physiological challenge to fingerlings. A considerable amount of research has been carried out on minimising stress and maximising fingerling transport densities for a number of species (Farrell, 2006; Golombieski et al., 2003; Paterson et al., 2003; Pavlidis et al., 2003; Rimmer, 1997), as live transport is an area where large gains can be made by making transport more economical and delivering fish in a better physiological state for on-growing (Wedemeyer, 1996). Most of this research has focussed on freshwater species with considerably lower metabolic rates than yellowtail kingfish, and probably different physiological stress tolerances considering the difference in athletic ability. This highlights the need to establish baseline data on the effects of live transport on yellowtail kingfish.

The main factors that affect fish health during transport are: low dissolved oxygen (O_2); elevated dissolved carbon dioxide (CO_2); ammonia (NH_3) toxicity; and physical damage (Wedemeyer, 1996). By far the most critical factor is low O_2. The use of bottled O_2, efficient diffusers and, more recently, automated delivery systems have ameliorated transport hypoxia (Rimmer et al., 1997). Elevated CO_2 levels remain an industry concern and limiting factor as stocking densities and transport time have increased with the development of the aquaculture industry, not only for road transport (Rimmer, 1997), but also for well-boat transport (Rosten et al., 2005). High ambient CO_2 levels increase the partial pressure of CO_2 in the blood (termed hypercapnia), which in turn affects the ability of haemoglobin to both bind and offload oxygen (Brauner & Randall, 1996). In addition, depending on the exposure time, hypercapnia is known to affect respiration and ventilation rate, reproduction, early development, and growth (Ishimatsu et al., 2005). Unlike O_2, very little is known about what constitutes a safe CO_2 level for either the live transport or husbandry of marine fish. This is due to a combination of factors including the historical prevalence of freshwater fish culture, the interactive nature of the effects of CO_2 with other water quality parameters, and

difficulties associated with measuring CO_2 directly. The pH of water is often used as a proxy for CO_2, as when CO_2 dissolves in water carbonic acid is formed and the pH decreases (Roy et al., 1993), though this can be somewhat problematic given the buffering capacity afforded to saltwater by the high carbonate concentration. The main method by which CO_2 is controlled during road transport is the use of chemical buffers or degassing (Rimmer, 1997), the latter being the most effective method for saltwater given the naturally high buffering capacity.

Ammonia toxicity occurs through the formation of high concentrations of the unionised form (NH_3), and is acutely toxic from around 0.5 mg l^{-1} (Ip et al., 2001). The ionised form of NH_3 (ammonium, NH_4^+) is not particularly toxic and the concentration of this form of NH_3 increases with H^+ concentration. The presence of NH_3 can be minimised by ensuring transported fish are post-digestive and the water pH remains below approximately 7.5. Accumulated CO_2 can lower water pH sufficiently to minimise the risk of ammonia toxicity, so for this reason NH_3 accumulation generally is not considered to be a problem for short to moderate periods of confinement (i.e. 0-2 days). Physical damage during live transport can be limited by the use of anaesthetics or fish pumps during loading to minimise handling damage, and using transport tanks with a smooth finish.

The purpose of this chapter was to investigate the physiological capacity of yellowtail kingfish fingerlings to withstand handling and confinement stress associated with live transport. All fingerlings produced to date at the NIWA Bream Bay Aquaculture Park have been transported to on-growing operations via road transport with varying degrees of success. Previous transports using the NIWA salmon smolt transporter have seen the pH of moderately stocked tanks (20 kg m^{-3}) decline to 6.8-7.2 despite the constant use of agitators. These figures represent low-moderate stocking densities and considerable pH decreases compared to salmon transports, indicating that yellowtail kingfish juveniles have a high CO_2 excretion rate. In order to increase loading densities and improve the survival and health of transported fish, it is important to understand how yellowtail kingfish fingerlings respond to elevated CO_2 levels, as this is the most likely factor that will limit the improvement of transportation. In order to test how well fingerlings cope with transport and hypercapnia, two trials were undertaken to simulate a five hour transport to a client for on-growing.

The first trial involved measuring physiological condition indices during and after the transport. Mortality is a crude and inadequate measure of the effectiveness of live

transport technologies in the finfish aquaculture industry when fish are required for on-growing. Compromised metabolic status has a significant effect on growth through induction of the endocrine stress response (Pankhurst & Van der Kraak, 1997). Accordingly, researchers have focussed on key indicators of primary and secondary stress in order to optimise protocols for handling and transport of fish (Frisch & Anderson, 2000; Pickering, 1998; Schreck et al., 1989). Although endocrine measurements are powerful primary indicators of the stress response, the neurogenic stress response involved in the release of corticosteroids and catecholamines can be so rapid that it is difficult to obtain useful control samples.

Blood glucose and plasma and muscle lactate were measured as these are potential secondary indicators of metabolic stress that are easy to measure (Wells & Pankhurst, 1999). Muscle buffering capacity was also measured. This parameter reflects the ability of tissues to suffer the insults of perturbation to pH, and may change during exposure to intense exercise or respiratory acidosis as H^+ accumulates in tissues and reduces buffering capacity. Athletic species like yellowtail kingfish have metabolic defences protecting against the excess production of acidic end-products of anaerobic metabolism that accumulate during burst swimming (*cf*. Castellini and Somero 1981). These are naturally buffered by the high proportion of histidine-containing proteins in the fillet muscles of the fish. Muscle buffering capacity has been manipulated in Japanese yellowtail (*S. quinqueradiata* Temminck & Schlegel 1845) using high histidine diets in order to improve swimming ability and taste (Ogata, 2002), but little is known about the effect of acute stress and recovery on muscle buffering capacity in fish.

The second trial involved measuring blood haematology at differing CO_2 levels in order to quantify the effect of hypercapnia on both the haematocrit (% of packed red blood cells in total blood volume) and red blood cell (or erythrocyte) haemoglobin content. Elevated concentrations of circulating blood H^+ levels resulting from hypercapnia can cause erythrocytes to swell due to differences in the intra- and extracellular osmotic gradient, and erythrocytes can also swell due to adrenergic effects (Gallaugher & Farrell, 1998). There is a corresponding loss in the oxygen carrying capacity per unit volume of blood, in addition to a reduction in haemoglobin binding and off-loading efficiency due to the Bohr effect (Burggren et al., 1991).

As well as measuring stress parameters during and after transport, a calculation was also made of the resting O_2 consumption, and CO_2 and NH_3 excretion rates of

yellowtail kingfish fingerlings based on the findings of Chapter 6. These are compared to rates known for other species in order to establish how much the metabolic rate of yellowtail kingfish fingerlings differs from other species, and gives an indication of the challenges that transport of this species represents.

Materials and Methods

The O_2 consumption and NH_3 excretion rate was calculated from the relationship between mass and resting oxygen consumption rate given in Figure 34 (Chapter 6) for a range of masses which were used for transport (5, 10 and 20 g). The molar oxygen consumption rate ($\dot{N}O_2$) was converted to a mass oxygen consumption rate ($\dot{M}O_2$) to allow for comparison with other studies, and the same was done for NH_3 excretion rate. The time to acute NH_3 toxicity (0.5 mg l^{-1}) (Ip et al., 2001) for a stocking density of 20 kg m^{-3} was calculated using 0.81% as the percentage of unionised NH_3 present at pH 7.4, 20°C and 30 ppt salinity (Wedemeyer, 1996). Previous live transports of yellowtail kingfish fingerlings using the NIWA salmon transporter had shown that the pH during transport varied from 6.6-7.6. The CO_2 excretion rate was estimated from stoichiometric relationships with O_2 consumption using a respiratory quotient of 0.84, which is approximately mid-way between lipid or carbohydrate (0.72 and 1.0 respectively) and protein (0.97) as energy substrates (Gnaiger, 1983). Data from Chapter 6 indicated that approximately 50% of aerobic metabolic energy requirements are met through protein catabolism for 10 g yellowtail kingfish. From this quotient it can be calculated that CO_2 production is approximated by:

$$\dot{M}CO_2 \text{ (mg h}^{-1}\text{ kg}^{-1}\text{)} = 1.16 \times \dot{M}O_2 \text{ (mg h}^{-1}\text{ kg}^{-1}\text{)}$$

The ability of yellowtail kingfish fingerlings to tolerate transport stress was assessed via two simulated transport trials representative of a trip to an on-growing operation five hours drive away. The first trial involved measuring a variety of factors related to stress responses (mortality, blood glucose, plasma lactate, muscle lactate, pH and buffering capacity) in transported and control fingerlings both during transport and in the recovery phase. The loaded transport tank was maintained at the NIWA Bream

Bay Aquaculture Park in order to observe fish continuously. A stocking density of 20 kg m^{-3} was chosen as this represented the most heavily stocked NIWA salmon transporter tanks. Degassing was carried out using compressed air from a dive cylinder equipped with 2-stage regulator, a 40 l min^{-1} flow meter and a ceramic diffuser. As the dive bottle became empty mid-trial a compressor was used instead, which took around 20 min to install.

Transportation was simulated using an 800 l insulated transport tank filled to 540 l with well-oxygenated seawater (20.4°C, 35‰). In addition to air, the facility for pure oxygen was supplied from a cylinder via a diffuser. Prior to loading, approximately 3200 post-digestive fish (purged for 24 h) were lightly sedated with 6 ppm AquiS (clove oil based FDA approved anaesthetic, AquiS New Zealand Ltd, Wellington, New Zealand) and weighed. A biomass of 11.19 kg (fingerling weight range 2-6 g) was introduced to the hauling tank while water was flushed through the tank at approximately 40 l min^{-1}. Approximately 50 fish were transferred to a separate flow-through holding tank and monitored as controls. Both pH and dissolved oxygen were measured with a TPS-90FL field meter (TPS Pty Limited, Australia), and oxygen saturation was maintained at 90-110% by controlling the flow of gaseous oxygen. The CO_2 concentration of the transport water was estimated from pH using equation 2.7 of Timmons et al. (2002) at an alkalinity of 100 mg l^{-1} $CaCO_3$ equivalent (estimated alkalinity of seawater). At the completion of the simulated transport fish were transferred to two holding tanks for observation and sampling at 24 h intervals during the recovery phase (see Table 3). The fish were fed soon after transfer to the holding tanks and again, 12 h later.

The sampling protocol followed the regime in Table 3. At each sampling time, fish were individually netted from either the hauling or control tank, immediately despatched by pithing and weight recorded. The fish could be caught with relative ease from near the water surface of the transport and control tanks, and there was little effect on the behaviour of other fish in the tank. The tail was then severed and the droplets of blood forming at the caudal vein were contacted with the test strips of an Accu-Chek Advantage blood glucose meter (Roche Diagnostics, Australia) and an Accusport Portable Lactate Analyzer (Boehringer Mannheim, Roche Diagnostics, Australia). Next, a cut was made with a scalpel into the white muscle behind the dorsal fin and a pH electrode (IJ44 spear electrode, Ionode Pty Ltd, Australia) inserted into the muscle and the pH recorded after 20 sec (WP80 pH meter, TPS Pty Limited, Australia). Finally, two portions of white muscle were removed (approximately 0.1-0.2 g each) for muscle lactate and muscle buffering capacity measurements, and stored on dry ice before

being transferred to a -70°C freezer. The entire sampling process was completed in less than 2 min. Exercised fingerlings were also sampled for comparison with control and transported fish. Fingerlings were exercised by chasing around a 20 l bucket for 5 min with a stick before sampling, a similar technique to that used by Yamamoto et al. (1980) for testing the haematology of exercise exhausted Japanese yellowtail. Ten fish were sampled per time period and treatment. Muscle lactate was determined using the method of Barnett and Pankhurst (1998) and expressed as mmol lactate kg^{-1} tissue mass. Muscle buffering capacity was determined using the method of Ogata (2002) and expressed as mmol NaOH pH^{-1} kg^{-1} muscle. Significant differences in physiological stress indicators between transported and control fish were assessed via *t* – tests with an alpha level <0.05.

Table 3. Sampling regime showing number of fish sampled at each period

Time(Hours)	Control Fish	Transported Fish	Exercised fish
0 (pre-transport)	10		10
2.5 (mid-transport)	10	10	
5 (pre-unloading)	10	10	
24	10	10	
48	10	10	
120	10	10	

The second transport trial involved measuring erythrocyte stress in response to varying levels of hypercapnia and subsequent recovery. To do this, three 50 l tanks were set up and 60 fingerlings (post-digestive, 10-15 g each) transferred into each tank under light sedation as previously mentioned. The tanks were flushed with water (2 l min^{-1}) for 30 min to allow the fish to recover (temperature 20.5 - 21.0°C, salinity 35‰), and the water flow stopped. Gaseous CO_2 was added to two of the tanks until the pH was 7.4 and 6.6, while one tank was kept as a control (pH 8.2). The CO_2 concentrations at pH 8.2, 7.4 and 6.6 were calculated as 1, 8 and 50 mg l^{-1}, respectively, using the method previously described. The fingerlings were maintained in the tanks for 5 h to simulate a transport, with gaseous oxygen added at a low flow rate in order to maintain oxygen saturation between 80-100%. In order to maintain the pH within ± 0.1 of the

treatment values the water was partially exchanged during experimentation to compensate for CO_2 excreted from respiration. Once the 5 h simulated transport was over, water flow was resumed, a feed administered and the fish allowed to recover for 31 h. A sample of 10 fish from each treatment were removed at 0, 2.5, 5, 10 and 36 h for haematology. Blood samples were collected by dispatching via pithing, severing the tail and collecting a drop of blood in a heparinized microcapillary tube, and sealing the microcapillary tube with plastercene before storing on ice. The haematocrit (Hct), blood haemoglobin concentration (Hb) and mean cell haemoglobin concentration (MCHC) were then quantified following the methods of Wells and Pankhurst (1999). A single sample of exercised fish (exercised as before) was also tested for comparative purposes. Significant differences between treatments at each time period were tested using t-tests with an alpha level <0.05.

Results

The estimated metabolic rates of yellowtail kingfish fingerlings at 5, 10 and 20 g are given in Table 4. At a stocking density of 20 kg m^{-3} and a pH of 7.4, it was estimated that NH_3 would reach toxic levels (0.5 mg l^{-1}) after 110, 118 and 128 h of confinement for 5, 10 and 20 g fish, respectively.

Table 4. Estimation of O_2 consumption and NH_3 and CO_2 excretion rate (mg h^{-1} kg^{-1} biomass) for yellowtail kingfish fingerlings.

Fingerling weight (g)	O_2 consumption	NH_3 excretion	CO_2 excretion
5	809	28	934
10	696	26	804
20	589	24	680

After the simulated transport of the first trial was initiated it soon became apparent that in order to stabilise the pH and CO_2 concentration it was necessary to have a high amount of aeration. Within 45 min the CO_2 concentration had increased to over 20 mg l^{-1}, even with increasing air flow (Figure 18). Approximately 1.5 h into the trial the dive tank was exhausted and the CO_2 concentration increased considerably. After an air

compressor was installed and a suitable level of degassing could be achieved, the CO_2 level stabilised at around 75 mg l^{-1} and slowly began to decrease. During the middle period of the transport many of the smaller fish were seen as inactive, rafting in the corners of the tank and yellowed.

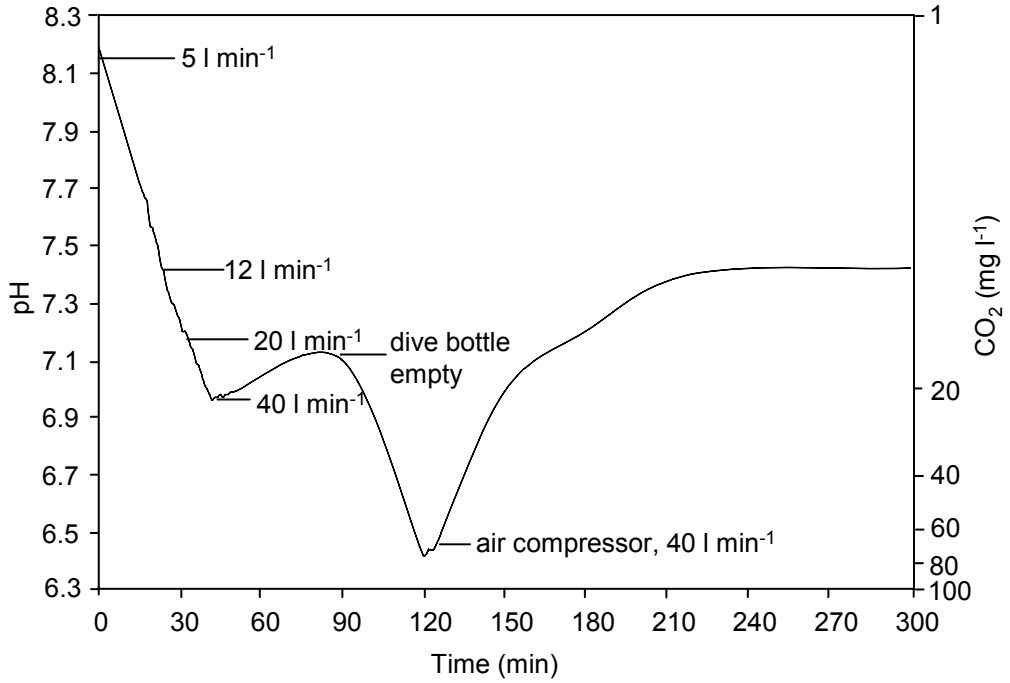

Figure 18. Profile of pH changes and estimated CO_2 concentration of transport tank during trial. Initially a dive tank was used to provide compressed air for degassing (air flow rates shown), though mid-trial a compressor was used as the dive tank ran empty.

At the completion of the transport five fish were found to have died (0.16% mortality), all of which were small with jaw and opercula deformities. One day after transport 12 more fish were found to have died in the recovery tank (0.38% mortality), of which nine were deformed. No further mortalities occurred.

Blood glucose in transported fingerlings was significantly elevated mid-way through the transport and was comparable to that of exercised fish (> 6 mmol l^{-1}, Figure 19). For the remainder of the trial blood glucose levels were similar to that recorded in control fingerlings.

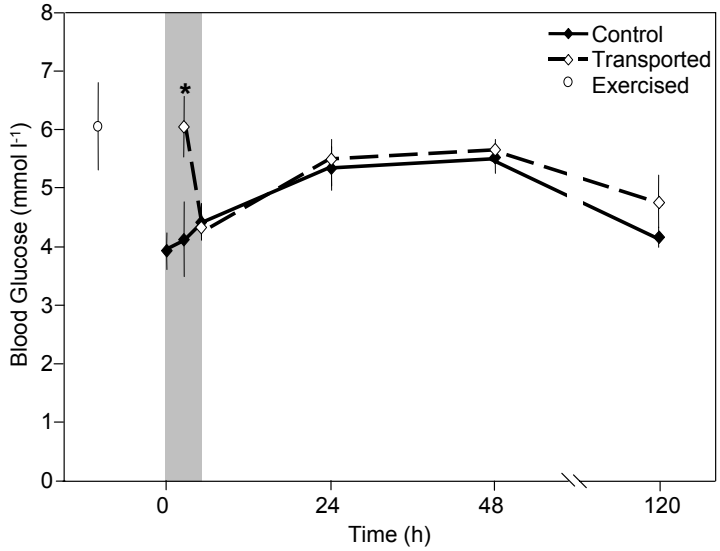

Figure 19. Changes in plasma glucose concentration (mean ± SE). Significant differences between transport and control treatments (p<0.05) are indicated by '*', and the grey bar indicates the transport period.

Plasma lactate varied between 0.9-2.7 mmol l^{-1} for both control and transport treatments, but was only significantly different at 24 h (Figure 20). Exercised specimens had an elevated plasma lactate level of 3.3 mmol l^{-1}.

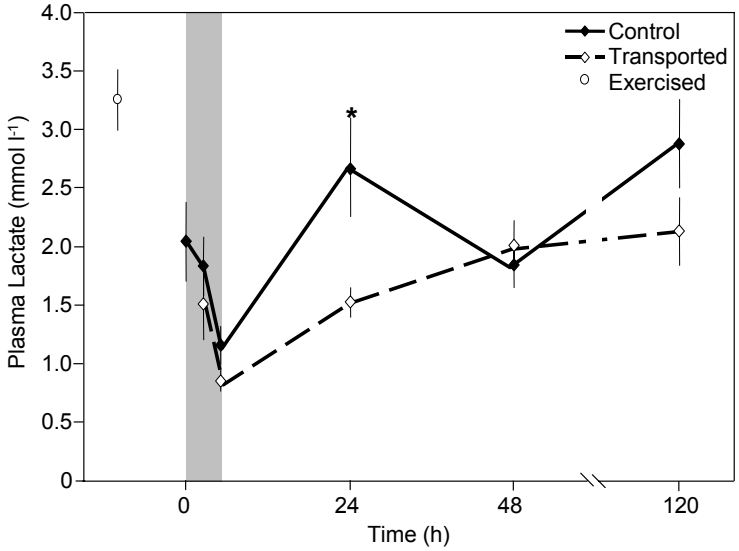

Figure 20. Changes in plasma lactate concentration (mean ± SE). Significant differences between transport and control treatments (p<0.05) are indicated by '*', and the grey bar indicates the transport period.

Mean muscle lactate content did not vary significantly between control and transported fingerlings (20.4–29.9 mmol kg^{-1}), though exercised individuals had greatly elevated muscle lactate levels (53.0 mmol kg^{-1}, Figure 21). Muscle pH of exercised fingerlings

was similar to that of the control treatment, which varied between 6.6-6.8 (Figure 22). Transported fingerlings had a substantially elevated muscle pH (7.2) at the end of the simulated transport, though were largely similar to the range of control values at other time periods.

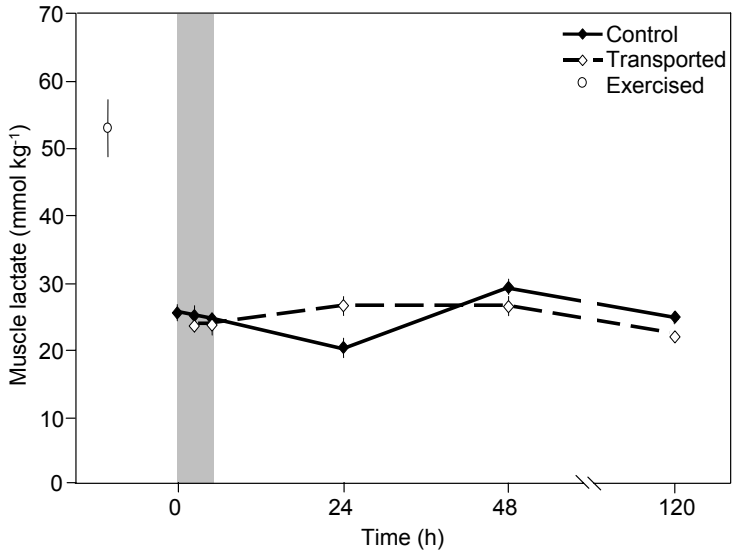

Figure 21. Changes in muscle lactate concentration (mean ± SE). Significant differences between transport and control treatments (p<0.05) are indicated by '*', and the grey bar indicates the transport period.

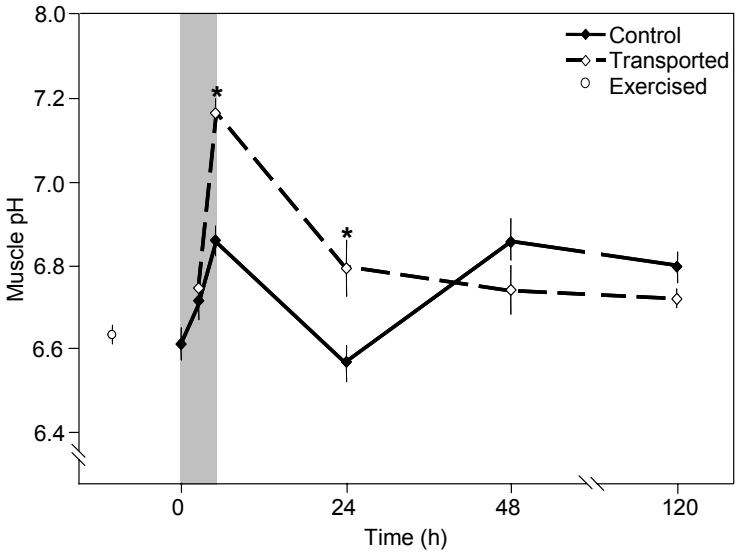

Figure 22. Changes in muscle pH (mean ± SE). Significant differences between transport and control treatments (p<0.05) are indicated by '*', and the grey bar indicates the transport period.

Muscle buffering capacity ranged between 41.5-47.6 mmol pH^{-1} kg^{-1} but was not significantly different between treatments (Figure 23).

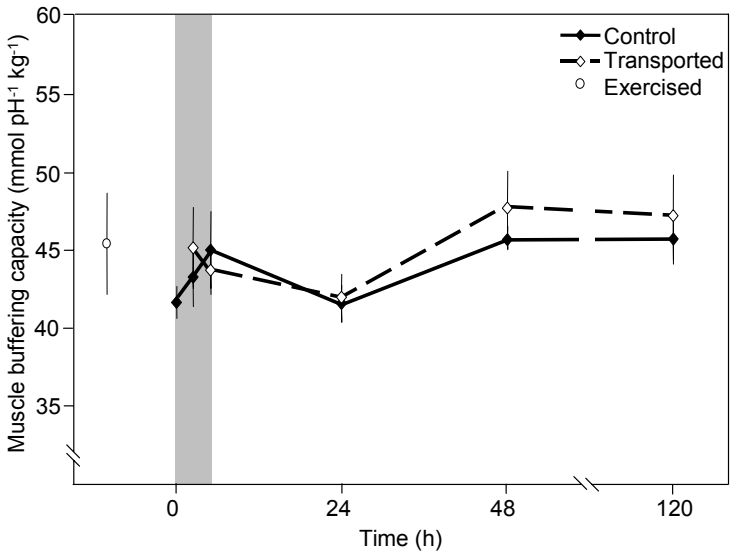

Figure 23. Changes in muscle buffering capacity (mean ± SE). Significant differences between transport and hypercapnic treatments (p<0.05) are indicated by '*', and the grey bar indicates the transport period.

There were no mortalities in the second transport trial, and behaviourally little difference between treatments. At the beginning of the trial all treatments showed a similar Hct value to that of forced exercise fish, between 43.3-46.3% packed volume. The Hct of fingerlings from the 50 mg CO_2 l^{-1} treatment were significantly elevated relative to the other treatments mid-way through the transport, and both hypercapnic treatments were significantly lower than the control treatment at 5 and 10 h (Figure 24). The Hct of all treatments were similar by 35 h (30 h post transport)

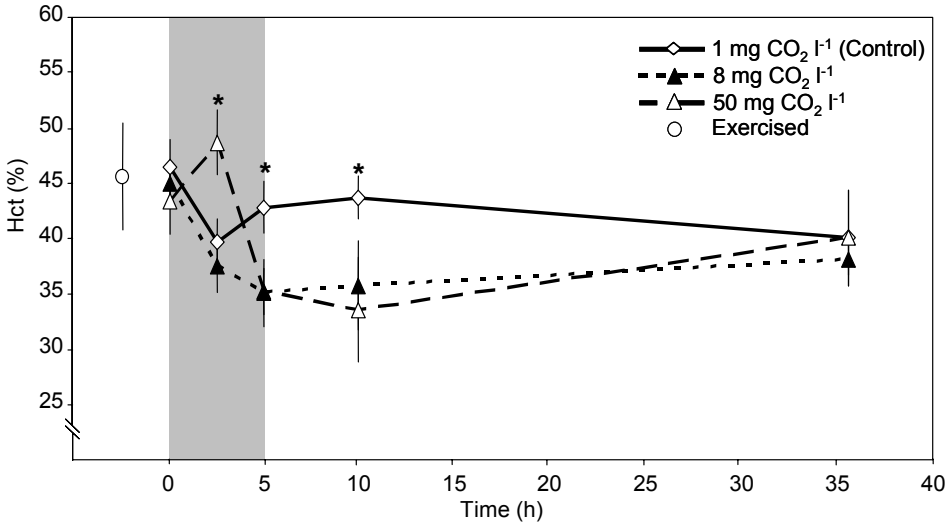

Figure 24. Changes in haematocrit (%, mean ± SE) at differing levels of hypercapnia. Significant differences between control and hypercapnic treatments (p<0.05) are indicated by '*', and the grey bar indicates the transport period.

The haemoglobin concentration of the blood decreased by approximately 30% in both hypercapnic treatments, though by the end of the simulated transport Hb had returned to control levels for the 8 mg CO_2 l^{-1} treatment (Figure 25). Hb in the 50 mg CO_2 l^{-1} treatment remained lower than control values until the end of the trial, at which point control and hypercapnic treatments were the same as pre-trial levels. Exercised fish had similar Hb values to that of pre-trial and recovered individuals (Figure 25).

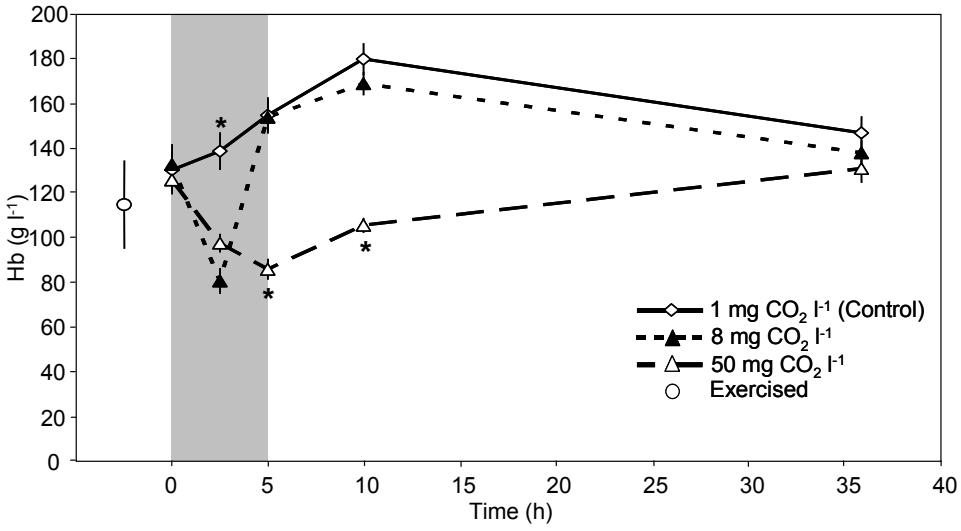

Figure 25. Changes in haemoblogin concentration (Hb, mean ± SE) of blood at differing levels of hypercapnia. Significant differences between control and hypercapnic treatments (p<0.05) are indicated by '*', and the grey bar indicates the transport period.

MCHC varied between 199.3–480.0 g l^{-1}, and mid-transport the hypercapnic treatments were significantly lower than the control (Figure 26). At the end of the transport and 10 h fingerlings from the 8 mg CO_2 l^{-1} treatment did not have a significantly different MCHC value to control fingerlings, and by 36 h both hypercapnic treatments had recovered to control values. Exercised fish had MCHC values similar to that of control and recovered individuals.

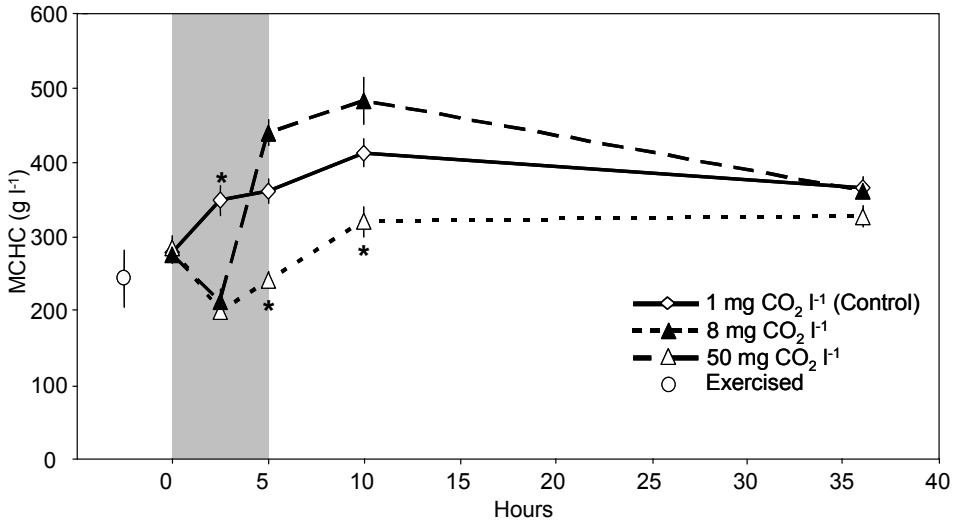

Figure 26. Changes in mean cell haemoglobin concentration (MCHC, g l^{-1}, mean ± SE) at differing levels of hypercapnia. Significant differences between control and hypercapnic treatments (p<0.05) are indicated by '*', and the grey bar indicates the transport period.

Discussion

Brett and Groves (1979) reviewed the resting or routine oxygen consumption rates for several species of fish at a variety of size and temperatures and found them to vary between 45-540 mg O_2 h^{-1} kg^{-1}. The estimates from Chapter 6 show that yellowtail kingfish juveniles have a higher resting $\dot{M}O_2$ than any of these species by a considerable margin (>580 mg O_2 h^{-1} kg^{-1}), and accordingly have a high CO_2 output and relatively high NH_3 excretion rate, illustrating the difficulty associated with the live transport of this species. Salmonids are used as model species for comparative fish physiology and also for specifying performance attributes of products for aquaculture. Yellowtail kingfish fingerlings have a $\dot{M}O_2$ approximately 1.5-2.0 times that of salmonids at equivalent sizes and temperatures (Brett & Groves, 1979), an important factor to take into account when designing facilities to culture this species. Likewise,

the estimated $\dot{M}CO_2$ of yellowtail kingfish is high compared to salmonids such as rainbow trout (Brauner et al., 2000; Lauff & Wood, 1997). The considerable CO_2 output of yellowtail kingfish was evident in the rapid pH drop in the first trial, emphasising the importance of constructing an efficient degassing system in order to maintain water quality. Estimation of $\dot{M}NH_3$ from Chapter 6 indicates that NH_3 toxicity at 20 kg m^{-3} stocking density would not become a concern until after four days of confinement. The decreased water pH resulting from the formation of carbonic acid is useful for reducing the possibility of NH_3 reaching toxic levels.

The rapid decrease in pH and the difficulty in maintaining a stable pH during the transport are similar to the problems encountered on larger scale commercial transports. The extremely low pH resulting from the dive cylinder running out and the high water turbulence from the ceramic diffuser may be considered a worst case scenario for transport. Given these conditions it is surprising there were so few mortalities, and the mortalities that did occur were the physiologically compromised fish with jaw and opercula deformities. Taken together with similar observations from previous commercial transports, it appears that kingfish are surprisingly tolerant to transport stress.

The physiological stress measures indicate a similar finding. There appeared to be no great fluctuations in the secondary stress responses, and indeed most indicators had returned to control levels after 24 h. It would be expected that acute stressors would cause a rapid increase in blood glucose and lactate and muscle lactate. There was, however, a slight increase in blood glucose mid-transport, by the end of the transport glucose had returned to pre-transport values. Blood lactate concentration decreased during transport and muscle lactate remained almost perfectly stable. Comparison of the levels of these blood metabolites to other athletic pelagic teleosts such as kahawai *Arripis trutta* (Davidson et al., 1997) and a number of scombrids (Wells et al., 1986) indicates the transported and control fingerlings exhibited little fatigue and a low secondary stress response. The low stress response is unlikely to reflect a depletion of metabolic fuels required for burst activity given the elevated levels of stress metabolites in exercised fish. Additionally, muscle pH increased during transport in both control and transported fish, indicating there was no acidosis occurring in the muscles. The muscle buffering capacity did not vary as a result of the transport, and values were very similar to those reported by Ogata (2002) for Japanese yellowtail. In general, the secondary stress indicators showed that yellowtail kingfish responded

much better to transport and confinement stress than other species exposed to similar treatments (Iwama et al., 1989; Paterson et al., 2003).

A possible explanation for the low stress response exhibited by the fish to the poor water quality conditions is the constant exposure to low level anaesthesia. When the fingerlings were initially transferred from the nursery tanks they were under a state of partial anaesthesia and hence did exhibit significant locomotory behaviour. Although the fingerlings were given time to recover in the transport tank the build up of CO_2 would have an additional anaesthetising effect, evident from the inactivity of the fish. The combination of these effects would cause blunted stress responses. Although it may have been useful to compare these results with a set of control fish that were not subjected to light sedation for netting and transfer into the transport tank, this would have made it difficult to separate the effects of sedation and transport. It is unclear whether there are long term effects on growth rates or immunity from a transport such as the one in this experiment and further research is highly desirable. However, given that the fish would take food immediately after transport and that there was very little mortality and little perturbation in physiological stress parameters, it appears that yellowtail kingfish are very robust to transport.

The mechanisms by which the yellowtail kingfish were able to cope with high levels of CO_2 were partially elucidated in the second transport trial. While the control and 8 mg CO_2 l^{-1} treatment represented moderate physiological challenges, the 50 mg CO_2 l^{-1} treatment caused considerable haematological disruption. At the beginning of the hypercapnia trial all treatments showed a similar Hct value to that of exercised fish, between 43.3-46.3%, meaning that the relative volume of circulating erythrocytes were largely similar. The Hb and MCHC values at the start of the trial were also similar to that of exercised fish, indicating that the stress of transferring the fish into the treatment tanks had likely affected the haematology of the test subjects. The MCHC decreased in the hypercapnic treatments mid-way through the transport, which is indicative of osmotic stress on erythrocytes resulting from hypercapnia. The MCHC of the 8 mg CO_2 l^{-1} treatment was not significantly different from control values at the end of the transport, probably due to the fact that the fingerlings had been able to compensate for any respiratory acidosis by increasing the bicarbonate concentration of the blood and adjusting the rate of Cl^-/HCO_3^- and Na^+/H^+ exchange through the gill membranes (Hayashi et al., 2004; Yoshikawa et al., 1991). Unlike the 8 mg CO_2 l^{-1} treatment, fingerlings from the 50 mg CO_2 l^{-1} treatment were not able to recover the MCHC to that of control values by the end of transportation, though were able to return

to pre-treatment levels 5 h after transportation. All treatments recorded similar haematology results 30 h post-transport, indicating that recovery from intense hypercapnia can be achieved within 10-30 h after exposure.

The Hct and Hb values for yellowtail kingfish in the current study (38.0-46.4% and 80-179 g l^{-1}, respectively) were within the range recorded for Japanese yellowtail exposed to environmental hypercapnia (Hct 25-50%, Lee et al., 2003), but higher than that recorded by Yamamoto (1991) and Yamamoto et al (1980) for rested Japanese yellowtail (26.1% and 77-80 g l^{-1}, respectively). The reason for the discrepancy between these closely related species is unclear, though the differing ontogenetic stages tested may be an important factor (the current study used 10-15 g fish whereas Yamamoto (1991) and Yamamoto et al (1980) used 400-900 g fish). The blood sampling method is also an important consideration, as the acute sampling method used in the current study tends to overestimate Hct compared to the cannulation technique employed by Yamamoto and coworkers (Gallaugher & Farrell, 1998).

These results show that yellowtail kingfish fingerlings are able to cope well with acute hypercapnia. Although little is known about what constitutes a safe CO_2 level for marine fish husbandry, 20 mg l^{-1} is generally taken as the upper limit for growth (Colt et al., 1991), and anaesthesia of carp and salmonids can be achieved within 5 min at 200-500 mg l^{-1} (Iwama & Ackerman, 1994). A study by Lee et al. (2003) found that 1.4 kg Japanese yellowtail exposed to water equilibrated to 1% CO_2 (~10 mg l^{-1} CO_2) did not suffer any mortality over 72 h, whereas at 5% CO_2 (~105 mg l^{-1} CO_2) there was complete mortality within 8 h. Cardiac failure appeared to be the main cause of death, brought about by acidosis of the myocardium and a resultant decrease in cardiac contractility (Lee et al., 2003). Yellowtail kingfish fingerlings were able to adjust blood haematology at moderate levels of exposure to CO_2 (8 mg l^{-1} CO_2), and quickly recovered from moderate-high levels (50 mg l^{-1} CO_2). The range of values recorded for both haematological and secondary stress parameters from exercised, transported and rested fish indicates that juvenile yellowtail kingfish have a wide tolerance to transport stress, much more so than most other fish species studied to date, including salmonids (Iversen et al., 2005; Paterson et al., 2003; Sandodden et al., 2001; Schreck et al., 1989). This is perhaps to be expected of an active pelagic carnivore which has evolved robust physiological mechanisms in order to cope with intense periods of exercise. The use of light anaesthesia to transfer and handle fish during loading into transport tanks undoubtedly contributed to the low stress response, and as previously mentioned, the

CO_2 that accumulates during transport is useful for keeping fingerlings slightly anaesthetised while confined, thereby lowering metabolism.

Although the accumulation of CO_2 in transport tanks appears useful and only transiently affects yellowtail kingfish fingerlings, there are two reasons to degas as much CO_2 as possible on route. Firstly, the direct use of CO_2 to lower activity and metabolism during live fish transport has been investigated previously with variable results and a high risk of mortality at useful levels of anaesthesia (Takeda & Itazawa, 1983). Yellowtail kingfish have a high metabolism and therefore excrete large amounts of CO_2, as was evident in the pH drop with only a moderate stocking density in the first trial transport. This means it would be difficult to control the concentration of CO_2. Secondly, as hypercapnia results in reduced binding and offloading of oxygen by haemoglobin, the scope for fingerlings to cope with oxygen stress is greatly reduced. In summary, yellowtail kingfish fingerlings are robust to the stressors imposed by confinement and transportation, as long as enough oxygen is supplied to meet the high metabolic rate, and CO_2 is effectively degassed.

Chapter 6: Ontogenetic Scaling of Metabolism in Yellowtail Kingfish

Introduction

One of the original studies that investigated the relationship between mass and metabolism was an intraspecific analysis of the relationship between heat production and weight in dogs by Rubner (1883), who reported that heat production scaled allometrically with mass to an exponent of ⅔. This study was followed by the now famous interspecific analysis of metabolism by Klieber (1932), which showed that the metabolic rate of mammals from a mouse to an elephant conformed to an allometric scaling exponent of ¾. The origin and range of mass variation has been an important consideration in the study of mass-metabolism relationships, and for many years researchers have sought to make a distinction between allometries derived from intraspecific mass ranges versus those derived from interspecific mass ranges (Wieser, 1984). One of the main reasons for making a distinction between interspecific and intraspecific metabolic allometry is that the scaling exponent often differs between the two (Glazier, 2005; Heusner, 1982b), which hinders one of the most sought after goals of comparative physiology: to find a fundamental law, preferably derived from first principles, that constrains the metabolism of an organism to its mass. The discrepancy between the two types of scaling exponents has been treated in a variety of ways, from cautionary notes about not comparing the two based on the fact they have been found to differ (Clarke & Johnston, 1999; Heusner, 1982b; Hou & Burggren, 1989), to theoretical and mathematical reasons why the two should not be compared (Calder, 1984; Heusner, 1982a; Kozlowski & Weiner, 1997; Witting, 1995; 1997). Most text books or reviews on the subject of scaling ignore ontogenetic metabolic allometry altogether (Dodds et al., 2001; Goolish, 1991; 1995; Hochachka & Somero, 2002; Peters, 1983; Schmidt-Nielsen, 1984; West et al., 2000). In addition, despite the empirical evidence to the contrary, there is often an assumption that functional explanations derived from interspecific allometry may also apply to ontogenetic allometry (Brown et al., 1997; West et al., 1997), though such claims are seldom made explicit (Kozlowski & Weiner, 1997). One interpretation of the treatment of this discrepancy could be that the underlying desire to put a figure on a single allometric scaling constant and subsequently derive a fundamental law has taken precedence over the uncomfortable fact that it is difficult to reason why such a law could only be applied to “adults” of species ranging from bacteria to blue whales.

An interesting development in the study of fish allometry has been the recent discussion of how metabolic rate varies with ontogeny (Bokma, 2004; Burggren, 2005;

Hunt von Herbing, 2005). Fish are particularly good candidates for studying the ontogenetic development of metabolism as, unlike other vertebrates such as mammals, birds or reptiles, most fish develop from small eggs released directly into the environment and grow quickly from a small size (Hunt von Herbing, 2005). In addition, the form of growth in fish is generally continuous with little metamorphic discontinuity. The continuous improvement of oxygen microsensor technology now allows researchers to accurately and precisely measure the metabolic rate of individual first hatch fish larvae less than 1 mg wet weight (ww), meaning it is possible to derive ontogenetic data sets that span the same mass range as the widely touted mouse to elephant example of allometric scaling. This is an important point, as historically intraspecific analyses only tended to cover 1-2 orders of mass magnitude (Heusner, 1982b; Smith, 1980), whereas interspecific analyses encompass up to 9 orders of magnitude, thus complicating the statistical comparison of the two (Brown et al., 1997; Smith, 1981).

A number of studies have been published on the ontogeny of fish metabolism, some from analyses that have combined respiration data sets from several sources over large mass range > 5 orders of magnitude (Bishop & Torres, 1999; Bochdansky & Leggett, 2001; Giguere et al., 1988; Gruber & Wieser, 1983; Kamler, 1992; Oikawa & Itazawa, 1992; Oikawa et al., 1991; Post & Lee, 1996; Rombough, 1988a; Wieser, 1995; Wieser & Forstner, 1986; Wuenschel et al., 2004). Most of these studies concluded that the metabolic scaling exponent varies during ontogeny, though there is disagreement over whether the metabolic scaling exponent changes abruptly or gradually. Oikawa et al. (1991) and Post and Lee (1996) proposed a biphasic relationship where the scaling exponent changes from near isometry during the larval phase to allometry during the juvenile phase and thereafter, which may in turn be linked to an ontogenetic change in the surface area to volume ratio of respiratory organs. In contrast, Bochdansky and Leggett (2001) proposed a curvilinear relationship between mass and metabolism for a combined ontogenetic data set of 25 species, and derived a single metabolic model to approximate the relationship. Quite which model is the most accurate is open for debate as there is no biological basis to argue either is better. The issue is further complicated by the fact that several authors propose that the scaling of metabolic rate varies as a function of many factors, including mass, developmental stage, level of activity, temperature and other environmental variables (Hochachka & Somero, 2002; Hunt von Herbing, 2005; Lovegrove, 2000; Wieser, 1995), most of which cannot be controlled for when comparing data sets from several sources.

This chapter investigates the ontogenetic development of aerobic metabolism in yellowtail kingfish (*Seriola lalandi* Valenciennes 1833). This respirometric study is unique in that it spans nearly the entire ontogenetic mass range, from 0.6 mg larvae to 2.2 kg sub-adult fish (adult weight 20-60 kg Gillanders et al., 1999), corresponding to the mass range between a mouse and elephant. While the terms intraspecific allometry and ontogenetic allometry are often applied interchangeably depending on the field of research (e.g. anthropology, medical science, paleobiology, physiology), for the purpose of this chapter ontogenetic allometry will be used as this most accurately describes the entire lifespan of a species. While the standard metabolic rate (SMR) is often used as the standard measure of metabolism in comparative physiological studies (Withers, 1992), it has been recognized as perhaps too narrow a measure of metabolism (Darveau et al., 2002). In this study the metabolic rate of fish were measured in a state that might, according to the International Union of Physiological Sciences (IUPS), be called the SMR as the fish were measured in a condition where the organism is rested (or as near to rested as is possible) and fasting (or post digestive) (IUPS Thermal Commission 2003). However, the definitions assigned by the IUPS Thermal Commission are probably too narrow to apply to fish as they are predominantly based on mammalian attributes such as thermoregulation. A more representative term for the metabolic rate used in this study would be resting metabolic rate (RMR), as the fish weren't measured in an inactive state, but were allowed to swim at the resting levels observed in large rearing tanks. It is recognized, however, that this definition of RMR does not fit into the mammalian-based definition given by the IUPS Thermal Commission (2003).

The RMR of individual larvae were measured via closed and intermittent flow respirometry utilizing a recently developed fluorimetric oxygen microsensor. The RMR of juveniles and sub-adults were measured using flume and flow-through respirometry, respectively, in order to match the behaviour and stress tolerance of different ontogenetic stages with the stress associated with different respirometry methods. In addition, the contribution of protein catabolism to ontogenetic energy requirements was investigated by measuring post-digestive ammonia excretion rates (Finn et al., 2002; Wood, 2001). Careful consideration was given to standardizing activity levels and temperature in order to minimize factors other than mass that influence metabolic scaling. The data were interpreted using a number of different models, including a traditional linear regression and the models developed by Post and Lee (1996) and Bochdansky and Leggett (2001). The models were evaluated using a number of

methods, including correlation, null hypothesis testing and information theory approaches. The results of this study are compared to other allometric studies in fish ontogenesis, and the findings are discussed in terms of the implications for the study of metabolic scaling in general.

Materials and Methods

Egg collection, larval rearing and general animal husbandry

All experiments were carried out at the National Institute of Water and Atmospheric Research Limited Bream Bay Aquaculture Park between April 2003 and March 2005. Fourteen wild-caught *Seriola lalandi* brood stock were held in an 80 000 l tank maintained under ambient photoperiod and sea water temperatures. The brood stock naturally spawned in the spring and summer months while the water temperature remained above 17°C. The positively buoyant eggs were collected at the surface by a boom and channelled into a separate 200 l tank. The eggs were collected in the morning, sterilised with chloramine-T and incubated at 18-20°C in 200 l semi-conical tanks. Hatched larvae were transferred to 13 000 l black tanks and raised under green water, semi-static conditions at 18-23°C and ambient photoperiod. Larvae were administered enriched rotifers for 3-12 d post-hatch (DPH), enriched *Artemia sp.* nauplii for 10-30 DPH, and weaned onto artificial feed from day 25. Juveniles were raised under ambient photoperiod and seawater conditions (19-21°C) and administered pelletised feed daily. Sub-adults (100 g-2.2 kg) were maintained in flow-through 17 000 l tanks on ambient photoperiod and seawater conditions (17-19°C). At all stages of animal husbandry oxygen saturation was maintained above 80% via the use of pure oxygen.

Larval and juvenile respirometry (0.6 mg-1.0 g)

For all fish below 10 g oxygen concentration was measured using a needle enclosed oxygen microsensor (diameter 0.8 mm) connected to a Microx TX3 fibre optic oxygen meter (PreSens Precision Sensing GmbH, Regensburg, Germany). The oxygen microsensor was calibrated using % air saturation on the morning prior to respirometry according to manufacturer's instructions and set to take readings every 10 s. The smallest test subjects were pre-feeding 2 DPH larvae (0.60 mg ± 0.04 SD). Approximately 100 larvae were removed from a 19°C rearing tank in a beaker and transferred to a 20 l bucket containing 1 µm filtered UV-sterilised water, and left to

acclimate to 20°C overnight. The temperature was kept constant via a thermostatically controlled laboratory and 250 W aquarium heater, and oxygen was supplied by gentle aeration with an air stone. The following morning the larvae were transferred to a fresh bucket of water, and 10 larvae individually transferred to 1 ml syringes for respirometric measurement. Experimental subjects were placed in the syringes by removing the plunger, partially filling the syringe with water and transferring an individual larva into the syringe barrel with a trimmed plastic pipette. The plunger was then replaced with care to exclude air. Measurements were made with the syringe sitting in the air and maintained at 20°C via the thermostatically controlled laboratory. The total length (L_T) of the larvae (4.4-4.8 mm) was smaller than the diameter of the syringe (5 mm) meaning the larvae could turn around if necessary. Larvae were given 2 h to settle in the respirometry chambers during which time the water was exchanged every 10 min by gently expelling and drawing around 0.8 ml of the volume. Measurements of the activity levels of pre-feeding larvae in stock tanks were made for comparison with confined individuals by making 20 observations of the amount of time a larva spent swimming in 1 min. The oxygen consumption rate was measured by slowly expelling the water volume down to the 0.1 ml mark and inserting the oxygen microsensor into the needle housing of the syringe in such a way that the larva could move around unimpeded by the needle (Figure 27).

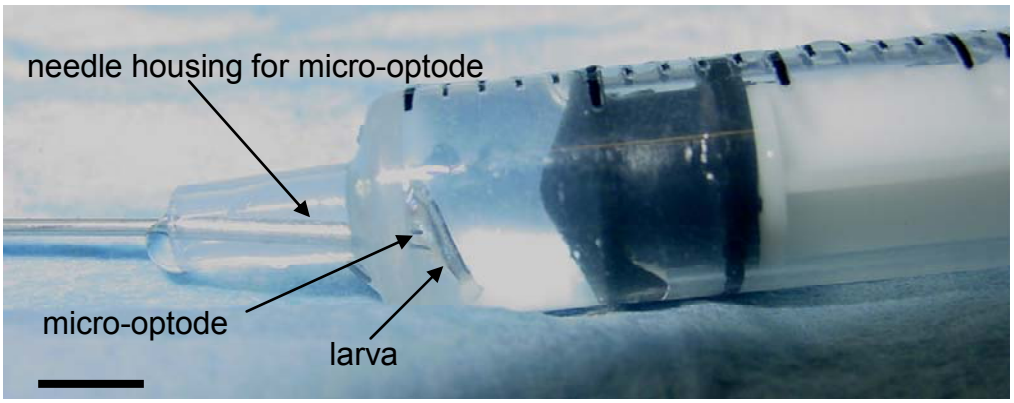

Figure 27. Larva in syringe respirometer with oxygen micro-optode inserted in needle housing. Scale bar represents 5 mm.

The decrease in oxygen was monitored for 5-12 min depending on the amount of time required to get a satisfactory linear oxygen decrease (Figure 28), after which the microsensor was withdrawn and freshly aerated water drawn into the syringe. The oxygen concentration never fell below 80% air saturation. A measurement was also made of larval activity levels during respirometry by measuring the amount of time the larva spent swimming in a 1 min period, and the total activity compared to non-

confined larvae using a *t*-test. The next larva was then measured until five respirometric measurements of the 10 larvae had been made (50 observations in total for the size class).

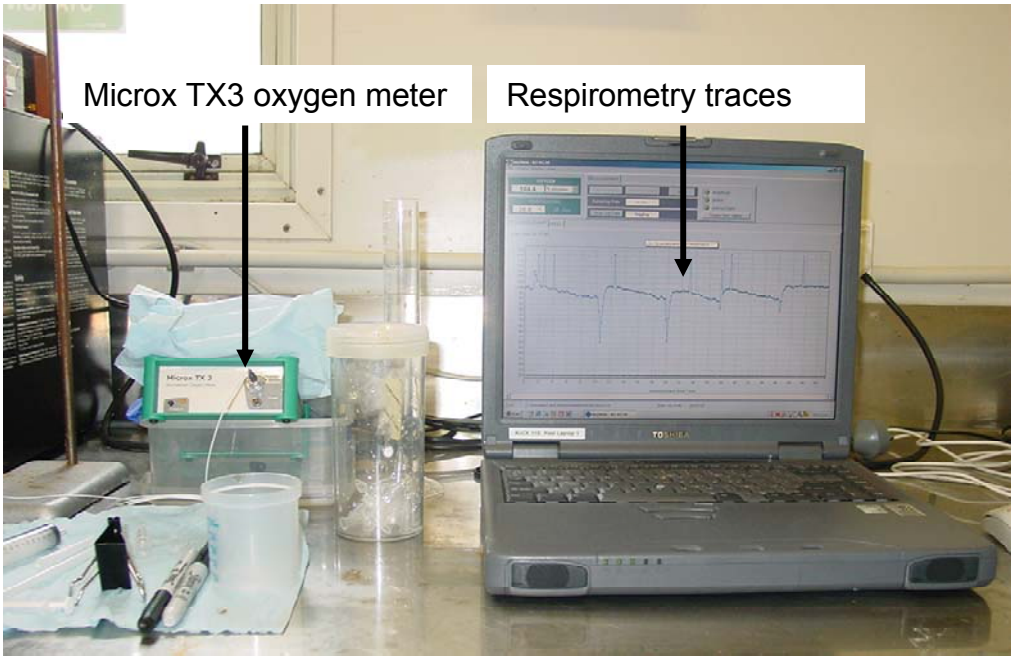

Figure 28. Microx TX3 oxygen meter and laptop showing oxygen decrease measured during respirometry.

It was not possible to measure the ammonia excretion rate of individual larvae given the small volume of water used for respirometric measurements. Throughout experimentation a control syringe was also measured which did not contain a larva to provide a measure of bacterial respiration. At the completion of the five measurements individual larvae were removed from the respirometers, dispatched using an overdose of isoeugenol, and blotted dry before weighing to the nearest 0.01 mg using an Oertling WA205 microbalance. Finally, the volume of water in the syringe at the 0.1 ml mark was accurately quantified by weight. The entire measurement process took approximately 10 h.

The next size class of test subjects were 8 DPH and 3.6 mg (± 1.0 SD) in weight (5.2-7.9 mm L_T). The respirometry procedure was identical to that above except that the respirometer was a 3 ml syringe adjusted to 0.5 ml for respirometry. Ammonia excretion was quantified by taking triplicate water samples at the beginning of the measurement period, and at the end of the measurement period using a 1 ml syringe and needle to take a single 200 µl water sample from the needle housing of the 3 ml respirometer. These water samples were immediately frozen at -20°C for ammonia

quantitation at a later date. Ammonia samples were analysed as total unionised ammonia following the methods of Bower and Holm-Hansen (1980).

The remainder of the juvenile size classes (0.052, 0.36 and 1.3 g) were measured in plastic containers modified into intermittent flow respirometers located inside a temperature controlled water bath set to 20°C. These chambers had three ports (2-10 cm long plastic tubes 2 mm in diameter) located in the screw-top lids through which the oxygen microsensor could be placed, water samples could be taken and freshly aerated water could be flowed through. Water flowed from a low-head aerated tank (20 l) into the respirometers during non-measurement periods at a rate of around one exchange min^{-1}, and flowed out the shortest port to waste (Figure 29). The water flow could be turned off to individual respirometers in order to make oxygen consumption measurements.

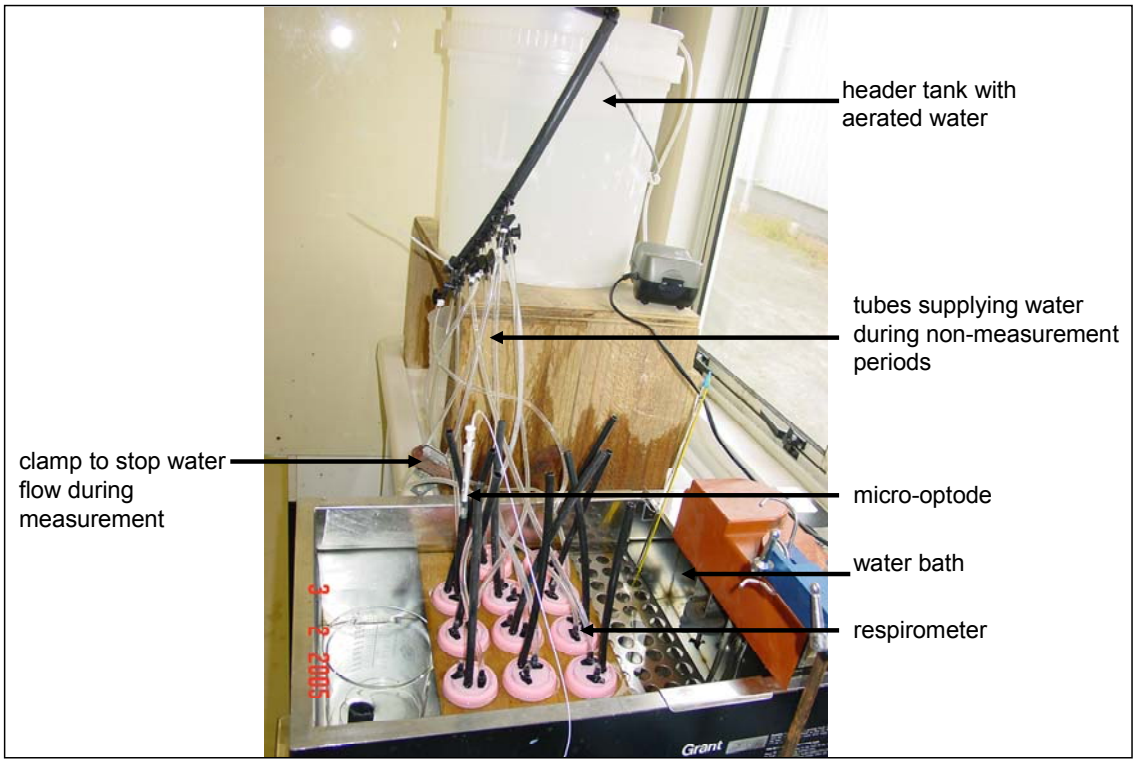

Figure 29. Respirometry apparatus for 1 g larvae.

The 10 test subjects of each size class were serially measured until five oxygen consumption rates had been recorded per individual, along with triplicate water samples for each measurement period in order to calculate the ammonia excretion rate. The volume of the respirometers for 0.052 g juveniles (± 0.010 g SD, 27 DPH, 14-17 mm L_T) were 15 ml, for 0.36 g juveniles (±0.09 g SD, 40 DPH, 28-38 mm L_T) 72 ml and for 1.3 g juveniles (±0.3 g SD, 55 DPH, 40-45 mm L_T) 258 ml. The water volumes

of respirometers were chosen on the basis that they provided enough room for the fish to turn and resulted in a 10-20% decrease in oxygen saturation over 5-15 min. This allowed enough time for a linear respirometry trace and meant oxygen saturation remained above 75%. Activity levels for these fish were measured according to that previously mentioned.

Juvenile respirometry (14 g)

The next size class for respirometry were juvenile fish 14.3 g (± 0.3 g SD, 119 DPH, 75-100 mm L_T). These fish are behaviourally distinct from the smaller size classes in that they continually swim. Fish observed in circular rearing tanks were found to normally maintain a swimming speed of 8-13 cm s^{-1} in a tank with 10-20 cm s^{-1} water velocity. It was decided that 10 cm s^{-1} was an appropriate water velocity to test the standard oxygen consumption rate. A Brett-style (Beamish 1978) enclosed swim tunnel was employed to measure the oxygen consumption rate of individual fish via intermittent respirometry (total volume 9.6 l, calculated from the sum of the component volumes, Figure 30).

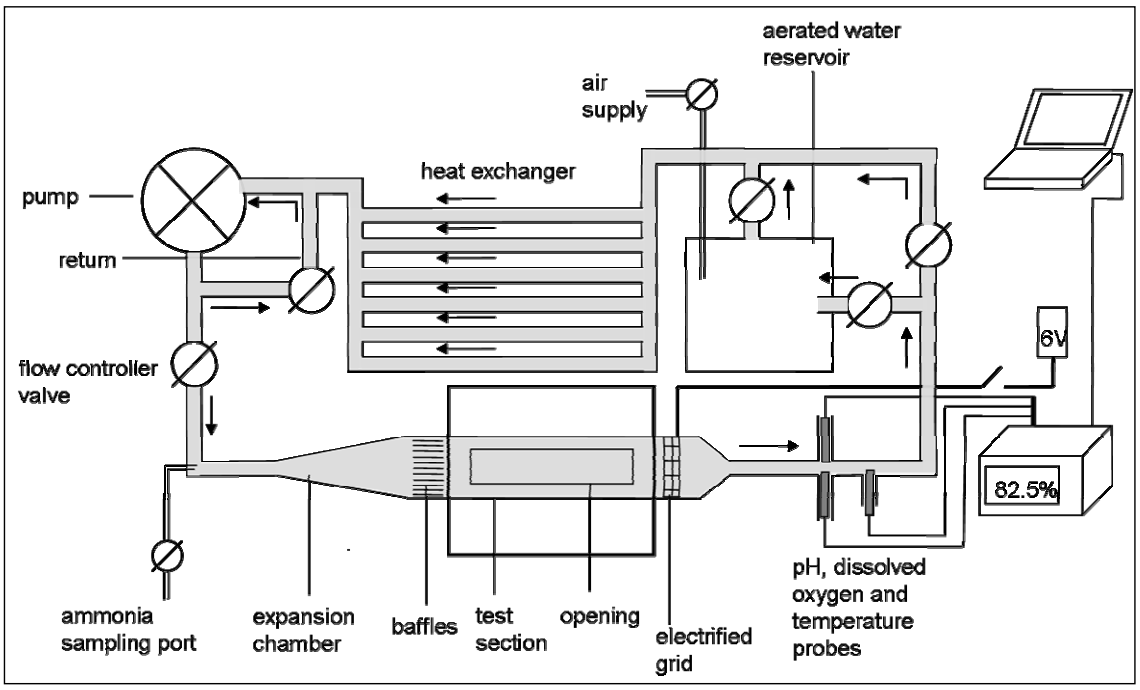

Figure 30. Schematic of flume respirometer used to quantify respirometric rate in 14 g kingfish. Diagram not to scale.

The flume could be either in a closed state for respirometric measurement, or in an open state supplying the test subject with aerated water during non-measurement periods. Water flow through the test section was made rectilinear through the use of

both and expansion cone and baffles (Figure 31a). Water velocity within the test section was controlled via a bypass loop configured around the pump, and the velocity calibrated to 10 cm s^{-1} before experimentation using a General Oceanics flow meter (model 2030, General Oceanics Inc. Miami, U.S.A., Figure 31b) installed in the test section, and dye flow tests. A flow rate of 10 cm s^{-1} was chosen as this was near the cruising speed of fish in 17 m^{-3} commercial rearing tanks. Oxygen concentration and temperature within the respirometer was measured using a YSI polarographic oxygen electrode (YSI Inc., Ohio, U.S.A.) connected to a TPS 90-FL meter (TPS Pty. Ltd., Brisbane, Australia, Figure 31c), which was calibrated daily. The entire respirometer was immersed in a salt water bath and maintained at 20°C via a 2 kW bar heater, and a heat exchanger installed in the respirometer to control for the heat given off by the pump (Figure 31d). Ten test subjects were taken from the rearing tanks (19-20°C) and placed into a separate holding tank to ensure there was no feeding 24 h prior to testing. To make respirometric measurements individual test subjects were loaded into the test section of the respirometer and the lid sealed. The pump was then turned on and the water circulated in the open state while the fish acclimated. If the fish did not exhibit the desired swimming behaviour within 20 min it was replaced. After a 2 h acclimation period the respirometer was closed and the oxygen concentration monitored until it fell below 80% saturation, after which it was opened (typically 30 minutes). Water samples for ammonia quantitation were taken in triplicate at the beginning and end of each measuring cycle from a water sampling port and stored at -20°C. Five measuring cycles were performed per individual before the test subject was removed, anaesthetised and weighed to the nearest 0.1 g. One individual was tested per day, and at the beginning and end of every day three blank runs were performed to test for bacterial respiration.

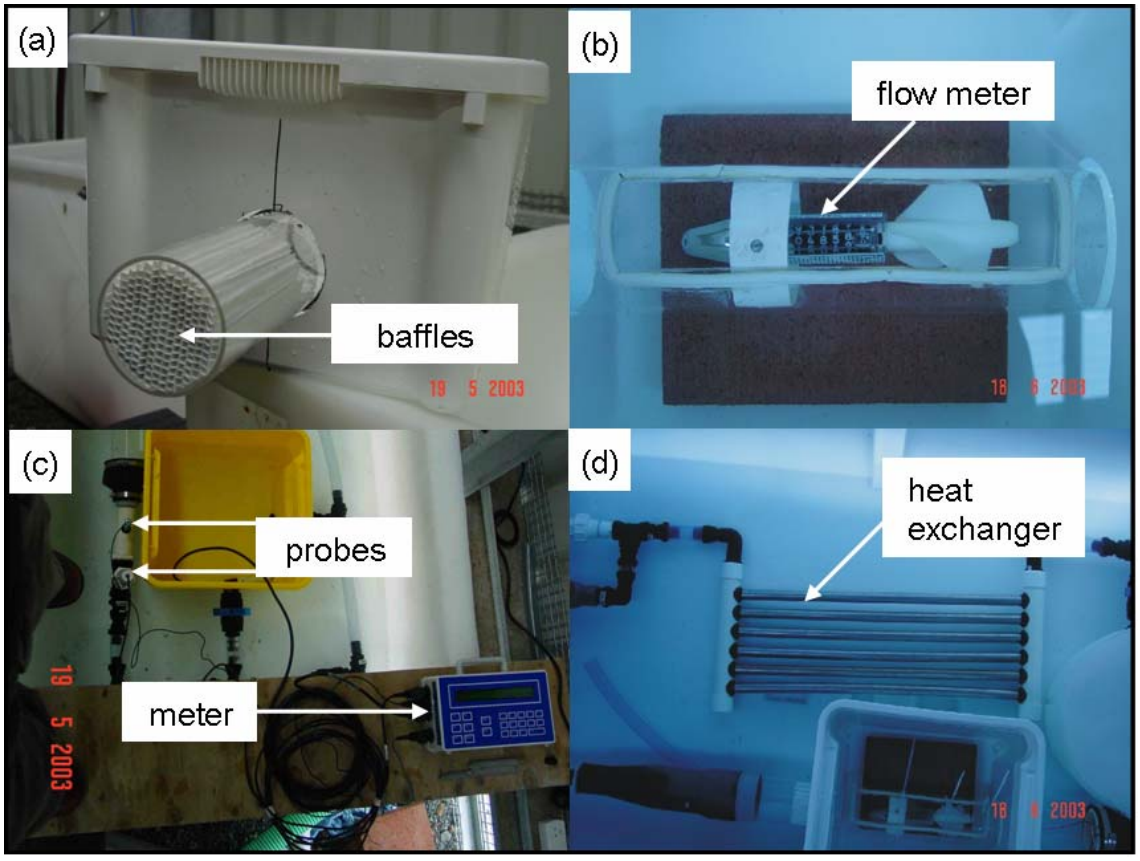

Figure 31. Detail of flume respirometer: (a) test section showing baffles; (b) flowmeter in test section for water velocity calibration; (c) oxygen and temperature probes and meter; (d) heat exchanger.

Sub-adult respirometry (0.56 and 2.20 kg)

Careful consideration was given to the method that would best allow an accurate determination of the standard metabolic rate of sub-adult fish. Experience from culturing yellowtail kingfish had shown that sub-adults (i.e. > 300 g) were not as amenable to handling as smaller fish, and a similar observation had also been made by Tsukamoto and Chiba (1981) during respirometric experimentation with the closely related species *S. quinqueradiata*. The method for quantifying oxygen consumption needed to be minimally intrusive, yet maximise accuracy in determining standard oxygen consumption rate. A swim tunnel did not fit this condition given the amount of handling needed to get fish into the test section and the fact that the naturally highly active fish would need to be anaesthetised. A flow-through tank respirometer was deemed the least intrusive method that allowed the fish to maintain a close to normal behavioural state. A 1000 l header tank supplied water under a constant head pressure to a 2700 l circular test tank in which the test subjects were kept. Four airlifts installed in the header kept the water well saturated with oxygen (98.5 ± 0.5%), and a

thermostatically controlled 2 kW bar heater ensured the temperature remained at 20 ± 0.5°C. The water flow rate was set to allow the tank to self-clean via a castle and standpipe mounted in the centre. An appropriate biomass of fish was added to allow for a detectable difference in oxygen concentration between the incoming water and the test tank based on estimated oxygen consumption rates. It was estimated that the biomass should consume around 15% of the available oxygen for standard metabolism plus another 5-10% for digestion, which allowed for the oxygen to remain above 70% saturation for the duration of the trial. The oxygen concentration of the header and respirometry tank was recorded using two TPS 90-FL meters set to measure oxygen saturation every 10 min during the measurement periods.

To begin the trial, 40 fish weighing an average of 490 g (approximately 7 months old) were anaesthetised in 15 ppm Aqui-S (Aqui-S Ltd, New Zealand), weighed and loaded into the respirometry tank with a water flow rate of 3010 l h^{-1}. The trial began four days after the transfer, at which point feeding had resumed to the pre-handling state. The fish were fed 1.5% body weight daily (0900 hours), and the respirometry tank cleaned in the afternoon if necessary. Daily oxygen measurements were recorded prior to feeding by taking the average of six oxygen saturation recordings made 10 min apart to account for the wash-out effect (Steffensen, 1989). In addition, triplicate water samples of both the header tank and respirometry tank were taken for ammonia analysis. The fish were not aware of the sampling process, and could be observed from a hidden viewpoint to observe activity during measurement. After ten days of measurement the fish were removed from the test tank, anaesthetised, weighed and placed into another tank. The test tank was left flowing but without fish for 24 h to measure bacterial respiration. The trial was again repeated with six fish weighing an average of 2.20 kg (approximately 13 months old) at a water flow rate of 2730 l h^{-1}. It took nine days for the fish to regularly resume feeding before oxygen consumption measurements were taken.

Oxygen consumption and ammonia excretion calculations

For the closed respirometry methods used for larvae and juveniles, the oxygen consumption rate was calculated using the equation:

$$\dot{V}_{O_2} = \frac{\Delta O_2 \cdot V}{t},$$

where $\dot{V}O_2$ is the oxygen consumption rate (ml O_2 consumed h^{-1}), ΔO_2 is the measured change in oxygen concentration (ml O_2 l^{-1}), *V* is the volume of the respirometer (l) and *t* is time (h). A volumetric measure of oxygen consumption was used for this study as the ontogenetic model of oxygen consumption developed by Bochdansky and Legget (2001) will only apply to such a measure. Ammonia excretion rate ($\dot{N}NH_3$, mmol NH_3 excreted h^{-1}) was calculated using the same equations except that ΔNH_3 (mmol l^{-1}) was substituted for ΔO_2. Ammonia samples were calculated as total unionised ammonia following the methods of Bower and Holm-Hansen (1980). The mean $\dot{V}O_2$ and $\dot{N}NH_3$ from the five replicates of each individual larva and juvenile were then used for further analysis. For the flow through respirometry method used for sub-adults, the oxygen consumption rate was calculated using the equation

$$\dot{V}_{O_2} = \frac{\Delta O_2 \cdot Q}{n},$$

where $\dot{V}O_2$ is expressed as above, ΔO_2 is the difference in oxygen concentration between the header tank and test tank (ml O_2 l^{-1}), *Q* is the water flow water rate (l h^{-1}) and *n* is the number of fish in the test tank. The data for the sub-adult respirometry were presented as a mean oxygen consumption and ammonia excretion rate due to the daily measurements being pseudoreplicates.

Ontogenetic variation in $\dot{V}O_2$

In addition to using the $\dot{V}O_2$ derived from the current study, data from Partridge et al (2003) was also included in the analysis as this represents the only other published study of respirometric rates in yellowtail kingfish. The relationship between mass and metabolism was described using a number of different models, but as the Bochdansky and Legget (2001) model was unit dependent the body mass data had to be transformed to µg dry weight (dw) by dividing the wet weight by 6 (Post & Lee, 1996) and the $\dot{V}O_2$ calculated as ml O_2 consumed d^{-1}. The first model represented a traditional analysis of the double log transformed data using ordinary least-squares (LS) regression in the form

$$\log \dot{V}_{O_2} = a + b \cdot \log dw$$

where *a* denotes the intercept and *b* the metabolic scaling coefficient (Schmidt-Nielsen, 1984).

The second model was a segmented regression function similar to that described by Post and Lee (1996). The break point was initially approximated using the "Piecewise linear regression function" in Statistica (v. 6, Statsoft Inc., Tulsa, USA), which estimates segmented regressions for the dependent variable. The estimated break point was then used as a starting value for deriving a segmented regression based on the independent variable from the "Custom loss function" of Statistica. Both quasi-Newton and Simplex algorithms were used for calculating a segmented linear regression, and were reported in the form

$$\log \dot{V}_{O_2} = a + B_1 \cdot \log dw + B_2 \cdot (\log dw < BP) \cdot (\log dw = BP),$$

where *a* represents the intercept, BP the break point, B_1 the slope (or metabolic scaling coefficient) of the segment below the break point and B_2 the difference in slope above the break point (i.e. the scaling coefficient of the second segment). The metabolic scaling coefficients are denoted by the lower case b_1 and b_2. The Simplex algorithm could not derive a useful segmented regression as the break point was below the smallest size category, effectively resulting in a linear regression. For this reason this model was not used in subsequent analyses.

The third function was the curvilinear model developed by Bochdansky and Legget (2001) to describe the ontogenetic development of metabolism in fish, which followed the form

$$\log \dot{V}_{O_2} = \log \left(\frac{1}{\frac{1}{10^{(-3.71 + \log dw)}} + \frac{1}{10^{(-2.40 + 0.67 \log dw)}}} \right),$$

The fourth function fitted was a LS regressed second order polynomial, which was used for comparative purposes as this represented a gradual change in the scaling exponent and, like the segmented regression and the Bochdansky and Legget (2001) model, had three parameters. The explanatory power and value of the models were evaluated in a number of ways. Firstly, the Pearson's R^2 correlation coefficient was calculated along with the adjusted R^2 ($\overline{R}^2$), which introduces a penalty based on the

number of parameters in a function. The residual sum of squares error (RSS) was used as an index of the error in a model, and the *F*-statistic

$$F^{H}_{n-K} = \frac{(\mathrm{RSS}\{K\text{-}H\} - \mathrm{RSS}\{K\})/H}{(\mathrm{RSS}\{K\})/(\mathrm{n} - K)},$$

used to evaluate whether complex models with *K* parameters were significantly more useful than simpler models with *H* fewer parameters (Hamilton, 1992). In this statistic RSS{*K*} denotes the RSS for the complex model, and RSS denotes the RSS for a model with *K* – *H* parameters. The derived *F*-statistic was then compared to a theoretical *F*-distribution with $df_1 = H$ and $df_2 = n\text{-}K$ degrees of freedom. The models were also evaluated using an information theory approach by employing the LS estimated Akaike's information criterion (AIC, Akaike, 1973) and Schwartz (or Bayesian) information criterion (SIC, Schwarz, 1978). The LS estimation of AIC is represented by

$$\mathrm{AIC} = n \log(\hat{\sigma}^2) + 2K,$$

where

$$\hat{\sigma}^2 = \frac{\mathrm{RSS}}{n},$$

though the modified AIC_c was used in the current study as this correction is best used when *n/K* is less than approximately 40 (Burnham & Anderson, 1998),

$$\mathrm{AIC}_c = \mathrm{AIC} + \frac{2K(K+1)}{n - K - 1},$$

The SIC is represented by

$$\mathrm{SIC} = n \log(\hat{\sigma}^2) + K \log(n),$$

with the main difference between the two criteria being that SIC imposes a stronger penalty on extra variables than AIC (Heij et al., 2004). Differences in the AIC_c values of the different models were compared using Δ_i

$$\Delta_i = \mathrm{AIC}_i - \mathrm{AIC}_{min},$$

and SIC was interpreted likewise (Burnham & Anderson, 1998).

Ontogenetic variation in $\dot{N}_{NH_3}$

Ontogenetic variation in post-digestive ammonia excretion was investigated by plotting $\dot{N}_{NH_3}$ against wet weight, along with the molar oxygen consumption rate for comparison. As no ammonia excretion measurements could be made on the smallest larvae (0.6 mg) this size class was excluded from the analysis. A statistical analysis of polynomial and linear regression functions showed the latter was adequate to describe the relationship between mass and $\dot{N}_{NH_3}$. The theoretical nitrogen quotient (*NQ*) was estimated from the linear and polynomial regressions of $\dot{N}_{NH_3}$ and $\dot{N}_{O_2}$, respectively, using

$$NQ = \frac{N\,NH_3}{N\,O_2},$$

The theoretical *NQ* values derived from the regression lines were compared to the actual values calculated for each of the respirometric measurements. In addition, the *NQ* values were also calculated as a relative contribution of amino acids as a respiratory substrate from stoichiometric relationships, and expressed as a percentage of aerobic metabolism based on lipid or carbohydrate as a co-substrate (Finn et al., 2002; Gnaiger, 1983).

Results

The two smallest size classes of larvae measured had no difference in activity level compared to non-confined larvae, while larger larvae were found to swim substantially less in the respirometers (Figure 32), and were observed to use more sculling action with the pectoral fins when confined.

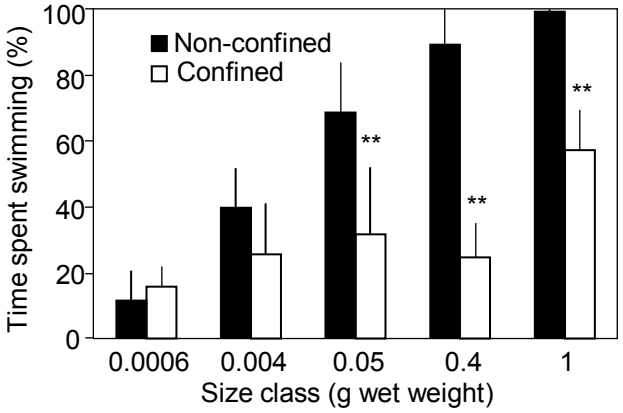

Figure 32: Comparison of activity rates for confined versus non confined larvae of yellowtail kingfish during respirometry. Significant differences are denoted by '**' (*t*-test, $p<0.01$).

Juveniles adapted well to swimming in the flume respirometer, with only three fish found not to settle into the flume satisfactorily for respirometric measurement. There was no observable effect of respirometric measurement on sub-adult fish, with the swimming and feeding behaviour similar to that seen in the 17 m^3 commercial stocking tanks.

There was a strong correlation between all of the $\dot{V}O_2$ data and the fitted functions. The linear regression gave a metabolic scaling exponent of 0.90 (Figure 33a), while the segmented regression derived from the quasi-Newton algorithm gave a scaling exponent of 1.06 below the break point (15488 µg dw, 93 mg ww), and an exponent of 0.76 above the break point (Figure 33b). The correlation coefficients for all LS models were close to 1 (R^2 > 0.99, $\overline{R}^2$ > 0.99), and there was little residual error from all models (RSS < 4.26, Table 5). The *F*-statistic indicated that the three parameter segmented regression and polynomial functions were a significant improvement over the two parameter linear regression (Table 5).

The Bochdansky and Leggett (2001) model fitted the data well in the lower mass range but underestimated the $\dot{V}O_2$ above approximately 10^5 µg dw (Figure 33c), and was not a significant improvement over the less parameterized linear regression. The polynomial function (Figure 33d) was the most informative model based on the AIC_c and SIC criteria (Table 5), though the low Δ_i values for the segmented regression indicate that this model still had a relatively high level of empirical support according to

the interpretation of Δ_i by Burnham and Anderson (1998). The other models all resulted in Δ_i values above 20 (Table 1) and therefore had a very low level of empirical support.

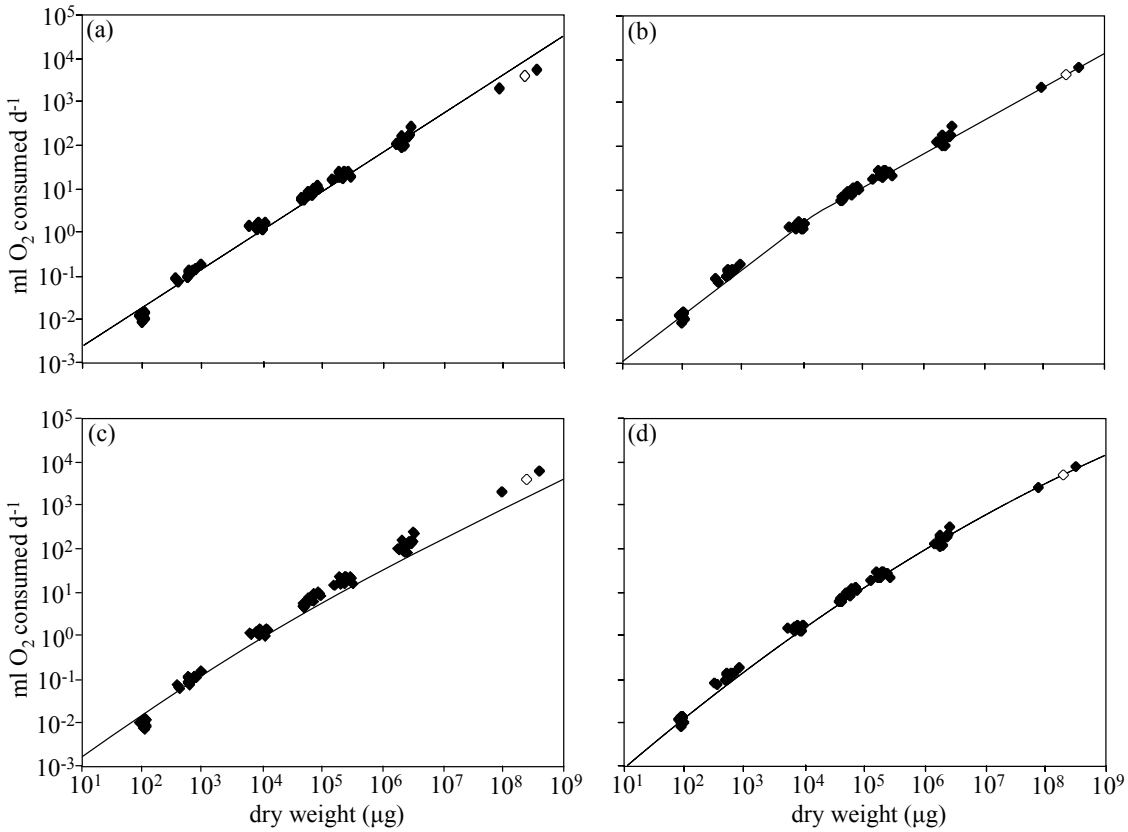

Figure 33. Oxygen consumption of yellowtail kingfish during ontogeny. Four functions were used to model the data: (a) LS linear regression; (b) LS segmented regression; (c) Bochdansky and Leggett (2001) model; (d) LS polynomial regression. See Table 5 for description of each model and goodness of fit. The unshaded points represent data from Partridge et al. (2003).

Table 5. Comparison of models for $\dot{V}O_2$ data. See Methods for description and computation of statistical tests and information criteria.

Model description	Function	Number of parameters	R^2	$\overline{R}^2$	RSS	F^1_{60}	AIC_c	$AIC_c\ \Delta_i$	SIC	SIC Δ_i
Linear regression	$\log R = -3.52 + 0.90 \cdot \log dw$	2	0.99	0.99	1.63		-225.88	69.97	-265.47	93.58
Segmented regression	$\log R = -4.03 + 1.06 \cdot \log dw - 0.30 \cdot (\log dw < 4.19) \cdot (\log dw \geq 4.19)$; $b_1 = 1.06$, $b_2 = 0.76$	3	1.00	1.00	0.54	120.74*	-293.15	2.71	-356.34	2.71
Bochdansky and Leggett (2001)	$\log R = \log\left(\frac{1}{\frac{1}{10^{(-3.71 + \log dw)}} + \frac{1}{10^{(-2.40 + 0.67 \log dw)}}}\right)$	3	n/a	n/a	4.26	-36.96	-163.37	132.48	-226.56	132.48
Polynomial	$\log R = -4.25 + 1.23 \cdot \log dw - 0.04 \cdot \log dw^2$	3	1.00	1.00	0.52	128.68*	-295.86	0.00	-359.05	0.00

'n/a' denotes not applicable; '*' denotes significant difference at $P<0.01$

There was strong positive correlation between $\dot{N}NH_3$ and mass (R^2 = 0.99), with the LS linear regression function resulting in a scaling exponent of 0.87 (Figure 34). The data clusters for each size range tested were more variable than that of $\dot{N}O_2$. The *NQ* values derived for each paired $\dot{N}O_2$ and $\dot{N}NH_3$ observation did not show a particularly strong correlation with mass, though the sub-adult fish were distinctly higher (mean = 0.16) compared to larvae and juveniles (mean = 0.08) in the contribution of protein catabolism to aerobic metabolism (Figure 35). The contribution of amino acids to metabolism calculated from the regression of $\dot{N}O_2$ and $\dot{N}NH_3$ reflected this difference, with amino acid catabolism estimated at 20-35% for 10^{-3} to 10^{2} g ww, and above this weight catabolism increased to 80% at 10^{4} g ww (Figure 35).

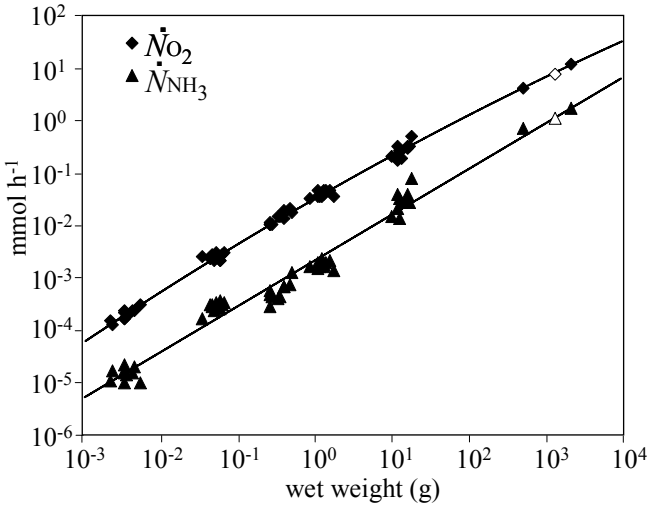

Figure 34. Comparison of $\dot{N}O_2$ and $\dot{N}NH_3$ for yellowtail kingfish throughout ontogeny. Oxygen consumption rate is represented by the function $y = -1.50 + 0.83 \cdot x - 0.03 \cdot x^2$, and ammonia excretion rate represented by the function $y = -2.68 + 0.87 \cdot x$. The unshaded points represent data from Partridge et al. (2003).

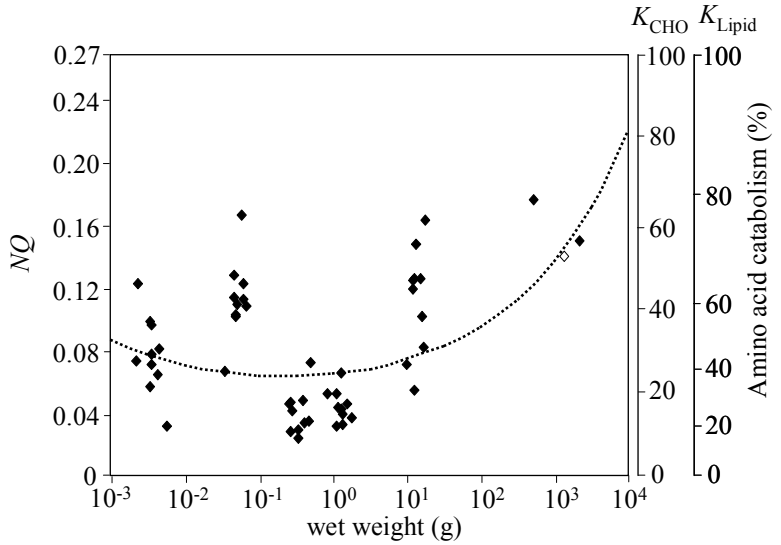

Figure 35. Ontogenetic variation in *NQ* values for paired $\dot{N}O_2$ and $\dot{N}NH_3$ measurements for yellowtail kingfish. The estimated contribution of amino acid catabolism to aerobic energy requirements based on carbohydrate (CHO) or lipid as a co-substrate is added for comparison. The line through the data represents the theoretical *NQ* based on the $\dot{N}O_2$ and $\dot{N}NH_3$ functions from Fig. 3, and the unshaded point represents data from Partridge et al. (2003).

Discussion

This study investigated the ontogenetic development of aerobic metabolism in yellowtail kingfish, and as with interspecific comparisons, an important consideration in interpreting the results is whether the measure of metabolism was comparable between groups and respirometric methods. It was necessary to employ an adaptable definition of standard metabolism in the current study given the wide range of forms and behaviours exhibited by the experimental subjects. The fact that larvae and juveniles <10 g were found to be no more active in confinement than in non-confinement indicates that the respirometric rates measured in these size classes can be presumed to be an accurate measure of RMR. Juveniles in the flume respirometer adapted easily to flume confinement and generally maintained position and swimming speed throughout measurement, meaning the fish were rested and respirometric measurements reflected the RMR. Though the method used to measure the respirometric rates of sub-adults was not particularly precise, the fact that the fish were

not apparently stressed during measurement undoubtedly meant that the RMR was at least accurately measured. The strong continuity in sub-adult $\dot{V}O_2$ and $\dot{N}NH_3$between the current study and that of Partridge et al. (2003), and the high correlation between mass and metabolism in general indicates that a comparable measure of metabolic rate was recorded between size classes.

The relationship between mass and $\dot{V}O_2$ was investigated using a number of models, all of which correlated the two variables well. The Bochdansky and Leggett (2001) model correlated well with larval $\dot{V}O_2$ but underestimated $\dot{V}O_2$ at higher masses. Bochdansky and Leggett (2001) do note, however, that their model was only tested on data sets less than 10^8 µg dw, and that there were likely to be species specific differences that depart from the predicted relationship. The three models derived via LS regression had more explanatory power than the Bochdansky and Legget model (as indicated by the lower RSS values). The simple linear regression resulted in a metabolic scaling exponent of 0.90, which is within the higher end of the range reported in other fish species (Brett & Groves, 1979; Clarke & Johnston, 1999; Goolish, 1995). Both the segmented regression and polynomial function were a significant improvement over the linear regression, with the latter model being slightly more informative based on AIC_c and SIC information criteria. The break point (0.085 g ww) and the slopes of the segmented regression (1.06, 0.76) were within the range reported by Post and Lee (1996) for common carp (*Cyrpinus carpio*), rainbow trout (*Oncorhynchus mykiss*) and sea bream (*Pagrus major*). The goodness of fit of both the segmented regression and polynomial models for yellowtail kingfish precludes any definitive statement about whether the metabolic scaling exponent changes gradually or biphasically during ontogeny, especially given the lack of consensus on the relationship for other fish species (Hunt von Herbing, 2005), and the poor understanding of the mechanisms underpinning allometry in general. What can be stated with certainty is that, in accordance with several other studies, larval fish metabolism scales isometrically (Finn et al., 1995b; Finn et al., 2002; Giguere et al., 1988; Wuenschel et al., 2004), and the metabolic scaling exponent changes during ontogeny (Bochdansky & Leggett, 2001; Giguere et al., 1988; Oikawa & Itazawa, 1992; Oikawa et al., 1991; Post & Lee, 1996; Rombough, 1988a; Wuenschel et al., 2004).

Such a finding is important for comparative purposes as most studies that aim to establish the species-specific scaling exponent do not survey the same mass range as that covered in the current study, which was one order of magnitude short of covering

the entire ontogenetic mass range. For example, if the scaling exponent for yellowtail kingfish was estimated from the juvenile to adult mass range the exponent would be close to 0.75, which is substantially different from the 0.90 scaling exponent derived for the entire ontogenetic size range. This illustrates two important points: 1) the ontogenetic scaling allometries assigned to species are contingent on the size range measured, and 2) the practice of computing ratios to normalise physiological data for variation in body size (Packard & Boardman, 1999) becomes more complicated when the scaling exponent changes over ontogeny. One of the few interspecific studies of fish scaling exponents to consider ontogenetic variation was that of Clarke and Johnston (1999), who reported no difference in the inter- and intraspecific scaling exponent of 69 fish species. Although this differs from the finding of the current study, the analysis of Clarke and Johnston (1999) was somewhat cursory given the intraspecific scaling exponent for each species was derived from two points.

The ammonia excretion rate scaled linearly with an exponent of 0.87, which is within the range found by Finn et al. (2002) for fasted larvae of Atlantic cod (*Gadus morhua*) in light (0.82-0.99), but considerably higher than that of Peck et al. (2004) for the same species (0.56). The latter study tested less than an order of magnitude in mass range, and given that $\dot{N}NH_3$ is somewhat more variable than $\dot{N}O_2$ (this study and Finn et al., 2002) a relatively large mass range is required to get an accurate estimate of the metabolic scaling exponent. The variability in $\dot{N}NH_3$data compared to $\dot{N}O_2$ data probably reflects differences in the energetic pathways used to fulfil the energy budget of each individual. The variability in *NQ* values from the current study matches the observations of Finn et al. (2002) and Peck et al. (2004) for Atlantic cod. It is interesting that the proportion of aerobic metabolism derived from amino acid catabolism is apparently so high in sub-adult yellowtail kingfish (~50-80%) given that for most species observed to date amino acid catabolism values are between 15-35% in the non-fed state (Wood, 2001). Though it is possible that the sub-adults were not completely purged of the previous feed at the time of measurement and were still utilising amino acids directly from the diet, unpublished data of yellowtail kingfish ammonia excretion rates two days after a feed of 1-2% body weight indicates that $\dot{N}NH_3$ remains relatively stable after the specific dynamic action excretion period, which is estimated to last 18-20 h post-feed (D. Moran unpublished data). Protein synthesis rates have been found to scale allometrically with mass in a number of fish species (Carter & Houlihan, 2001), meaning that the increased protein catabolism rate observed in larger yellowtail kingfish may be related to a decrease in protein synthesis.

The data from this study show unequivocally that the metabolic rate of yellowtail kingfish does not scale to ⅔ or ¾, but is best approximated linearly for $\dot{V}O_2$ and the $\dot{N}NH_3$ by the exponents 0.90 and 0.87, respectively. The effects of temperature and activity had been accounted for throughout the study, and the high correlation of the data with all models tested indicated the data set was robust. This has important implications for the study of mass-metabolism relationships, as it cannot be argued that differences in mass magnitude range largely account for the discrepancy between intra- and interspecific metabolic scaling exponents (Brown et al., 1997). These findings lend weight to the idea that new theories on the relationship between mass and metabolism will be stimulated by the use of comparative developmental physiology to study the way organ systems and energy demand develops during ontogeny (Burggren, 2005), combined with more encompassing measures of metabolism other than SMR (Darveau et al., 2002) and a move away from the focus on a single universal scaling exponent.

Chapter 7: General Discussion

Overview

This thesis describes a range of metabolic and physiological variables that are important in the ontogenetic development of cultured yellowtail kingfish (*Seriola lalandi* Valenciennes 1833). A particular strength of this body of work is that a wide range of developmental stages were surveyed, from newly fertilised eggs through to sub-adults. The resources required to carry out such a study are only really available through a commercial fish production facility such as the NIWA Bream Bay Aquaculture Park, and allows experiments that would otherwise be impossible using wild specimens. This enables studies that not only advance the aquaculture of yellowtail kingfish, but also considerably improves our knowledge of the ecophysiology of this species, and fish in general. It is therefore useful to discuss both the scientific and applied significance of the results separately, and to outline research ideas that are worthy of further investigation.

Scientific significance of results

Chapters 2 and 3 investigated how temperature affected the early development of yellowtail kingfish prior to first feeding. As emphasised throughout this thesis, temperature is probably the most important abiotic variable to affect fish, both in the wild and in aquaculture. The typically small size of fish eggs and larvae combined with an undeveloped physiology makes this ontogenetic phase particularly vulnerable to temperature variation. This was evident from the experiments in Chapter 2, where developmental rate and larval length at hatch were highly correlated with incubation temperature. Larval length is often used as a condition index and is thought to be an important determinant of recruitment success in the wild (Miller et al., 1988; Pepin & Meyer, 1991). The negative relationship found between length at hatch and temperature for yellowtail kingfish larvae could be interpreted to mean that eggs that develop at lower temperatures result in larvae with a higher condition index. However, larval size at first feeding was comparable between temperatures, thereby indicating that larval growth tended to occur within the egg chorion at lower temperatures, and on the yolk sac at higher temperatures. There was an apparent decoupling of ontogeny and growth, where higher temperatures appeared to advance ontogeny ahead of growth. The proposed explanation of this finding was that larval yellowtail kingfish

hatched smaller at warmer temperatures because not only was the formation of the hatching glands advanced, but also the elevated activity of the hatching enzymes and embryo caused earlier disruption of the chorion. This work builds on the observation by Fuiman et al. (1998) that for larval fish, temperature appeared to advance ontogeny ahead of growth. The decoupling of ontogeny and growth not only has important implications for studies of early fish development, but offers a more general explanation for why ectotherms at cooler latitudes seem to grow bigger, mature later and live longer (Atkinson & Sibly, 1997; van der Have & de Jong, 1996).

The physiological data from Chapter 3 supported this hypothesis, with a negative correlation found between total embryonic oxygen consumption and temperature. This finding was very much unexpected, and required reconsideration of the traditional methods used to interpret temperature-dependent developmental time. The proportion of time to hatch was found to be a suitable unit to represent relative developmental time, and will provide a useful measure for future studies combining egg development and temperature. The metabolic energy requirements of yellowtail kingfish eggs appeared to be almost entirely dependent on catabolism of free amino acids (FAA). While pelagic fish eggs are known to be strongly dependent on FAA for energy (Fyhn, 1989; Fyhn & Serigstad, 1987; Rønnestad et al., 1992a; Rønnestad et al., 1994; Rønnestad et al., 1998; Sivaloganathan et al., 1998), the degree to which yellowtail kingfish eggs were reliant on this fuel source was surprising as it was considerably more than that recorded for other species, where lipid is also used as a co-substrate (Sivaloganathan et al., 1998). The pattern of substrate utilisation during embryogenesis was not found to vary too much between 17-21°C, however, at 23°C ninhydrin positive substances (a proxy for FAA) decreased at a faster rate. This possibly indicates that 23°C exceeds the temperature limit for normal physiological development. Unlike most other studies on metabolic fuel use in fish eggs, this study is unique in that it simultaneously tested the effect of temperature on respiration *and* metabolite flux in fish eggs. This made comparisons with other species difficult, though did offer some insight into what effect varying sea temperatures might have on early development in fish.

Chapter 4 presented some particularly interesting results on the interaction between growth potential and aggression in modifying the rate at which juvenile yellowtail kingfish grew. Intra-cohort size heterogeneity greatly increased as larvae and juveniles progressed through the different types of feeds, and large individuals developed strong aggressive tendencies, with smaller juveniles the main targets of the aggression. While

the development of aggression is fairly well studied for a number of fish species, what is not well known is the degree to which aggression affects an individual's growth rate. Chapter 4 used a grading trial to reduce size heterogeneity and aggression in order to quantify just how well small, medium and large juveniles, all of the same age, were able to grow. Using both weight gain and RNA:DNA ratio as condition indices it was evident that large individuals were not necessarily larger due to inherently better protein deposition rates, as all growing juveniles had similar RNA:DNA ratios at any given weight. It was hypothesised that the large juveniles benefited from more efficient food capture or conversion traits. The small juveniles were on an apparently degenerate growth trajectory irrespective of the aggressive environment. When the size grade distribution was calculated as a proportion of the population, it was estimated that the small juveniles composed around 40% of a cohort and most would not survive until adulthood. The differentiation of the growth potential between size grades not only has impacts for the culture of this species, but has wider implications for ecological or fisheries recruitment models. Not all juveniles are created equal apparently, and if reproductive output is to be linked to fecundity, the loss of 40% of the larval/juveniles within a cohort simply due to developmental inadequacies, rather than the more traditional explanations of predation or starvation (Fuiman & Cowan Jr, 2003; Fuiman et al., 2005; Miller et al., 1988), is an important factor in recruitment.

The metabolic rate of fingerling yellowtail kingfish makes the confinement and transport of this species difficult compared to salmonids, the benchmark of fish ecophysiology. Fortunately, yellowtail kingfish were found to be a physiologically robust species with a strong tolerance to elevated carbon dioxide levels (hypercapnia) associated with transport. Erythrocyte swelling resulting from hypercapnia was compensated for relatively quickly, and there was virtually no change in secondary stress indicators (blood glucose and lactate, muscle lactate and pH) to transport and hypercapnia. The ability to endure physiological conditions similar to that of intense exercise (e.g. respiratory acidosis) is undoubtedly related to the fact that this species is an active pelagic predator and is known to migrate distances over 1000 km (McGregor, 1995).

The ability to sample the respirometric rate of yellowtail kingfish at a variety of life stages, and the advent of new oxygen microsensor technology, offered a unique opportunity to investigate ontogenetic allometry. The relationship between mass and metabolism was explored for a mass range comparable to that of a mouse to an elephant, an often cited interspecific allometry that has formed the basis of an intense

discussion in the past decade (Brown et al., 1997; Cates & Gittleman, 1998; Cates & Gittleman, 1997; Glazier, 2005; Kozlowski & Weiner, 1997). Chapter 6 investigated two main questions relating to how the standard metabolic rate changes with age. Firstly, a number of models have been put forward to explain how oxygen consumption varies during ontogeny in fish (Bochdansky & Leggett, 2001; Post & Lee, 1996), however, all have lacked suitable methods of comparing the fit of models. This thesis employed a number of techniques to compare different models, from traditional correlation and hypothesis testing methods to an information theory approach. The goodness of fit of all models indicated that caution must be taken when making functional statements based on the trend line fitted to an allometric data set, especially if the data set does not cover the ontogeny of an organism. The second main question addressed in Chapter 6 was whether the ontogenetic allometry of yellowtail kingfish matched that seen in interspecific allometries over similar mass ranges. This was definitely not so, and this data set is a key piece of evidence in the argument over functional explanations of allometry. It is evident that whatever theory is used to describe the ¾ scaling law, it must also allow for a changing mass-metabolism scaling exponent during ontogeny.

As can be seen from the preceding passages, the work contained within this thesis has significantly improved our understanding of how the ecophysiology of yellowtail kingfish changes with age. Perhaps more importantly, this work has also contributed to the study of fish biology in general, with a number of key ideas concerning ways in which to understand the influence of temperature on ontogeny, growth and metabolism, the impact of individual growth variation on fitness and survival, and the relationship between metabolism and age. The next section will discuss the importance of the findings in an aquaculture context.

Significance of the results to aquaculture

The practical significance of the findings can be considered from both a species-specific aspect, and also in the importance to aquaculture in general. The results of Chapter 2 and 3 indicate that differences in incubation temperature between 17-21°C had little obvious effect on the condition of yellowtail kingfish during the endogenous feeding period. The use of temperatures at the lower end of this range may extend the first feeding window as the oil droplet was conserved somewhat at 17°C, and first feed larvae are known to catabolise lipid for energy (Hilton, 2002). At 23°C the metabolic

development pattern appeared to change considerably, therefore incubation at or above this temperature should be avoided. Yellowtail kingfish are heavily dependent on FAA for early development, perhaps the most reliant of all fish species studied to date. Therefore, it is particularly important that amino acids are considered when formulating broodstock and first feed diets not only for this species, but also for *Seriola* spp. in general.

There were a number of important applied findings in Chapter 4. Firstly, the development of aggression in yellowtail kingfish is more delayed than that of related species such as Japanese yellowtail (*S. quinqueradiata* Temminck & Schlegel 1845). Grading can therefore be carried out before aggression becomes intense, and the negative effects of size heterogeneity and aggression reduced. As the large individuals, which represent around 8% of the pre-weaned population, carry out virtually all of the aggression it is only necessary to target these individuals for grading initially. An important finding from Chapter 4 was that a significant portion of a pre-weaned population (~40%) is on a degenerate developmental pathway, and will probably die irrespective of the aggressive or nutritional environment. Given that similar relationship between the development of aggression, size heterogeneity and mortality are observed in other fish species (Kestemont et al., 2003) it is almost certain that this observation (though not necessarily the proportion) is true for finfish in general.

Yellowtail kingfish are a species resistant to the stressors imposed by live transport, and providing that adequate oxygen is supplied and efficient degassing systems are installed in transport tanks, there is no reason to believe that high densities of fingerlings (>50 kg m^{-3}) cannot be successfully transported. The stress physiology data from Chapter 5 is useful as a baseline for other high metabolism species such as the southern bluefin tuna (*Thunnus maccoyii* [Castelnau, 1872]), which is currently being targeted for larviculture in South Australia. Such work is also useful as a starting point for formulating water quality parameters for general husbandry of warm water fish species, and especially useful for planning recirculation systems.

The profile of oxygen consumption and ammonia excretion across a wide mass range given in Chapter 6 will be of importance to several areas of aquaculture. Although simple, this represents one of the most complete sources of information on age-related metabolism of a non-salmonid species available. Many of the goods that are designed for aquaculture are specified to the requirement of salmon, and often results in an

underestimation of the requirements for the increasing number of warm water species being cultured. The data contained in Chapter 6 is vital for estimating oxygen demand in cages or tanks, and for calculating nitrogen output for biofiltration or environmental impact assessments. It can also be used to estimate feed requirements based on energy output.

Yellowtail kingfish are rapidly becoming one of the better studied finfish species used in aquaculture, and the data contained within this thesis has significantly improved our understanding how particular aspects of physiology vary during ontogeny in this species. Yellowtail kingfish are also a useful model species for a high metabolism finfish, and no doubt techniques and research methodologies formulated to overcome problems in the culture of this species will be applied to the development of other carangid and scombrid species. It is therefore pertinent to briefly discuss future directions of research.

Future directions of research

The effect of temperature on the rate of ontogeny and growth would be a very interesting research topic. To test whether there is a general effect it would be best to use a range of ectotherms, and important to carefully define ontogenetic stages. It would also be useful to carry out more work on the influence of temperature on metabolite flux during early development of fish. So little information is available on this topic that there are many avenues to be explored, all of which would add detail to respirometry and energy budget studies. The intriguing question of why there is such variability in growth potential between larvae and juveniles begs an answer. Separating the effects of genetic and non-genetic factors would be important, as would be investigating proximate reasons for growth differences such as food capture and assimilation variation. Before explanations for mass-metabolism relationships can fully be explored it is necessary to develop a better picture of ontogenetic allometry, as work in this area is greatly lacking. Fish make great study subjects for this, though future respirometry work at different life stages should aim to use a more diverse measure of metabolism that simply standard metabolic rate.

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